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# Impact of foliar application of nano micronutrient fertilizers and titanium dioxide nanoparticles on the growth and yield components of barley under supplemental irrigation

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## ABSTRACT

Nano-fertilizers are new generation of the synthetic fertilizers which contain readily available nutrients in nano scale range. Nano fertilizers are preferred largely due to their efficiency and environment friendly nature compared to conventional chemical fertilizers. To evaluate the effects of foliar spray of micronutrient nano-fertilizer (iron and zinc) and nano-titanium dioxide ($nTiO_2$) solution on grain yield and its components in barley under supplemental irrigation conditions, a field experiment was carried out in the semi-arid highland region of Maragheh, Iran. Barley plants were separately treated with of chelated nano-scale zinc oxide (ZnO) and ferric oxide ($Fe_2O_3$) suspensions during tillering stage, booting and milky stages. Results revealed that days to anthesis and maturity significantly increased after application of both nano-fertilizers. Furthermore, a considerable improvement was observed in grain mass, spike length, number of the grains per spike, chlorophyll content, grain yield and harvest index by application of nano-fertilizer. However the impact of nano zinc fertilizer was more prominent than iron. Foliar application of $nTiO_2$ positively affected some morphophysiological characteristics like as days to anthesis, chlorophyll content and straw yield. The results suggest that the delivery of Zn into barley seedling through spray of nano-fertilizer can be an efficient nutrient management strategy in semi-arid regions. Overall, our result indicated that the integration of nanotechnology in fertilizer products can improve fertilizer use efficiency and significantly increase of barley yield. However, plant response to nanoparticles significantly depend on concentration and time of application as well as size, shape, and surface functionalization of the particles.

**Key words:** chemical fertilizer, exogenous application, nano ferric oxide, $TiO_2$ nanoparticles, nano zinc oxide

## IZVLEČEK

### VPLIV FOLIARNEGA DODAJANJA NANO MIKRO GNOJIL IN NANODELCEV TITANOVEGA DIOKSIDA NA RAST IN PRIDELEK JEČMENA V RAZMERAH NAMAKANJA

Nano gnojila so nova generacija sintetičnih gnojil, ki vsebujejo hitro razpoložljiva hranila v nano območju. Priljubljenost nano gnojil temelji na njihovi učinkovitosti in okolju prijazni naravi v primerjavi s konvencionalnimi mineralnimi gnojili. Za ovrednotenje učinka foliarnega pršenja z mikro nano gnojili (železo in cink) in raztopino nano titanovega dioksida ($nTiO_2$) na pridelek zrnja ječemena in njegove komponenete je bil na semiaridnem višavskem območju Maragheha, Iran izveden poljski poskus ob sočasnem namakanju. Rastline ječmena so bile ločeno tretirane s suspenzijo nano delcev cinkovega (ZnO) in železovega oksida ($Fe_2O_3$) v fazah razraščanja, bilčenja in mlečne zrelosti. Rezultati so pokazali, da se je število dni do anteze in zrelosti značilno povečalo po uporabi obeh nano gnojil. Še več, po uporabi nano gnojil je bilo opazno znatno izboljšanje v masi zrnja, dolžini klasa, številu zrn na klas, vsebnosti klorofila, pridelku zrnja in v žetvenem indeksu.Večji učinek na te lastnosti je imela uporaba cinkovega gnojila. Foliarno dodajanje $nTiO_2$ je pozitivno vplivalo na nekatere morfo-fiziološke lastnosti kot so število dni do anteze, vsebnost klorofila in pridelek slame. Rezultati nakazujejo, da je vnos Zn v kalice ječmena s pršenjem nano gnojil učinkovit način uravnavanja hranil v semi-aridnih območjih. Ti rezultati nakazujejo, da vključitev nanotehnologije v proizvodno gnojil lahko izboljša učinkovitost njihove uporabe in znantno poveča pridelek ječmena. Kakorkoli, odziv rastlin na nano delce je značilno odvisen od njihove koncentracije, časa uporabe, kot tudi od njihove velikosti, oblike in površinske funkcionalnosti.

**Ključne besede:** mineralna gnojila, zunanja uporaba, nano železov in cinkov oksid, semi-aridna območja, $TiO_2$ nanodelci

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## 1 INTRODUCTION

Barley (*Hordeum vulgare* L.) is a major cereal grain. It was one of the oldest cultivated grains and now it is one of the most widespread cereals. Barley yield and quality predictions are of major interest to the growers and it is because of current consumer interest in nutrition and health. Improvement of the barley quality and quantity may help restore barley's status in the human diet. In Iran barley as second important crop and it has been estimated that its production in Iran is exceeded 3.2 million tones from an area of 1.6 million ha land (FAOSTAT. 2013). In Iran barley is used almost exclusively as animal feed. In northwest of Iran barley is planted almost on dry-farmed lands, located on mountain slopes.

However, the main reasons of low yield of barley in semi-arid regions of the Mediterranean basin are adverse environmental conditions, nutritional imbalance, cultivation of local cultivars with low potential yield and lack of other production inputs. Terminal drought and high temperature are two environmental phenomena frequency occurring during critical reproductive growth stage. Heat stress during anthesis and post-anthesis period can significantly decrease grain yield and quality in barley. Furthermore, climate change during the last decades intensified these abiotic stresses in rainfed areas, as increased the temperatures and changed the patterns of precipitation and water availability (Batisani and Yarnal, 2010). Although in barley production there are many management strategies for improving yield and grain quality, supplement irrigation and adequate plant nutrition, involving the application of fertilizers, especially micronutrient is seen as having a crucial role in food productivity/quality particularly in semi-arid area (Ryan et al., 2012). In order to achieves an acceptable grain yield in barley providing the micro and macro nutrients are fundamentals. Fertilizer management can intensely affect crop productivity in semi-arid region where the terminal drought stress is very prevalent, thus, the addition of nutrients can either enhance or decrease plants resistance to drought or have no effect at all, depending on the level of water availability (Boorboori et al., 2012; Fahad et al., 2014).

Accordingly, a balanced fertilization strategy with macro and micronutrients in plant nutrition is very imperative for crop production in this areas. Although micronutrients are needed in small quantities, they play vital roles in development of plants. The great importance of micronutrients is because of their stimulatory and catalytic effects on metabolic processes and their positive effects on yield and quality (Hänsch and Mendel, 2009; Marschner, 2012). However, among the micronutrients, iron is essential for crop growth and food production. Iron is involved in the production of chlorophyll, photosynthesis, mitochondrial respiration, hormone biosynthesis (ethylene, gibberellic acid, jasmonic acid), production and scavenging of reactive oxygen species and osmoprotection (Hänsch and Mendel, 2009). Also it is a component of many important enzymes associated with energy transfer, nitrogen reduction and fixation, lignin formation and pathogen defense. Fahad et al. (2014) reported that iron deficiency is a dominant problem in semi-arid zone with alkaline soils. Furthermore, some soils with low organic matter also may be iron-deficient. In these area foliar spray has been recommend as a more efficient way to correct iron deficiency (Galavi et al. 2012; Boorboori et al., 2012).

Moreover, zinc is a necessary component of various enzyme systems for energy production, protein synthesis, energy production, maintains the structural integrity of biomembranes and growth regulation (Hänsch and Mendel, 2009). Like to iron, zinc deficiencies are mainly found on sandy soils low in organic matter and on alkaline soils. Uptake of zinc also is adversely affected by high pH, high levels of available phosphorus and iron in soils (Ghasemi-Fasaei and Ronaghi, 2008).

In the last few years, some researchers tried to examine the potential of nanotechnology to improve fertilizer use efficiency. These efforts led to design and development of nano-fertilizer. Nanotechnology-based fertilizers could be more soluble or more reactive than their bulk counterparts (Nair et al., 2010; DeRosa et al., 2010; Naderi and Danesh-Shahraki, 2013; Rameshaiah and Jpallavi, 2015). Application of nano-fertilizers may improve solubility and dispersion of insoluble nutrients in soil, reduce nutrient immobilization (soil fixation) and increase the bio-availability (Naderi and Danesh-Shahraki,

2013). Nano formulated fertilizers can be easily absorbed by plants and they may exhibit prolonged effective duration of nutrient supply in soil or on plant (Rameshaiah and Jpallavi, 2015). Zhang et al. (2006) investigated the effects of slow/controlled-release fertilizers cemented and coated by nanomaterials on crop. It was found that these nanocomposites were safe for wheat seed germination, emergence and growth of seedlings and they can provide a regulated, responsive and on time delivery of nutrients to plants. Also several studies show that exogenous application of some nanoparticle can significantly improve plant growth (Mandeh et al., 2012; Song et al., 2013). Titanium dioxide nanoparticles ($nTiO_2$) are promising as efficient nutrient source for plants to improve biomass production due to enhanced the nitrogen assimilation, photoreduction activities of photosystem II and electron transport chain, scavenging of reactive oxygen species, and (Morteza et al., 2013; Raliya et al., 2015). In the northwestern part of Iran zinc and iron deficiencies are nutritional disorders in most of the plants grown in dryland condition. Since these two nutrients are meaningfully involved in grain yield production and their deficiency may occur frequently in semi-arid regions; the present investigation was undertaken to improve understanding about the foliar application of nano chelated iron and zinc fertilizers as well as $nTiO_2$ on yield and yield components of winter barley.

## 2 MATERIALS AND METHODS

The trials presented here were carried out at the research field, College of Agriculture, University of Maragheh (37°23' N; 46°16' E), Maragheh, in northwest of Iran (Figure 1). Maragheh has an average annual rainfall of 375 mm consisting of 73 % rain and 27 % snow falling through winter and early spring. Based on Koppen's classification, this region has semi-arid and cold temperate. Some climatic parameters during this research are given in Table 1. The soil texture of the experimental site is sandy loam, comprising of 53 % sand, 31 % silt and 16 % clay. It contains 0.14 % organic matter (OM) with a pH of 7.87, with electrical conductivity (EC) = 1.96 ds $m^{-1}$, 0.058 % nitrogen (N) and 5.67 available phosphorus (mg $kg^{-1}$). Soils of the arid and semi-arid zones are rich in potassium, as amount of available potassium (K) was 342 mg $kg^{-1}$.


**Figure 1:** The experimental field was located in Maragheh which is a town in East Azerbaijan Province, North West of Iran.

The field was mouldboard-ploughed and twice disked before seed sowing. After primary and secondary tillage, the seeds were hand planted on 3 December, 2014. The facultative barley (*Hordeum vulgare* 'Sahand') was used in the experiment. The cultivar is a two-row barley with approximately long awns that is cold tolerant and appropriate for highlands. Each plot was 9 $m^2$ consisting of eight rows, 3 m long and 10 cm apart. Seeds were sown 4 cm apart at 5 cm depth. Phosphorus was applied at the rate of 30 kg $ha^{-1}$ as basal dose and urea (46.6 % N) as nitrogen fertilizer was utilized at the rate of 100 kg $ha^{-1}$. It was added into two equal portions as rational application, the first part was utilized in sowing time and the second part was applied in jointing stage. There was no incidence of pest or disease on plants during the experiment. Weeds were controlled by systemic selective chlorophenoxy herbicides including 2, 4-D and MCPA. Plants were grown under rain-fed condition that received natural rainfall and only two supplemental irrigations (surface or flood irrigation) were applied during jointing and heading stages. The amount of irrigation water was calculated to restore water content in the root zone to field capacity. Depth of net irrigation water fraction was ~110 mm. All other inputs and agronomic practices were carried out uniformly.

**Table 1:** Monthly temperature and precipitation during the growing season in 2014-2015.

| | maximum temperature (°C) | Minimum temperature (°C) | Humidity (%) | Total precipitation (mm) | $ET_0$ |
|---|---|---|---|---|---|
| December | 7.36 | 2.15 | 61.25 | 14.46 | 64.38 |
| November | 9.08 | 3.53 | 48.64 | 31.24 | 76.16 |
| January | 8.07 | -2.26 | 59.53 | 22.72 | 52.23 |
| February | 10.07 | -0.21 | 59.55 | 21.5 | 61.24 |
| March | 13.49 | 2.47 | 52.78 | 24.5 | 96.31 |
| April | 19.24 | 6.43 | 46.70 | 16.38 | 14.93 |
| May | 25.30 | 11.62 | 42.00 | 38.71 | 206.13 |
| June | 32.21 | 18.00 | 26.95 | 1.8 | 267.04 |
| July | 37.00 | 23.45 | 22.00 | 0.28 | 348.00 |

$ET_0$=reference evapotranspiration,

The investigated treatments included micronutrient fertilizers (iron nano chelate, zinc nano chelate, control) and foliar application of titanium dioxide nanoparticles ($nTiO_2$) at two concentrations: 0 and 2000 ppm. Micronutrients nano-fertilizers applied three times during initiation of tillering stage, booting and milky stage. $nTiO_2$ solution was sprayed on the plants leaves at the end of vegetative growth (double ridge stage) and during the inflorescence emergence (reproductive stage). Plants sprayed with distilled water served as the control. Treatments were applied according to randomization complete blocks design (RCBD) under factorial with three replicates. Nano-titanium ($nTiO_2$) was purchased from the Pishgaman Nano, Iran. Nano chelated fertilizers were obtained from the Sepeher Parmis Company, Iran, which contained zinc oxide or iron oxide nanoparticles. Synthesized nano particles had been characterized morphologically by transmission electron microscopy (Figure 2). Chlorophyll content was measured by "SPAD 502" portable chlorophyll-meter system at the beginning of booting stage for fully expanded upper-canopy leaves. Crop phenology was monitored at 1–2 day intervals throughout the season and number of days from sowing to initiation of anthesis and day to maturity was determined. At maturity stage, yield contributing parameters and morphological traits, such as plant height, number of fertile tillers, straw mass, panicle length, number of grains/panicle, 1000 grain mass and grain yield evaluated. The statistical analysis of experimental data utilized the SAS program. Each experimental value was compared to its corresponding control. Statistical significance was accepted when the probability of the result assuming the null hypothesis, p is less than 0.05 (level of probability). Correlation analysis and principal component analysis (PCA), based on the rank correlation matrix and biplot analysis were performed by SPSS ver. 16, STATISTICA ver. 8 and Minitab ver.16.

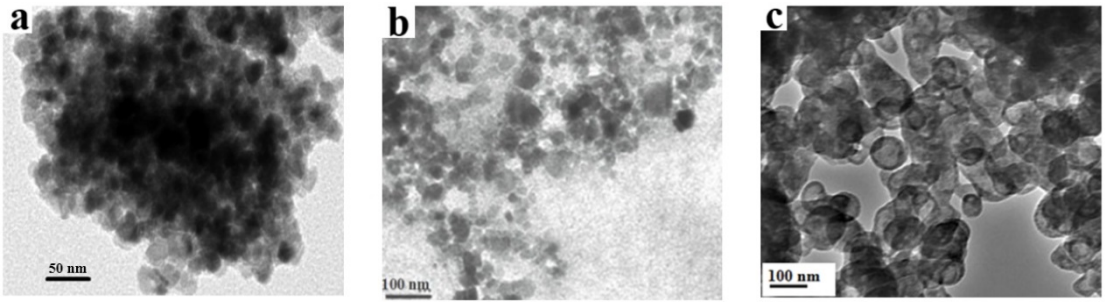


**Figure 2:** Transmission electron microscopy (TEM) micrograph of synthesized $TiO_2$ nanoparticles (a), nano zinc oxide (b) and iron oxide nanoparticles (c) nanoparticles.

## 3 RESULTS AND DISCUSSION

The results pertaining to effect of nano-fertilizers and nano- treatments on morphological characteristics are depicted in Table 2. Variance analysis showed that plant height (PH), peduncle length (PL) and stem diameter (SD) were not affected by foliar application of nano-fertilizers and $nTiO_2$. Although the micronutrients could numerically increase plant height (PH) up to 8 % compared to control, this increase was not statistically significant. The similar trend was recorded for $nTiO_2$ application. Although, these results differ from some published studies (Jam et al., 2011; Fahad et al., 2014), they are consistent with those of Abbas et al. (2009) who found that the various levels of Zn fertilizer not significantly affect the plant height. Based on the fact that the Fe and Zn have important roles in physiological and biochemical processes of development, it was predictable that application of micronutrients can increase the leaves growth and improve straw yield (Table 3).

Literature reports that the number of tillers per plant is an important yield component which varies considerably with variation in environmental conditions (seed-bed preparation, availability of soil moisture at sowing, nutrition managements, bird or insect damage on sown seeds, etc.) and with genotype. However, it seems that investigated variety tended to be more stable for tiller number. Likewise high planting density may affect this parameter. It appears that the number of tillers per plant has apparently high phenotypic plasticity under dense canopy, so that they cannot response to nutrient managements efficiently. These results are consistent with those of other studies suggesting that effects of foliar and soil application of Fe, Zn, Cu and Mn fertilizers on number of tiller was not statistically significant (Boorboori et al., 2012). Results from Table 2 show that foliar application with Zn and Fe did not increase the number of fertile tillers. Similarly $nTiO_2$ application had no any significant effect on number of fertile tillers. It is encouraging to compare this figure with that found by Arora and Singh (2004) and Bouis (2003) who suggested that number of tiller in wheat and barley is controlled by genetic factors and nutrition has a minor effect on this trait.

**Table 2:** Effect of foliar application of micro-nutrients and nano-titanium on some morphological traits of barley.

| **micro-nutrients** | PH | SD | NTP | NFT | GWP | NGS | PL |
|---|---|---|---|---|---|---|---|
| | ns | ns | ns | ns | * | * | ns |
| control | 68.00 a | 22.65 a | 3.58 a | 1.91 a | 0.64 b | 14.95 b | 12.46 a |
| Fe | 72.95 a | 23.07 a | 3.63 a | 2.09 a | 0.71 ab | 16.93 a | 12.75 a |
| Zn | 73.50 a | 23.85 a | 3.67 a | 2.02 a | 0.73 a | 16.61 a | 13.91 a |
| **nano- titanium** | ns | ns | ns | ns | ns | * | ns |
| non | 71.66 a | 22.66 a | 3.47 a | 1.92 a | 0.72 a | 15.38 b | 13.34 a |
| with | 71.52 a | 21.77 a | 3.79 a | 2.10 a | 0.68 a | 16.95 a | 12.71 a |
| CV % | 11.34 | 20.73 | 14.00 | 11.49 | 10.42 | 7.95 | 5.77 |

PH= plant height (cm), SD=stem diameter (mm), NTP=number of tillers per plants, NFT=number of fertile tillers, GWP= grain mass per plant (g), NGS=number of grain per spike, PL=peduncle length (cm). Mean values of the same category followed by different letters are significant at $p \leq 0.05$ level.

Result showed that foliar application of micronutrients considerably affected the thousand grains mass (TGW) and grain mass per plant (table 2 and 3). Application of zinc nano-fertilizer increased the grain mass up to 6 % over to control. The higher thousand grain mass indicates increased individual grain sink strength. The sink strength can be depicted as the output of sink activity and sink size (Yang et al., 2003). Sink activity is controlled by enzymes involved in starch biosynthesis and degradation (Bihmidine et al., 2013). It is assumed that phytohormones, particularly cytokinins play a major role in increasing sink size by promoting cell division during the early phase of seed filling. However, more recent work has shown that grain mass and endosperm cell number in wheat are closely associated (Saalbach et al., 2014). Besides in cereals, endosperm cell number is regulated by assimilate supply during the first two weeks after anthesis (Yang et al., 2003). With regards to positive effects of micronutrients on spike length and chlorophyll content (Table 2), it appears that micronutrient application improved the grain mass through the increasing assimilate supply, levels of cytokinins and sink size.

Assessment of the grain number per spike (NGS) revealed that it affected by micronutrient and $nTiO_2$ application with 95 % confidence level. So that foliar application of the Fe and Zn improved grain number up to 11 % and 13 %, respectively, in compared to control. This finding corroborates the ideas of Tarafdar et al. (2014), who suggested that application of zinc nano-fertilizer on pearl millet (*Pennisetum americanum* L.) significantly improved shoot length, root length, root area, chlorophyll content, total soluble leaf protein, plant dry biomass, and increased the grain yield by 37.7 %. On the other hand spray of $nTiO_2$ increased grain number per spike up to 10 % over to control. It is recognized that the grain number per spike is strongly depended on assimilates allocation to the spike and it is limited by post-phloem assimilate supply (Saalbach et al., 2014). Nano-titanium dioxide ($nTiO_2$) with photocatalytic property can increase photosynthesis by promoting cyclic and linear photophosphorylation (Gao et al., 2013) and it can result in enhancement of photoassimilates supply in leaves (i.e., increasing source capacity). However, positive effect of $nTiO_2$on grain number per spike in this study corroborates these earlier findings of Rezaei et al. (2015). Although theoretically there are some compensating effects between grain number per spike and grain mass, in current study application of nano-fertilizers increased both parameters.

Investigation the effect of nano-fertilizer and $nTiO_2$on chlorophyll content (CHL) showed that both of them significantly affected this parameter with 99 % confidence level. Different researchers feel that micronutrients play critical roles in the synthesis of chloroplast proteins and thus may interfere with chlorophyll synthesis. It has been revealed that lack of micronutrients inhibit the formation of chlorophyll through inhibition of protein synthesis (Marschner, 2012; Hänsch and Mendel, 2009). However, the findings of the current study do not support some result of Klingenfuss (2014), who reported that chlorophyll content of the Zn treatment was significantly smaller to the control. Besides the same research reported a positive effect of nano-titanium on

chlorophyll content in wheat seedling. These findings further support the idea of Morteza et al., (2013) that foliar utilization of $nTiO_2$ can improve plant growth and grain yield by facilitating the manufacture of pigments and transformation of light energy to active electron and chemical activity and increases photosynthetic efficiency.

**Table 3:** Effect of foliar application of micro-nutrients and nano- titanium on some morphological traits of barley.

| **micro-nutrients** | SL | TGW | DA | DM | CHL | GY | SY | HI |
|---|---|---|---|---|---|---|---|---|
| | ** | * | ** | ** | ** | ** | * | * |
| control | 9.50 b | 26.96 b | 187.33 b | 204.01 b | 52.00 b | 1771 b | 4145 b | 29.92 b |
| Fe | 10.12 b | 27.80 ab | 197.50 a | 221.00 a | 60.62 a | 2017 a | 4519 a | 30.77 ab |
| Zn | 11.06 a | 28.31 a | 192.66 a | 216.66 a | 57.93 a | 2088 a | 4344 ab | 32.46 a |
| **nano- titanium** | ns | ns | ** | ns | ** | ns | * | ns |
| non | 10.35 a | 27.37 a | 188.33 b | 214.00 a | 54.37 b | 1980 a | 4055 b | 32.68 a |
| with | 10.12 a | 28.01 a | 196.66 a | 213.33 a | 59.31 a | 2035 a | 4390 a | 31.64 a |
| CV % | 4.89 | 5.99 | 5.68 | 3.14 | 12.72 | 10.68 | 17.58 | 9.32 |

SL=spike length (cm), TGW=1000 grain mass (g), DA= days to anthesis, DM= days to maturity, CHL= chlorophyll content (SPAD unit), GY= grain yield (Kg $ha^{-1}$), SY= straw yield (Kg $ha^{-1}$), HI= harvest index (%). Mean values of the same category followed by different letters are significant at $p \leq 0.05$ level.

Likewise the evaluation of the phenological characteristics i.e. day to anthesis (DA) and day to maturity (DM) showed that application of micronutrients and $nTiO_2$ significantly affected them. It has been documented that plants under optimum condition tend to increase growth period as much as possible which can result in improved leaf photosynthesis, light-use potential and higher yield (Cui et al., 2015). This study showed that nano-fertilizers and nano $TiO_2$ considerably influenced the straw (SY) and grain yield (GY), which corroborate the findings of a great deal of the previous work (Gao et al., 2013; Morteza et al., 2013; Klingenfuss, 2014; Tarafdar et al., 2014). Foliar utilization of micronutrients improved straw yield and grain yield up to 7 % and 16 % over to control, respectively. Moreover, spray of $nTiO_2$ enhanced the straw yield by 8 %. A significant increase of grain yield in response to nano-fertilizer application statistically improved the harvest index (HI) in comparison with control.

Result of our study suggested that yield component of barley can be positively affected by of $nTiO_2$and nano chelated micronutrient fertilizers and it can be introduced as a safe nano-nutrient. In plant nanoparticles may adsorb to plant surface and taken up through natural nano or micrometer scale openings. The results of other previous studies demonstrated $nTiO_2$ as an efficient photocatalyst by improving the photosynthetic complexes, and nitrogen metabolism can enhance cell growth as well as fresh and dry mass of plant (Morteza et al., 2013; Gao et al., 2013; Klingenfuss, 2014; Rezaei et al., 2015; Raliya et al., 2015). However evidence from this study suggested that $nTiO_2$ along with nano-fertilizers can effectively influence both vegetative and reproductive characteristics under supplemental irrigation in semi-arid region. It appears that $nTiO_2$ may play a significant role in activation of defense mechanism and modulating the biosynthesis of phytohormones such as cytokinins and gibberellin (Mandeh et al., 2012).

The correlations between different traits are presented in Table 4. Grain yield observed to be significantly and positively correlated with grain mass (TGW), number of grain per spike (NGS), straw yield (SY) at 99 % confidence level. However peduncle length showed a high negative correlation with yield components. Furthermore a significant correlation was recorded between the number of tiller (NTP), number of the grain per spike (NGS) and chlorophyll content (CHL) with straw yield. Grain yield (GY) also showed a positive correlation at 95 % confidence level with number of days from sowing to anthesis and chlorophyll content. Harvest index (HI) also revealed a very significant and positive association with stem diameter (SD), grain mass (TGW), spike length (SL) and grain yield (Table 4). This trend was confirmed by principle component analysis (PCA). The PCA described a suitable amount of the total variation. The correlation coefficient between any two traits is approximated by the cosine of the angle between their vectors. In the

Figure 3, the most prominent relations are: a very positive association among grain yield (GY), grain mass (TGW), harvest index (HI), stem diameter (SD) number of the fertile tiller (NFT), spike length (SL); among straw yield (SY), days to maturity (DM); among day to anthesis (DA), chlorophyll content (CHL), number of the grain per spike (NGS) as indicated by the small obtuse angles between their vectors (r=cos 0=+1). There was a negative correlation between peduncle length (PL) and plant height (PH) with most of grain yield components (Figure 3) as indicated by the near perpendicular vectors (r=cos180=-1).

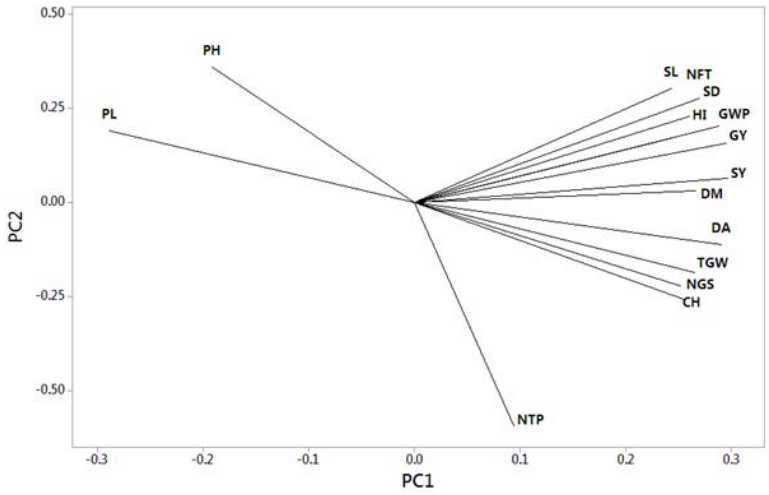


**Figure 3:** Plot of the first two PCAs showing relation among various agronomical traits of barley.

**Table 4:** Pearson's correlation coefficients among agronomical traits of barley

| | PH | SD | NTP | NFT | GWP | NGS | PL | SL | TGW | DA | DM | CH | GY | SY |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SD | -0.41 | | | | | | | | | | | | | |
| NTP | -0.01 | -0.11 | | | | | | | | | | | | |
| NFT | -0.43 | 0.46 | 0.60 | | | | | | | | | | | |
| GWP | -0.09 | 0.70 | 0.52 | 0.61 | | | | | | | | | | |
| NGS | -0.12 | 0.38 | 0.73 | 0.79 | 0.85 | | | | | | | | | |
| PL | 0.21 | -0.71 | -0.58 | -0.74 | -0.98 | -0.87 | | | | | | | | |
| SL | 0.27 | 0.68 | 0.03 | 0.07 | 0.79 | 0.41 | -0.68 | | | | | | | |
| TGW | -0.29 | 0.74 | 0.57 | 0.76 | 0.90 | 0.75 | -0.96 | 0.59 | | | | | | |
| DA | -0.25 | 0.22 | 0.77 | 0.77 | 0.71 | 0.96 | -0.75 | 0.19 | 0.62 | | | | | |
| DM | -0.26 | 0.70 | 0.09 | 0.47 | 0.77 | 0.72 | -0.73 | 0.63 | 0.55 | 0.65 | | | | |
| CH | -0.40 | 0.46 | 0.68 | 0.77 | 0.83 | 0.94 | -0.87 | 0.36 | 0.76 | 0.95 | 0.76 | | | |
| GY | -0.11 | 0.62 | 0.63 | 0.73 | 0.98 | 0.93 | -0.99 | 0.67 | 0.91 | 0.81 | 0.75 | 0.89 | | |
| SY | -0.06 | 0.34 | 0.80 | 0.73 | 0.87 | 0.98 | -0.89 | 0.46 | 0.77 | 0.94 | 0.66 | 0.93 | 0.94 | |
| HI | -0.20 | 0.94 | 0.13 | 0.52 | 0.85 | 0.54 | -0.86 | 0.81 | 0.87 | 0.34 | 0.68 | 0.55 | 0.89 | 0.53 |

Critical values of correlation $P < 0.05$ and $P < 0.01$ are 0.79 and 0.90, respectively. PH= plant height, SD=stem diameter (mm), NTP=number of tillers per plants, NFT=number of fertile tillers, GWP= grain mass per plant, NGS=number of grain per spike, PL=peduncle length, SL=spike length, TGW=1000 grain mass, DA= days to anthesis, DM= days to maturity, CHL= chlorophyll (SPAD unit), GY= grain yield, SY= straw yield, HI= harvest index.

In order to know with which combination type of agro-morphological traits the barley would attain high grain yield PCA was performed (Table 5). The Scree plot of the PCA (Figure 4) shows that the first four eigenvalues correspond to the whole percentage of the variance in the dataset. The first four main PCAs are extracted from the complicated components, the total cumulative variance of these five factors amounted to 98 % and these components had eigenvalues >1. The PCA simplifies the complex data by transforming the number of associated traits into a smaller number of variables as PCAs. The first principal component (PC1) is grain yield (GY) and yield components that explained 69 % of total variability. The traits, which contributed more positively to PC1, were grain mass (TGW), days to anthesis (DA), days to maturity (DM), stem diameter (SD), number of fertile tiller (NFT) and straw yield (Table 5). The second principal component (PC2) explains 14 % of total variability and among the property vectors of PC2 vegetative growth parameter like as plant height (PH), spike length (SL) and number of the fertile tiller (NFT) had higher values. The third principal component (PC3) is plant vegetative characters that explain about 11 % of total variability. Among the property vectors of PC3 plant height (PH), number of the grain per spike (NGS) and number of tiller per plant (NTP) had higher values. The forth principal component (PC4) was related to green area duration (chlorophyll content and days to maturity) and explains about 5 % of total variability (Table 5).

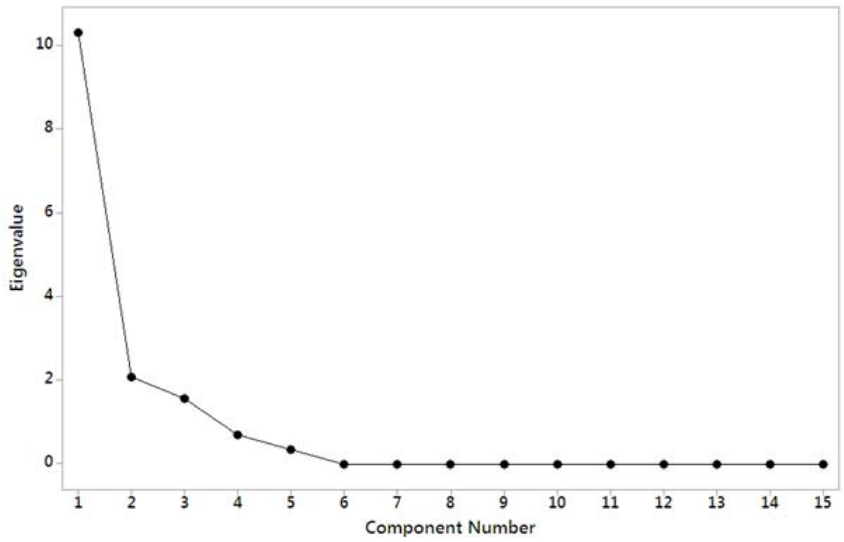


**Figure 4:** Scree plot showing eigenvalues in response to number of components for the estimated variables of barley.

**Table 5:** Loadings of PCA for the estimated traits of barley.

| Variable | PC1 | PC2 | PC3 | PC4 |
|---|---|---|---|---|
| PH | -0.19 | 0.36 | 0.42 | 0.20 |
| SD | 0.26 | 0.23 | -0.17 | -0.42 |
| NTP | 0.10 | -0.59 | 0.33 | -0.04 |
| NFT | 0.27 | 0.28 | -0.18 | 0.17 |
| GWP | 0.29 | 0.20 | -0.03 | -0.07 |
| NGS | 0.25 | -0.22 | 0.32 | 0.35 |
| PL | -0.29 | 0.19 | -0.14 | 0.15 |
| SL | 0.24 | 0.30 | 0.34 | 0.12 |
| TGW | 0.27 | -0.19 | 0.14 | -0.48 |
| DA | 0.29 | -0.11 | -0.21 | 0.07 |
| DM | 0.27 | 0.03 | -0.21 | 0.44 |
| CHL | 0.26 | -0.26 | -0.23 | 0.37 |
| GY | 0.30 | 0.16 | 0.15 | -0.09 |
| SY | 0.30 | 0.07 | -0.22 | -0.10 |
| HI | 0.25 | 0.17 | 0.43 | 0.00 |
| Eigenvalue | 10.31 | 2.09 | 1.57 | 0.69 |
| Proportion | 0.69 | 0.14 | 0.11 | 0.05 |
| Cumulative | 0.69 | 0.83 | 0.93 | 0.98 |

PH= plant height, SD=stem diameter (mm), NTP=number of tillers per plants, NFT=number of fertile tillers, GWP= grain mass per plant, NGS=number of grain per spike, PL=peduncle length, SL=spike length, TGW=1000 grain mass, DA= days to anthesis, DM= days to maturity, CHL= chlorophyll (SPAD unit), GY= grain yield, SY= straw yield, HI= harvest index.

## 4 CONCLUSION

Nanotechnology is the developing technology during recent years and operating in all fields of agriculture. Nano micronutrients fertilizers stand out as one of the most useful materials, due to their high efficiency, functionalities, convenient and easy applications. Although the effects of nano-titanium and nano-chelated micronutrients have been evaluated separately in some studies, here, their combined effects were evaluated on barley under semi-arid region with terminal drought stress. From the present study, it can be concluded that TiO2 nano particles and micro nutrients nano-fertilizers at investigated concentrations does not exhibit any significant phytotoxicity and could increases the chlorophyll content, vegetative growth and yield component of barley under supplemental irrigation condition in semi-arid regions with Mediterranean climates. Contrary to expectations, this study did not find a significant effects of $nTiO_2$ on yield component. This may be due to utilized concentration, time or frequency of application. Additionally, the findings suggest that the foliar application of nano-chelated micronutrients can be resulted in sustainable and high crop production. It can be ascribed to structure and small size of nano-fertilizers which are insoluble in water and the particles are rapidly adsorbed by plant tissue. Rapid delivery of the required elements in different subcellular parts can enhance application efficiency. Foliar application of micro nutrients in semi-arid region can solve the immobilization of element in soil. However, actual movement of nano-particles through the cuticle depends on the nutrient concentration, molecular size, chelating structure, time of application and plant species and environmental condition. Given that nanotechnology industry is growing in a very fast way, there is a crucial urgency to perform further studies about instructions for application of nano-fertilizers, consumption rates, synergistic, antagonistic or neutral interactions and its consequences on the cellular and molecular level.

## 5 ACKNOWLEDGMENTS

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**Agris category code**: F01, Q04

# Physicochemical properties and antioxidant activities of five Iranian pomegranate cultivars (*Punica granatum* L.) in maturation stage

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**ABSTRACT**

The aim of this study was to compare the physicochemical properties and antioxidant activity of five different pomegranate cultivars. Fruit mass ranged from 109.27 to 78.07 g. Peel thickness of the fruit was recorded from 5.05 to 2.70 mm. The pH, total soluble solids content, the titratable acidity content were within the range of 4.23 to 4.36, 20.00 (◦Brix) to 14.05 (◦Brix), 0.04 to 0.007 mg per 100 g of juice, respectively. Ascorbic acid content was from 4.73 to 1.98 mg per 100 g of juice. The amount of total phenolics in pomegranate cultivars was between 6.36 and 1.78 mg GAE/100 ml. The total flavonoids content also ranged between 4.93 to 2.24 mg GAE/100 ml. The level of antioxidant activity was varied from 86.77 % to 79.54 %. Reducing sugar content ranged between 5.81 to 1.72 mg/100g. Glucose content was found from 3.48 to 1.14 mg/100g. In total based on these results, the cultivar is the main parameter which influences the physic-chemical properties and antioxidant activity in pomegranates.

**Key words:** ascorbic acid; maturity index; phenolic compounds; physicochemical properties; pomegranate

**IZVLEČEK**

**FIZIKALNO-KEMIJSKE LASTNOSTI IN ANTIOKSIDACIJSKA AKTIVNOST PETIH IRANSKIH SORT GRANATNEGA JABOLKA (*Punica granatum* L.) V ČASU ZRELOSTI**

Namen raziskave je bil primerjati fizikalno-kemijske lastnosti in antioksidacijsko aktivnost petih sort granatnega jabolka. Masa plodov je bila med 109.27 in 78.07 g. Debelina olupka je znašala od 5.05 do 2.70 mm. pH, celokupna vsebnost topnih snovi in vsebnost titrabilne kislosti so bili v območju 4.23 do 4.36, 20.00 (◦Brix) do 14.05 (◦Brix), 0.04 do 0.007 mg na 100 g soka. Vsebnost askorbinske kisline je bila med 4.73 in 1.98 mg na 100 g soka. Vsebnost celokupnih fenolov je bila med 6.36 in 1.78 mg GAE/100 ml, vsebnost celokupnih flavonoidov pa med 4.93 in 2.24 mg GAE/100 ml. Antioksidacijska aktivnost je variirala med 86.77 % in 79.54 %. Vsebnost reducirajočih sladkorjev je bila med 5.81 in 1.72 mg/100g, vsebnost glukoze pa med 3.48 in 1.14 mg/100g. Iz rezultatov sledi, da je sorta najpomembnejši dejavnik, ki vpliva na fizikalno-kemijske lastnosti in antioksidacijsko aktivnost granatnega jabolka.

**Ključne besede:** askorbinska kislina; indeks zrelosti; fenolne spojine fizikalno.kemijske lastnosti; granatno jabolko

## 1 INTRODUCTION

Pomegranate (*Punica granatum* L.) is an important commercial fruit that is cultivated in many tropical and subtropical climates including Asia, North Africa, the Mediterranean and the Middle East (Khoshnam et al., 2007; Sarkhosh et al., 2006). Iran is one of the most important pomegranate producer and exporter in the world (Anonymous, 2005; Tehranifar & Mahmoodi-Tabar, 2009). Pomegranate cultivation has a long tradition in Iran. It is considered one of the most important fresh fruits in this country (Barone et al., 2001). It is now widely grown in many tropical and

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subtropical regions in Iran. The total production of pomegranate in Iran was 670,000 tons in 2005 (Anonymous, 2005).

Different parts of pomegranate trees and the fruit have been used directly for their medicinal properties and for other purposes such as juice, jams, syrup and sauce (Al-Maiman & Ahamad, 2002; Rania et al., 2008). Considerable amounts of acids, sugars, vitamins, polysaccharides, polyphenols, important minerals and high antioxidant activity extracts have been obtained ?? from different part of pomegranate fruit such as peel, juice and seeds (Ercisli et al., 2007; Gil et al., 2000; Ozgen et al., 2008; Singh et al., 2002).

Olaniyi et al., (2010) evaluated the physicochemical and antioxidant properties of three cultivars of pomegranate grown in the South Africa. The results showed that there is a significant difference in physicochemical properties and antioxidant activity among cultivars. The compounds of pomegranate fruit are highly dependent on the cultivar type, growing region, climate, maturity and cultural practice (Melgarejo et al., 2000; Olaniyi et al., 2010; Ozgan et al., 2008; Ozken et al., 2002).

Various reports have shown that cultivar influences the antioxidant activity and other physicochemical properties, such as peel and juice percentage, dry matter, pH, total soluble solids (TSS), total sugars, titratable acidity (TA), total phenolics, anthocyanins, organic acids and water-soluble vitamins composition (Al-Maiman & Ahmad, 2002; Al-Said et al., 2009; Kulkarni & Aradhya, 2005; Mousavinejad et al., 2009; Melgarejo & Artes, 2000; Tezcan et al., 2009).

Martinez et al., (2006) studied five varieties of pomegranate from Southeast Spain. They measured the morphological and chemical characteristics such as pH, TSS, maturity and harvest index. They showed that there was significant difference among the cultivars and that cultivar plays an important role in determining physicochemical properties.

This research is focused on the variation among pomegranate fruits of six cultivars from the perspective of morphological and physiochemical properties. The aim of the present study was to determine and compare the variability in the juice and peel physicochemical characteristics and antioxidant activity and other physical and chemical properties.

## 2 MATERIALS AND METHODS

### 2.1 Plant material

Pomegranate fruit harvest criteria was based on local and commercial harvest time. The fruits were harvested at commercially ripe stage in september 2012 from five main cultivars such as ‘Rubbab’, ‘Ghand’, ‘Shishe-Kap’, ‘Shahvar’, ‘Shalghami ’ from different mature trees (10 to 12-year-old) randomly selected in Ferdows, Khorasan Jonoubi, Iran (between 39-32 degrees up to 42-43 degrees north latitude and 5-75 up to 55-58 degrees east longitude is located). The city due to its geographical location and the proximity to the desert has tropical climate. The average rainfall in this city is about 155 mm per year. General characteristics of the cultivars are as follows: ‘Rubbab’: sour-sweet, red and thickened skin; seed color is red, average fruit mass: 95.012 g. ‘Ghand’: sweet, white and thickened skin, seed color is white, average fruit mass: 70.08 g. ‘Shishe-Kap’: sour-sweet, red and relatively thin skin, seed color is pink, average fruit mass: 109.027 g. ‘Shahvar’: sweet, red and thin skin, seed color is candy, average fruit mass: 80.94 g. ‘Shalghami’: sour, red and relatively thin skin, seed color is red, average fruit mass: 78.42 g. After harvest, fruits were quickly transported in cold bag to the research laboratory at Gorgan university of agricultural sciences and natural resources. The ripe fresh fruits were from different mature trees randomly (completely randomized design of four trees per variety in a sample of twelve fruits per replications) selected to represent the population of the plantation.

### 2.2 Morphological Properties

Harvested fruits were sorted for size and uniformity of shape and mass. All fruits were first flushed by tap water before the peel, pulp and seed

fractions were carefully separated. The peel and pulp were separated manually after measurement of fruit fresh mass, volume and fruit density. Fruits were weighted in the air on a balance of accuracy of 0.001 g. Fruit volume was calculated by a liquid displacement method. The mass density of the fruit was obtained by the ratio of mass to volume. The length and diameter of the fruit and arils, length and diameter of calyx were measured with a digital vernier calliper. The measurement of fruit length was made on the polar axis, i.e. between the apex and the end of stem. The maximum width of the fruit, as measured in the direction perpendicular to the polar axis, is defined as the diameter. Then peel thickness was measured by a digital calliper with 0.01 mm accuracy and oven-dried (105 ° C) to constant mass to calculate its moisture content. Aril, juice and seed mass were measured as above. Then the arils and juices were analyzed for major chemical compositions and antioxidant activity.

### 2.3 Chemical Analysis

The total soluble solids (TSS) in the juice (°Brix) were determined with a digital refractrometer (060279, Ceit, Belgium), at 20 °C, calibrated using distilled water. Titrable acidity was estimated by juice titration with 0.1 N NaOH to the titration end point of pH 8.3, monitored with a pH meter (Labtron) and expressed as citric acid per 100 g of juice. The pH measurements were performed using a digital pH meter (Labtron, Iran) at 21 °C, calibrated using distilled water). For electrical conductivity (EC, dSm 1) determination, the sample was measured with a conductivimeter (ABB-100).

Ascorbic acid was determined by employing the method described by Kashyap & Gautam (2012). Results were expressed as mg per 100 g of juice.

Reducing sugars were measured according to the method of Maccready et al. (2000). Glucose and fructose were determined by Miller (1959) and Ashwell (1957), respectively and expressed as mg sugar 100 g of juice.

Total phenolics were measured calorimetrically at 760 nm by using the Folin-Ciocalteu reagent (Singleton & Rossi, 1965). Gallic acid was used as a standard. The results were expressed as mg Gallic acid equivalent in 100 g of fruit juice (mg GAE/100 g of juice).

The total flavonoids content was measured by using Yang method (Yang et al., 2009). 250 µl of pomegranate juice sample was mixed with 5 % sodium nitrite solution (75 µl), then the mixture was mixed with aluminium chloride (10 %, 150 µl), sodium hydroxide (1 M, 500 µl) and distilled water (775 µl). The absorbance of the mixture was measured spectrophotometrically at 510 nm. Total flavonoid content was expressed as mg per 100 g of juice.

Antioxidant activity was assessed according to the method of Sun & Ho. (2005). 30 µl of pomegranate juice was mixed with 2 ml of 0.1 mM DPPH in methanol and the mixtures was 1 ml. After 15 minutes, absorbance of the resulting solution was measured at 515 nm by a spectrophotometer. The antioxidant activity was calculated using the following equation: antioxidant activity (%) = [1− (sample 515 nm/control 515 nm)] × 100.

### 2.4 Statistical Analysis

Data were analyzed by Statistical Analysis System (SAS) software and mean comparison was done using Least Significant Difference (LSD) test in level 5 %.

## 3 RESULTS AND DISCUSSION

### 3.1 Physical properties

A considerable variation was observed in some of the physical-chemical and antioxidant properties of studied pomegranate cultivars. The physical characteristics of five pomegranate cultivars analyzed are described in Tables 1. Significant difference were detected in length, length/diameter, juice volume, calyx length, peel thickness, length of aril, seed diameter and moisture ($P < 0.01$), mass and peel moisture ($P < 0.05$), while there were not showed significant differences in traits of diameter, width, volume, density and calyx diameter of fruits.

The maximum fruit mass of pomegranate cultivars was in 'Shishe-Kap' (109.27 g) and the minimum fruit mass were found in 'Ghand', 'Shalghami', 'Shahvar' (78.07, 78.42, 80.94 g, respectably) (Table 2). Main comparison of the results (Table 2) indicated that the highest of length/diameter ratio was in 'Shishe-Kap' (1.29 mm) and the lowest of length /diameter ratio were in 'Shahvar' (0.91 mm), 'Ghand' (0.89 mm) and 'Shalghami' (0.86 mm). The highest fruit length (109.27 mm) and the lowest fruit length (78.42 mm) were recorded in 'Shishe-Kap' and 'Shalghami', respectably (Table 2).

'Shalghami' and 'Ghand' fruits had the maximum (17.99 mm) and minimum (9.62 mm) calyx length, respectively and showed a significant difference to other studied cultivars (Table 2).

'Ghand' had the highest aril length (11.98 mm) and aril diameter (8.38 mm) while 'Rubab' and 'Shalghami' had the lowest aril length (10.61 mm and 10.70 mm respectively), The minimum of aril diameter was observed in 'Shahvar' (6.93 mm) (Table 2). The fruit peel thickness varied from 5.05 ('Ghand' and 'Rubab') to 2.70 mm ('Shahvar') (Table 2). The highest peel moisture was in 'Shalghami' (58.88 %), 'Shishe-Kap' (57.51 %) and 'Shahvar' (56.89 %). There were no significant differences among these three cultivars in terms of peel moisture. The lowest peel moisture was found in 'Ghand' (51.15 %) (Table 2). 'Ghand' and 'Rubab' fruits had the maximum (68.37 %) and minimum (56.55 %) seed moisture, respectively. In several studies, a wide variation was showed that the fruit mass, fruit length, fruit diameter, calyx length and calyx diameter of pomegranate fruits grown in Iran are between 169.89 g - 315.28 g; 69.49 mm - 81.46 mm; 64.98 mm - 86.88 mm; 13.45 mm - 24 mm; 12.52 mm - 23.96 mm respectively (Thehranifar et al., 2010; Sarkhosh et al., 2006). Our results in general were close to these studies and showed these differences among cultivars. These differences may be related to the design or selection of appropriate packaging for fruit handling and storage (Valero & Ruiz-Altisent, 2000). According to reports, the existence of significant differences in morphology is relevant to development of the fruit (Zarei et al., 2011). Shulman et al. (1984) reported that these differences of fruit could be attributed to the cultivars type and ecological condition. The juice percentage ranged between 30.21 % ('Shahvar') and 16.46 % ('Rubab') (Table 2). Tehranifar et al. (2010) stated the juice percentage varied from 26.95 % to 46.55 %. The juice percentage is one of the most important parameters from an industrial point (Tehranifar et al. 2010).

**Table 1:** Analysis of variance of fruit mass (FM), fruit length (FL), fruit diameter (FD), fruit length/diameter (F l/d), fruit width ($FW_i$), fruit volume (FV), fruit densities (FDs), juice volume (JV), calyx length (CL), calyx diameter (CD), peel moisture (PM), peel thickness (PT), aril length (AL), aril diameter (AD), seed moisture (SM) of the studied Iranian pomegranate cultivars

| Source of variation | df | MS | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | FM (g) | FL (mm) | FD (mm) | F l/d | $FW_i$ (mm) | FV ($cm^3$) | $FD_S$ (g/cm3) | JV (%) | CL (mm) | CD (mm) | PM (%) | PT (mm) | AL (mm) | AD (mm) | SM (%) |
| Cultivars | 4 | $52.32^{*}$ | $12.23^{**}$ | $60.73^{ns}$ | $0.20^{**}$ | $36.300^{ns}$ | $4500.68^{ns}$ | $0.062^{ns}$ | $231.44^{**}$ | $53.89^{**}$ | $178.28^{ns}$ | $60.20^{**}$ | $5.22^{**}$ | $2.094^{**}$ | $3.086^{**}$ | $41.03^{**}$ |
| Error | 28 | 6293.19 | 68.17 | 55.71 | 0.0029 | 64.31 | 5109.63 | 0.048 | 16.14 | 12.37 | 157.084 | 16.54 | 0.557 | 0.29 | 0.237 | 9.90 |
| CV % | | 22.5 | 9.31 | 8.35 | 5.40 | 9.33 | 20.12 | 21.92 | 18.66 | 28.53 | 63.73 | 7.31 | 19.90 | 4.89 | 6.18 | 5.19 |

Note: (ns) No significant differences, (** & *): significant difference at 1 % and 5 % level, respectively

**Table 2:** fruit mass (FM), fruit length (FL), fruit diameter (FD), fruit length/diameter (F l/d), fruit width (FWi), fruit volume (FV), fruit densities (FDs), juice volume (JV), calyx length (CL), calyx diameter (CD), peel moisture (PM), peel thickness (PT), aril length (AL), aril diameter (AD), seed moisture (SM) of five Iranian pomegranate cultivars

| Cultivars | FM (g) | FL (mm) | FD (mm) | F l/d | $FW_i$ (mm) | FV ($cm^3$) | $FD_S$ ($g/cm^3$) | JV (%) | CL (mm) | CD (mm) | PM (%) | PT (mm) | AL (mm) | AD (mm) | SM (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $P<0/0001$ | $P<0/0001$ | $P<0/0001$ | $P<0/0001$ | $P<0/0001$ | $P<0/0001$ | $P<0/0001$ | $P<0/0001$ | $P<0/0001$ | $P<0/0001$ | $P<0/0001$ | $P<0/0001$ | $P<0/0001$ | $P<0/0001$ | $P<0/0001$ |
| 'Ghand' | $78.07^{c}$ | $97.68^{b}$ | $90.46^{a}$ | $0.89^{c}$ | $85.35^{a}$ | $333.25^{a}$ | $0.233^{a}$ | $20.58b^{c}$ | $9.62^{c}$ | $9.62^{a}$ | $51.15^{b}$ | $5.05^{a}$ | $11.98^{a}$ | $8.38^{a}$ | $68.37^{a}$ |
| 'Shahvar' | $80.94^{c}$ | $98.94^{b}$ | $89.50^{a}$ | $0.91^{c}$ | $84.55^{a}$ | $336.13^{a}$ | $0.240^{a}$ | $30.21^{a}$ | $12.83^{b}$ | $10.83^{a}$ | $56.89^{a}$ | $2.70^{c}$ | $11.09^{b}$ | $6.93^{b}$ | $61.58^{b}$ |
| 'Shishe-Kap' | $109.027^{a}$ | $109.27^{a}$ | $88.19^{a}$ | $1.29^{a}$ | $83.65^{a}$ | $330.00^{a}$ | $0.302^{a}$ | $16.79^{c}$ | $12.76^{b}$ | $10.76^{a}$ | $57.51^{a}$ | $3.60^{b}$ | $11.06^{b}$ | $8.35^{a}$ | $61.12^{b}$ |
| 'Rubab' | $95.012^{b}$ | $98.006^{b}$ | $91.14^{a}$ | $1.065^{b}$ | $85.63^{a}$ | $331.28^{a}$ | $0.262^{a}$ | $16.46^{c}$ | $13.50^{b}$ | $9.50^{a}$ | $55.66^{ab}$ | $5.05^{a}$ | $10.61^{c}$ | $8.24^{a}$ | $56.55^{c}$ |
| 'Shalghami' | $78.42^{c}$ | $78.42^{c}$ | $90.69^{a}$ | $0.86^{c}$ | $84.29^{a}$ | $335.00^{a}$ | $0.234^{a}$ | $21.95^{b}$ | $17.99^{a}$ | $10.99^{a}$ | $58.88^{a}$ | $3.89^{b}$ | $10.70^{c}$ | $7.39^{b}$ | $59.80^{cb}$ |

Note: The dissimilar letters in each column indicate significant differences between them

### 3.2 Chemical properties

The chemical characteristics of five pomegranate cultivars analyzed are described in Tables 3. Chemical properties of studied pomegranate fruits showed significant differences in all parameters ($P < 0.01$) except the fructose. The highest of pH were in 'Ghand' (4.36) and 'Shishe-Kap' (4.23). The lowest of pH were in 'Rubab' (3.78) and 'Shalghami' (3.78) (Table 4). This parameter defines the acidic taste of pomegranate juice (Zarei et al., 2011). The pH values observed in the present study are higher than values that reported by Cam et al. (2009) on pomegranate cultivars (from 2.82 to 0.81). The results of our study are indicative of the lower levels of $H^+$.

The maximum of electrical conductivity (Ec) was found in 'Shalghami' (48.2 S/m) and the minimum was in 'Ghand' (32.3 S/m) (Table 4). Akbarpour et al. (2009) reported that EC value of some pomegranate cultivars in Iran was between 3.41 and 5.11 23 mmohs/cm. The total soluble solid values ranged between 20.00 °Brix ('Rubab') to 14.05 °Brix ('Shahvar') (Table 4). Our results were in agreement with values (15.17–22.03 °Brix) reported by Akbarpour et al. (2009), while our values were higher than values observed (11.37–15.07 °Brix) by Tehranifar et al. (2010). The titrable acidity content varied from 0.040 mg per 100 g of juice ('Shahvar') to 0.007 mg per 100 g of juice ('Ghand') (Table 4). Citric acid is the predominant acid in pomegranate (Varidi, 1992). Kulkarni & Aradhia (2005) stated that acidity decreases at the time of maturation and is associated with increasing in the sugar content. The highest content of ascorbic acid was in 'Shalghami' (4.73) and the lowest in 'Rubab' (1.98 mg per 100 g of juice), 'Ghand' (2.26 mg per 100 g of juice) and 'Shishe-Kap' (2.09 mg per 100 g of juice) (Table 4). According to our results, the cultivars have a very important role in the amount of total soluble solids, pH and titratable acidity and vitamin C (Tehranifar et al., 2010).

According to Table 2, there are significant differences among the studied cultivars. Some researchers used maturity index for classifying of pomegranate cultivars (Cam et al., 2009; Martinez et al., 2006; Melgarejo et al., 2000, Tehranifar et al., 2010). Martinez et al. (2006) stated that the maturity index in some Spain varieties of pomegranate were in the range of 25/16 to 94/56. Other researchers have noted wide ranges for this parameter. For example, Sharman & Bist (2005) reported that MI value was 95.16. Following classification was proposed by Martinez et al. (2006) for the values of maturity index in Spanish cultivars: maturity index (MI) = 5 - 7 for sour, MI = 17 - 24 for sour - sweet and MI = 31 - 98 for sweet cultivars. Based on these results, all our studied cultivars can be classified as sweet, because their maturity index range were between 98 to 31. The ratio of sugar to acid is a determining factor in fruit flavors. Quality of the cultivars depends in this factor. Cam et al. (2009) and Martinez et al. (2006) stated the maturity index (TSS/TA) is responsible for the taste and flavor of pomegranate.

**Table 3:** Analysis of variance of pH, electrical conductivity (EC), total soluble solid (TSS), titrable acidity (TA), maturity index (MI), ascorbic acid (A), total phenolic(TPh), total flavonoids (TFl), antioxidant activity (AA), reducing sugar(RS), glucose(Gl), fructose (Fr) of the studied Iranian pomegranate cultivars.

| Source of variation | df | MS | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | pH | EC (S/m) | TSS (°B) | TA (mg/100 gr) | MI | A (mg/100 ml) | TPh (mg GAE/ 100 ml) | TFl (mg GAE/ 100 ml) | AA (%) | RS (mg/100g) | Gl (mg/100g) | Fr (mg/100g) |
| Cultivars | 4 | 0.43$^{**}$ | 2.43$^{**}$ | 33.72$^{**}$ | 0.0010$^{**}$ | 20.07$^{**}$ | 6.64$^{**}$ | 23.76$^{**}$ | 10.47$^{**}$ | 58.062$^{**}$ | 22.089$^{**}$ | 8.63$^{**}$ | 0.8$^{**}$ |
| Error | 28 | 0.017 | 0.101 | 3.72 | 0.009 | 1.29 | 1.66 | 3.71 | 34.29 | 0.93 | 0.93 | 0.423 | 0.582 |
| CV % | | 3.207 | 7.97 | 11.60 | 56.21 | 13.62 | 15.66 | 35.47 | 50.08 | 7.035 | 28.74 | 27.12 | 88.25 |

Note: (ns) No significant differences, (** & *): significant difference at 1 % and 5 % level, respectively

**Table 4:** pH, electrical conductivity (EC), total soluble solid (TSS), titrable acidity (TA), maturity index (MI), scorbic acid (A), total phenolic(TPh), total flavonoids (TFl), antioxidant activity (AA), reducing sugar(RS) glucose(Gl) fructose (Fr) of five Iranian pomegranate cultivars

| Cultivars | pH | EC (S/m) | TSS (°B) | TA (mg/100 gr) | MI | A (mg/100 ml) | TPh (mg GAE/ 100ml) | TFl (mg GAE/ 100 ml) | AA (%) | RS (mg/100 g) | Gl (mg/100 g) | Fr (mg/100 g) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *P*<0/0001 | *P*<0/0001 | *P*<0/0001 | *P*<0/0001 | *P*<0/0001 | *P*<0/0001 | *P*<0/0001 | *P*<0/0001 | *P*<0/0001 | *P*<0/0001 | *P*<0/0001 | *P*<0/0001 |
| 'Ghand' | 4.36$^{a}$ | 32.3$^{c}$ | 16.27b$^{c}$ | 0.0078$^{c}$ | 100.59$^{a}$ | 2.26$^{c}$ | 1.78$^{c}$ | 3.30$^{ab}$ | 83.43$^{bc}$ | 5.81$^{a}$ | 3.48$^{a}$ | 1.11$^{a}$ |
| 'Shahvar' | 4.03$^{b}$ | 44.3$^{b}$ | 14.05$^{b}$ | 0.040$^{a}$ | 73.11$^{d}$ | 3.30$^{b}$ | 2.41$^{c}$ | 2.24$^{b}$ | 79.54$^{c}$ | 3.27$^{b}$ | 3.29$^{a}$ | 0.67$^{a}$ |
| 'Shishe-Kap' | 4.23$^{a}$ | 43.6$^{b}$ | 16.38$^{bc}$ | 0.018$^{bc}$ | 80.44$^{c}$ | 2.09$^{c}$ | 4.34$^{b}$ | 5.14$^{a}$ | 8131$^{bc}$ | 1.72$^{c}$ | 1.14$^{c}$ | 0.68$^{a}$ |
| 'Rubab' | 3.78$^{c}$ | 43.5$^{b}$ | 20.00$^{a}$ | 0.028$^{ab}$ | 81.4$^{c}$ | 1.98$^{c}$ | 6.36$^{a}$ | 4.93$^{a}$ | 86.77$^{a}$ | 1.83$^{c}$ | 1.25$^{c}$ | 1.28$^{a}$ |
| 'Shalghami' | 3.78$^{c}$ | 48.2$^{a}$ | 17.07$^{b}$ | 0.027$^{ab}$ | 89.98$^{b}$ | 4.73$^{a}$ | 3.99$^{b}$ | 4.30$^{ab}$ | 80.12$^{ab}$ | 4.80$^{a}$ | 2.30$^{b}$ | 0.86$^{a}$ |

Note: The dissimilar letters in each column indicate significant differences between them.

Reducing sugar content was the highest in 'Ghand' (5.81 mg/100 g) and the lowest in 'Shishe-Kap' (1.72 mg/100 g) and 'Rubab' (1.83 mg/100 g). 'Ghand' had the maximum of glucose (3.48 mg/100 g) and the lowest content of glucose were found in 'Shishe-Kap' (1.14 mg/100 g) and 'Rubab' (1.25 mg/100 g) (Table 4). Glucose is the predominant sugar in pomegranate and amount of glucose is more than of fructose in this fruit (Melgarejo, 2000). The results of our research were lower than value (5.7 - 7.6 %) reported by Gadze et al. (2012). These results showed that the levels of reducing sugar, glucose and other physicochemical properties were different among various cultivars of pomegranate that could be due to existence of high genetic heterogeneity within the cultivars (Tehranifar et al., 2010).

'Rubab' had the highest content of total phenolics (6.36 mg GAE/100 ml) and total flavonoids (4.93 mg GAE/100 ml). The lowest content of total phenolics was found in 'Ghand' (1.78 mg GAE/100 ml) and 'Shahvar' (2.41 mg GAE/100 ml). 'Shahvar' had the minimum of total flavonoids (2.24 mg GAE/100 ml) (Table 4). Tehranifar et al. (2010) found a significant difference in total phenolics concentration among the twenty varieties of pomegranate (295.79 to 985.32 mg GAE 100 g−1). The highest and the lowest antioxidant activity were detected in 'Rubab' (86.77 %) and 'Shahvar' (79.54 %) (Table 4). The reported levels of antioxidant activity in other researches were 10.37–67.46 % for seven cultivars of pomegranate juices in Turkey (Tezcan et al., 2009) and 18.6–42.8 % for eight pomegranate juices from Iran (Mousavinejad et al., 2009).

The differences in the genetic variability led to the variation in the biosynthesis of phenolic compounds among cultivars. There is a close correlation between total phenolic content and antioxidant activity (Tehranifar et al., 2010).

## 4 CONCLUSIONS

This study showed significant differences in some physical and chemical properties of five pomegranate cultivars grown in Iran. Among the five cultivars studied, the highest values of morphophysiological characteristics were observed in the 'Shishe-Kap' and 'Rubab'. The highest content of total phenolics and total flavonoids and the highest antioxidant activity was found in 'Rubab' cultivar. 'Ghand' had the maximum of reducing sugar level and glucose. Thus could be concluded, 'Shishe-Kap', 'Rubab' and 'Ghand' are appropriate for fresh consumption and health benefits. Provided information on the physicochemical properties of pomegranate cultivars can be useful for developing fruit processing industry and selection of superior desirable pomegranate genotypes for commercial cultivation. This research provides important information about physicochemical properties in some pomegranate cultivars grown in Iran. Since Iran has a high genetic variation, however, more studies of physical and chemical properties of pomegranate are required.

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**Agrovoc descriptors**: *Artemisia,* drought, drought stress, drought resistance, salicylic acid, plant growth substances, in vitro experimentation, plant physiology, growth, phenylalanine ammonia-lyase

**Agris category code**: F60, F62

# The impact of salicylic acid on some physiological responses of *Artemisia aucheri* Boiss. under *in vitro* drought stress

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**ABSTRACT**

Salicylic acid (SA) is an important plant regulator which is involved in growth, development, and response to stress. This study was aimed to evaluate some physiological and biochemical responses of *Artemisia aucheri* Boiss. under drought stress after exogenous SA treatment. Experiment was performed *in vitro*. Polyethylene glycol (PEG/6000) with 0, 2 and 4 % (w/v) was used in MS medium to simulate drought stress and different concentrations of SA (0, 0.01 and 0.1mM) were added. After four weeks, SA alleviated the negative effects of PEG on dry and fresh mass as well as chlorophyll and carotenoid contents. Under drought stress, application of SA decreased storage polysaccharides and increased soluble carbohydrates respectively. Although PEG had no significant effect on flavonoid content, it increased significantly anthocyanin and total phenol content, total antioxidant capacity, PAL (phenylalanine ammonia-lyase) and TAL (tyrosine ammonia-lyase) activity and SA treatment improved these parameters significantly. According to the current data, it was concluded that SA increased drought tolerance of *Artemisia aucheri* by increasing biosynthesis of phenolic compounds, improvement of TAL and PAL activity as well as also by increased content of soluble carbohydrates.

**Key words:** *Artemisia aucheri* Boiss., salicylic acid, drought stress; polyethylene glycol, growth, phenylalanine ammonia-lyase, tyrosine ammonia-lyase

**IZVLEČEK**

**VPLIV SALICILNE KISLINE NA NEKATERE FIZIOLOŠKE ODZIVE VRSTE PELINA (*Artemisia aucheri* Boiss.) NA SUŠNI STRES V IN VITRO RAZMERAH**

Salicilna kislina je pomemben rastlinski hormon, ki je vključen v uravnavanje rasti, razvoja in odziva na stres. Cilj raziskave je bil ovrednotiti nekatere fiziološke in biokemične odzive vrste pelina *Artemisia aucheri* Boiss. v sušnem stresu po zunanjem dodajanju salicilne kisline. Poskus je potekal *in vitro,* v MS gojišču (Murashige and Skoog., 1962) z dodatkom polietilen glikola (PEG/6000, 0, 2 in 4 % (w/v)) za simulacijo sušnega stresa in dvema različnima koncentracijama salicilne kisline (SA) (0, 0.01 in 0.1 mM). Po štirih tednih je salicilna kislina zmanjšala negativne učinke polietilen glikola na suho in svežo maso kot tudi na vsebnosti klorofila in karotenoidov. V sušnem stresu je uporaba salicilne kisline zmanjšala vsebnost založnih polisaharidov in povečala vsebnost topnih ogljikovih hidratov. Čeprav polietilen glikol ni imel značilnega učinka na vsebnostn flavonoidov je značilno povečal vsebnost antocianinov, celokupnih fenolov, celokupno antioksidacijsko sposobnost, aktivnost PAL (fenilalanin amonium-liaza) in TAL (tirozin amonium-liaza), obravnavanje s salicilno kislino je te parametre značilno izboljšalo. Glede na te rezultate je bilo zaključeno, da salicilna kislina povečuje strpnost na sušo pri vrsti *Artemisia aucheri* s povečanjem biosinteze fenolnih snovi, z izboljšanjem aktivnosti TAL in PAL kot tudi s povečanjem vsebnosti topnih ogljikovih hidratov.

**Ključne besede:** *Artemisia aucheri* Boiss., salicilna kislina, sušni stres; polietilen glikol, rast, fenilalanin amonium-liaza, tirozin amoniium-liaza

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## 1 INTRODUCTION

The genus *Artemisia* belongs to Asteraceae family. There are 500 species of *Artemisia* in Asia, Europe and North America. Thirty four species of this family are known as wild spicies all over Iran. One of these species is *Artemisia aucheri* Boiss which has limited ecological distribution, it is endemic to mountainous areas of Iran and surroundings (Mozaffarian et al., 2010). This plant has many medicinal properties. In traditional medicine it is used as astringent and disinfectant and has an antileishmanial, antiparasitic, and antioxidant activities (Asghari et al., 2012). Verbenone, camphor, 1, 8-cineole, trans-verbenol, chrysanthenone, mesitylene, α-pinene, acyclic monoterpenes, and monoterpene hydroperoxides are bioactive compounds extracted from this plant (Rustaiyan et al., 1987).

Drought stress is one of the major environmental factors limiting plant growth and productivity (Nazar et al., 2015). Drought stress induces the massive generation of reactive oxygen species (ROS). The accumulation of ROS inhibits normal function of lipids, proteins and DNA and finally reduces plant growth and development (Asada, 1999). Plants expose to drought display several morphological, physiological and molecular responses (Jiménez et al., 2013).

Salicylic acid (SA) is a phenolic compound that acts as important phytohormone. Several studies have demonstrated that SA participates in many physiological processes such as growth and development, respiration, stomatal aperture, senescence, seed germination, seedling growth and thermo-tolerance (Vicente and Plasencia, 2011). Moreover, many previous studies have shown that SA plays a role in many biotic and abiotic stresses (Vicente and Plasencia, 2011). This phytohormone can regulate responses to salinity (Idrees et al., 2012) and cold (Siboza et al., 2014), drought (Shen et al., 2014) and the toxicity of heavy metals (Tamás et al., 2015). Moreover, research has shown that SA levels and/or SA signaling played a positive regulatory role in plant response to polyethylene glycol (PEG)-simulated drought stress (He et al., 2014). It has been found that plants treated with SA generally exhibited more tolerance to water deficiency, SA alleviates the negative effects of drought stress on growth of Zoysiagrass (*Zoysia* Willd.)(Chen ret al., 2014). Singh and Usha (2003) showed that SA increases dry weight and chlorophyll content in wheat seedlings in wheat seedlings under water stress. In addition, SA improves photosynthesis and growth of mustard under drought stress (Nazar et al., 2015). Foliar spray with SA positively affected physiological characteristics of fennel genotypes such as chlorophyll, carotenoid contents and soluble carbohydrate and increased drought tolerance (Askari and Ehsanzadeh, 2015).

Biotic and abiotic stresses induce the production of secondary metabolites, which are involved in the defense against harsh environmental conditions and enhance significantly the antioxidant activity of plant tissues. The phenylpropanoid pathway is one of the important pathways in plant secondary metabolite production including, phenolics and among them flavonoids (flavanols, anthocyanins and flavan-3-ols) compounds. These are also considered as antioxidant molecules because they are involved in scavenging of free radical (Pourcel et al., 2007). Key enzymes in the phenylpropanoid pathway are phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL). There has been few reports on activity of TAL in plants treated with SA under drought stress, while PAL activity has frequently been studied. Increase in PAL activity in wheat seedlings treated with SA under salinity stress leading to increase in phenolics and flavonoids and subsequently caused an improvement in antioxidant defense system (Saleh and Madany, 2015). On the other hand, Bandurska and Cies´lak (2013) showed a positive correlation between increase of SA content and PAL activity in leaves and roots of barley, under drought stress and UV-B radiation. Hence, there has been a positive relationship between the key enzymes activity in the phenylpropanoid pathway and production of flavonoids, among them anthocyanines and generally all phenolic compounds.

*Artemisia aucheri* has always been of great interest for botanical and pharmaceutical aspects. The effect of drought stress on physiological and biochemical responses in *Artemisia aucheri* has not been studied yet. Since, tissue culture technology is a rapid and fast method in assessment of

physiological responses of the plant consequently; the present study was aimed to evaluate the mechanism of responses of *A. aucheri* to *in vitro* drought stress treated with salicylic acid (SA).

## 2 MATERIALS AND METHODS

### 2.1 Plant material and growth conditions

The *Artemisia aucheri* Boiss. plants were obtained from stock shoot culture of Department of Biology, University of Isfahan. Isfahan, Iran. Single node stem sections were propagated on MS (Murashige and Skoog., 1962) medium supplemented with 30 g/l sucrose and 8 g/l agar with adjusted pH, to 5.8. Plants were grown in the culture room at 25±1 °C with 16/8 h photoperiod under 44 µmol phot.$m^{-2}$.$s^{-1}$ light.

In treatment experiment SA was added to the media at following concentrations: 0, 0.01 and 0.1 mM. For drought stress treatments, PEG (polyethylene glycol, MW 6000) was used in 0, 2 % and 4 % (w/v) concentrations. PEG was added to MS medium according to diffusion based method described by Girma and Kreig (199). Based on the Factorial Design of the experiments, three explants were cultured per jar (each jar was one replicate) and after 4 weeks, explants were harvested for analysis of different physiological and biochemical parameters. Plants were grown in three different medium, 1) medium without SA and PEG (control), 2) medium with SA and PEG separately, 3) medium with combination of PEG and SA.

### 2.2 Growth measurement

Fresh mass was measured directly after harvesting of plants and dry mass was measured after drying the plant materials at 70 °C for 24 hours. Shoot relative water content was measured according to Weatherly (1950) as described by Bandurska (2000) and it was calculated by the following formula: RWC = [(fresh mass – dry mass)/ (fresh matter at full turgor – dry mass)] × 100. TW is turgid mass after saturating the fresh sample with water for 4 h.

### 2.3 Determination of chlorophyll and carotenoid content

For photosynthetic pigment measurements, leaves (0.1 g) of the plants were grounded in 80 % cold acetone and centrifuged at 5000 g for 10 min. The absorbance of the purified chlorophyll samples were measured at 470, 646, and 663 nm (Shimadzu, Japan). Chlorophylls and carotenoid contents were calculated according to Lichtenthaler and Wellburn (1983).

### 2.4 Soluble carbohydrates and storage polysaccharides

Water soluble carbohydrates were determined based on the phenol-sulfuric-acid method (Dubois et al., 1956). To prepare carbohydrate extract, 10 mg of dry leaf and stem was homogenized with 10 ml deionized water. The samples were centrifuged and supernatant used to determine soluble sugars. Sample (0.5 ml) was mixed with 0.5 ml of phenol (5 %) and then mixed with 2.5 ml sulfuric acid (96 %). The samples were vortexed slowly for 30 min then soluble carbohydrates were measured at 490 nm. To determine the storage polysaccharides, sediment of carbohydrate extract was weighted and again homogenized with deionized water and boiled for one hour. Finally, storage polysaccharides were measured at 490 nm.

### 2.5 Anthocyanin content

Total anthocyanins were extracted and determined based on the method described by Laby et al (Laby et al., 2000) with minor modifications. Leaves (0.1 g) were grounded in 99:1 methanol:HCl (v/v) and incubated at 4º C for 16 h. Then, samples were centrifuged at 4º C and the absorbance of the supernatants were measured at 530 and 657 nm. Total anthocyanin content was expressed as $A_{530}$ $g^{-1}$ FW.

### 2.6 Phenolic content

The Folin-Denis method was applied to estimate total phenols contents in the supernatant (Singleton et al., 1999). Leaf samples (0.1 g) were homogenized with 10 ml of methanol 80 %. The homogenates were centrifuged at 12,000 g for 10 min then 1/5 ml Folin–Ciocalteu reagent (10 %) and 1 ml $Na_2CO_3$ (7.5 %) was added to 0.5 ml methanol extract. The absorbance of samples was measured at 760 nm. The total phenolic

compounds were calculated from the standard curve, using gallic acid as a standard and expressed as mg gallic acid (Sigma, USA) $g^{-1}$ FW.

### 2.7 Flavonoid content

To determine the flavonoid content, 0.1 g of fresh leaf tissue homogenized in 80 % methanol and centrifuged at 10000 g for 10 min. The reaction mixture containing 0.2 ml of 80 % methanol, 0.2 ml of aluminum chloride (10 %), 0.2 ml of sodium acetate and 0.1 ml leaf extract. After 30 min, the absorbance of the samples was measured at 415 nm. The quercetin (Sigma, USA) was used for the standard curve and results were expressed as mg $g^{-1}$ FW (Chang et al., 2002).

### 2.8 PAL and TAL assay

To estimate the PAL and TAL activity, leaf samples (300 mg) were grounded in a mortar at 4 ºC with 4 mL buffer (50 mmol/l Tris pH 8.5, 14.4 mmol/l 2-mercaptoethanol, 5 % w/v polyvinylpolypyrrorolidone) and was centrifuged at 6,000 g for 10 min at 4 ˚C. The supernatant was collected and centrifuged at 10,000 g for 10 min at 4 ˚C and was used to assay enzyme activity. The total protein concentration in soluble enzyme extracts was determined using the Bradford (1976) method. The reaction mixture contained 500 µmol of Tris- HCl buffer (pH 8), 100 µl of enzyme extraction and either 6 µmol of L-phenylalanine for measuring PAL activity (EC 4.3.1.5) or 5.5 µmol of L-tyrosine (Sigma, USA) for measuring TAL activity (EC 4.3.1). After 60 min at 40°C, the reaction was stopped by the addition of 0.05 ml 5 N HCl. The amounts of trans-cinnamic and p-coumaric acids were determined by measuring absorbance at 290 and 333 nm, respectively. The PAL and TAL activities were expressed as nmoles (cinnamic or coumaric acid) $h^{-1}$ $mg^{-1}$ protein (Beaudoin-Eagan and Thorpe., 1985).

### 2.9 Total antioxidant capacity

Leaf samples (0.1 g) were homogenized with 80 % methanol in cold mortar and pestle and centrifuged at 18000 g for 10 min. The extrications were used to measure total antioxidant capacity by ferric reducing antioxidant power (FRAP) method (Benzie and Strain., 1996) This method is based on the reduction of ferric tripyridyltriazine (FeIII_TPTZ) complex to ferrous (FeII) form that makes blue color with maximum absorption at 593 nm. FRAP working solution consisted with 25 ml of acetate buffer (300 mM, pH 3.6), 2.5 ml TPTZ (Sigma, USA) solution (10 Mm in 40 mM HCl) and 2.5 ml of $FeCl_3$ (20 mM) solution.1.5 ml of FRAP reagent was added to 50 µl plant extract and mixed well. The absorbance was measured at 593 nm after 5 min. Standard curve was prepared using the similar procedure with ascorbic acid as standard.

### 2.10 Statistical analysis

All experiments were carried out with at least three replicates and the results were expressed as mean ± standard deviation (SD). ANOVA was performe to determine the significance of differences between means by Duncan's test ($p < 0.05$).

## 3 RESULTS AND DISCUSSION

Plants have developed mechanisms to alleviate negative effects of drought stress to increase their chance for survival. SA as a phytohormone can be effective in modulating physiological and biochemical responses leading to adaptation of plants to unfavorable environments such as drought stress (Kang et al., 2013; Nazar et al., 2015).

The obtained data indicate that fresh and dry mass of control (without SA and PEG) plants supplemented with 0.01 mM SA were increased significantly, while fresh and dry mass of 0.1 mM SA treated plants was comparable to untreated controls. Fresh mass decreased dramatically by 57 % and 76 % when control plant treated with 2 and 4 % PEG respectively, and 2 and 4 % PEG decreased dry mass by 53 % and 72.5 % comparing to the control respectively. Application of SA (0.01 and 0.1 mM SA) significantly improved both parameters when compared to PEG treated controls without SA treatment. The maximum increase of fresh and dry mass was observed at 0.01 mM SA (56 and 59 % in fresh mass under 2 % PEG and 51.5 and 43 % in dry mass under 4 % PEG) (Figure. 1). Furthermore, as

it is shown in the Figure. 2, SA (0.01 mM) increased photosynthetic pigments in control plant without PEG treatment. On the other hand, treatment of *A.aucheri* with PEG reduced chlorophyll and carotenoid contents compared with untreated plants. Under 2 % PEG treatment, a similar increase in total chlorophyll and carotenoid concentrations was measured when plants were treated with 0.01 and 0.1 mM SA, respectively. However, the effect of SA treatment on pigment contents was insignificant when plants were pretreated with 4 % PEG. The results in our study are in agreement with those reported by other authors. It is known that exogenous SA application enhanced the growth and photosynthetic pigments in several plant species treated under water stress such as wheat (Singh and Usha, 2003), *Nigella sativa* (Kabiri et al., 2014), Zoysiagrass (Chen et al., 2014), mustard (Nazar et al., 2015). Increase in fresh and dry mass by SA application under drought stress can be related to the positive effect of SA on photosynthetic pigments which led to the improvement in growth. In other words, the low water availability reduces photosynthesis, resulting in a reduction of carbohydrate accumulation, limiting overall plant growth (Chaves and Oliveira, 2004). In the control plant, the RWC remained at similar levels when plant was treared with SA. RWC decreased progressively under water deficit as compared with control plants, both concentration of salicylic acid increased RWC as compared to plant without application of SA under drought conditions (Fig. 3). Similar result were observed in pot experiments with barley (Habibi, 2012) and *Arabidopsis* (Khokon et al., 2011). It was suggested that the observed increase in RWC may be due to SA induced stomatal closure which reduces water loss. However, these results cannot be directly compared to our observations, since *in vitro* cultivated plants have quite unique water balance regulation. Their control of transpiration is poor due to very thin cuticle and malfunctioning of the stomata (Sutter, 1988). In this respect the results of our study only prove the involment of SA in plant response to drought, but have limited direct ecological relevance.

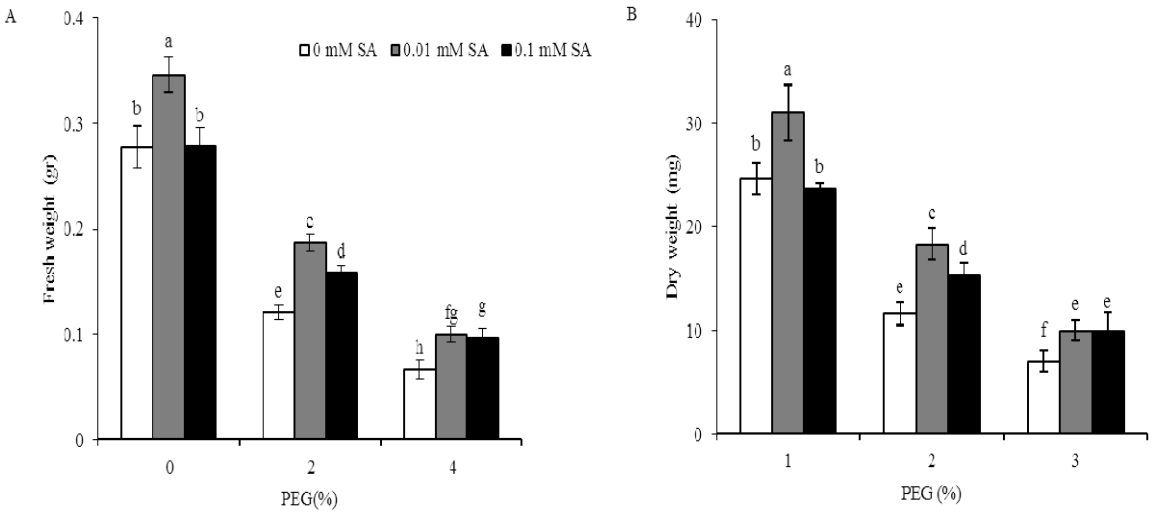


**Figure 1:** Effects of SA and PEG on (A) fresh and (B) dry mass of *Artemisia aucheri*. Data are means ± SD. Different letters indicate significant differences ($P < 0.05$) based on Duncan's test.

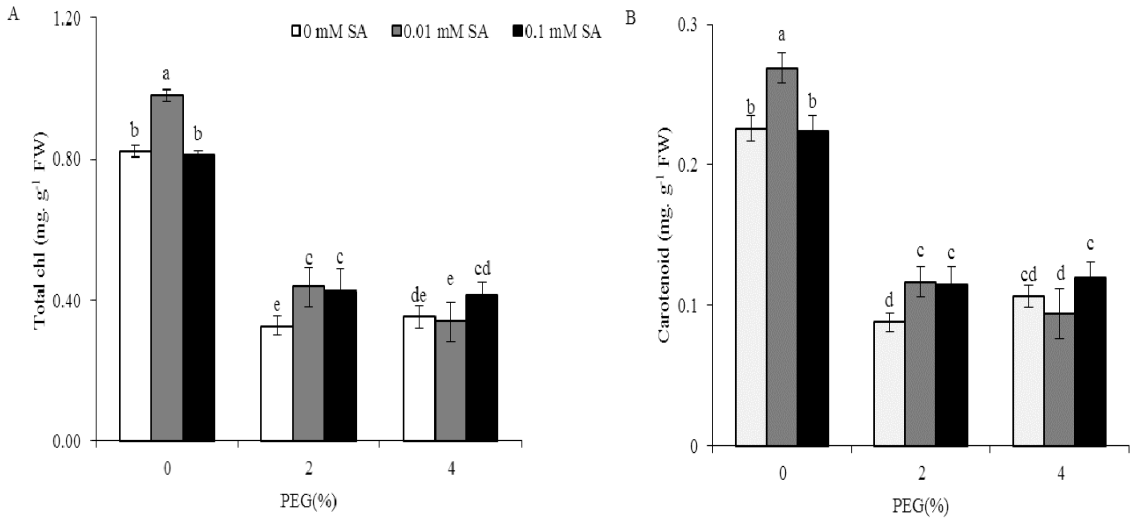


**Figure 2:** Effects of SA and PEG on (A) chlorophyll and (B) carotenoid of *Artemisia aucheri*. Data are means ± SD. Different letters indicate significant differences ($P < 0.05$) based on Duncan's test.

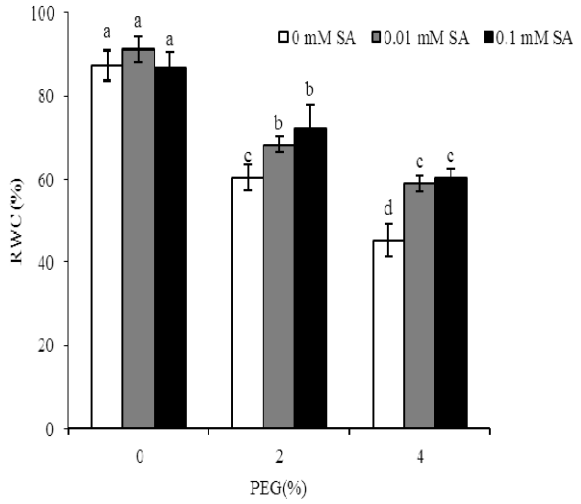


**Figure 3:** Effects of SA and PEG on relative water content (RWC) of *Artemisia aucheri*. Data are means ± SD. Different letters indicate significant differences ($P < 0.05$) based on Duncan's test.

Changes of carbohydrate and storage polysaccharides are presented in Figure 4. Drought stress (2 and 4 % PEG) declined the content of storage polysaccharides compared with untreated plants, and maximum decreased was observed at 4 % PEG. However it had no significant effect on soluble carbohydrates. Under 2 % and 4 % PEG, SA treatments decreased storage polysaccharides in the same manner (Figure. 4B), while soluble carbohydrates were increased (Figure. 4A). Moreover, both forms of carbohydrates in control plant were enhanced with 0.01 mM SA treatment. Soluble carbohydrates act as compatible solutes which support osmoregulation and are the main source of energy when plants are exposed to unfavorable environmental conditions (Patakas and Noitsakis, 2001). Increase of total soluble carbohydrates and storage polysaccharide content with 0.01 mM SA in control plants reflects stimulating role of SA in plant growth.

Interestingly, when SA treated plants have been exposed to drought stress, it has shown reverse trends in soluble sugars and polysaccharide contents as a result of graduate increase in SA treatment. It seemed that SA has facilitated conversion of polysaccharide to soluble carbohydrates and resulted in osmotic adjustment and resistance under drought. Accumulation of compatible solution is as an effective response to dehydration (Patakas and Noitsakis, 2001).

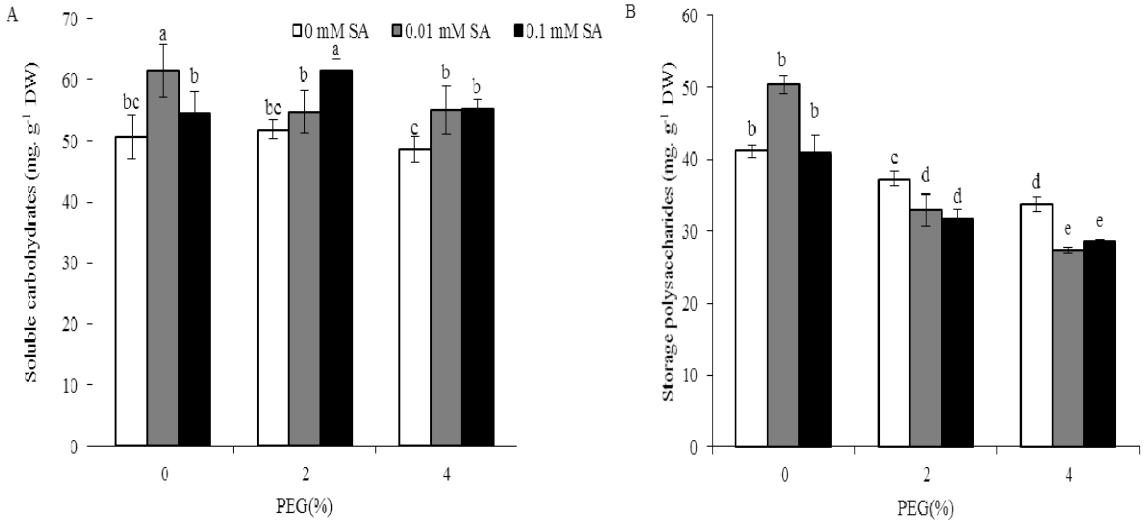


**Figure 4:** Effects of SA and PEG on (A) soluble carbohydrates and (B) storage polysaccharides content of *Artemisia aucheri*. Data are means ± SD. Different letters indicate significant differences ($P < 0.05$) based on Duncan's test.

According to our results, SA as a single treatment is able to increase phenolic compounds. Drought stress significantly elevated total phenolic contents compared with untreated plants. Moreover, both concentrations of SA (0.01 and 0.1 mM) increased the total phenol in 2 % PEG treated plants while SA with 0.1 mM increased phenol content in 4 % PEG (Figure. 5A). Drought stress induced by PEG had no significant effect on flavonoid levels compared with untreated plants. SA treatments (0.01 and 0.1mM SA) were remarkably effective on increasing of flavonoid content under 4 % PEG. In addition, flavonoid content was enhanced in response to 0.1 mM SA compared to control plants (Figure. 5B). Application of SA in the culture medium increased anthocyanin content in both PEG-treated and untreated plants. SA treatments in SA (0.1 Mm) + PEG (2 %) and SA (0.01 mM) + PEG (4 %) showed significant difference compared with the same PEG levels without SA (Figure. 5C).

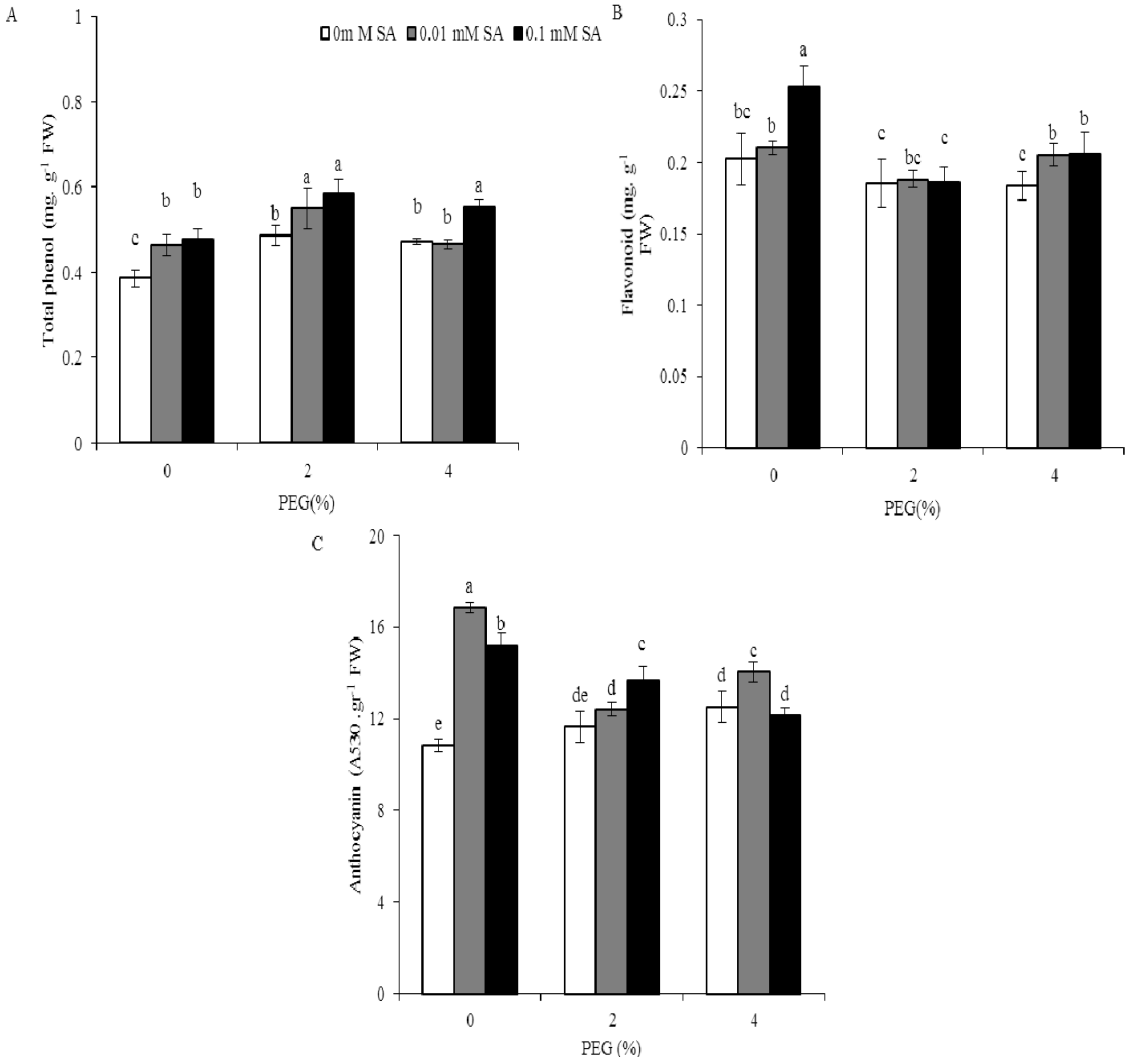


**Figure 5:** Effects of SA and PEG on (A) total phenol and (B) flavonoid and (C) anthocyanin content of *Artemisia aucheri*. Data are means ± SD. Different letters indicate significant differences ($P < 0.05$) based on Duncan's test.

PAL and TAL are two key enzymes in the phenylpropanoid pathway that catalyse the conversion of L-phenylalanine and L-tyrosine to trans-cinnamic acid and p-coumaric acids, respectively (Schroeder et al., 2008). Drought stress induced by PEG in combination with SA treatments remarkably enhanced PAL and TAL activity (Fig. 6). Notably, the increase of PAL activity in SA (0.01 and 0.1 mM) treated plants was also observed. The plants treated with 2 % and 4 % PEG showed an increase in PAL activity in 0.1 mM SA (Figure. 6A). Moreover, SA (0.1 mM) elevated TAL activity under 2 % PEG treatment, while SA treatments had no significant effect on TAL activity when plants were exposed to 4 % PEG (Figure. 6B). Our results are in agreement with the previous findings, that the increase in total phenolic content, such as phenol and flavonoids is accompanied by the induction of PAL and TAL activities that are involved in the defense system against biotic and abiotic stresses (S′wieca, 2015). The increase in level of anthocyanin, flavonoid and total phenol content by SA treatment was associated with TAL and PAL activity. Similar observation was reported by Dogbo et al (2012). They also showed exogenous salicylic acid induced PAL and TAL activity in cassava cell suspensions. Furthermore, elicitation using hydrogen peroxide in lentil sprouts enhanced PAL and TAL activity and subsequently elevated phenolic levels (S′wieca, 2015). Several studies have demonstrated that the production of ROS

especially hydrogen peroxide is induced by SA (Miura and Tada, 2013). Although under PEG treatments flavonoid contents did not change significantly, but anthocyanin as a phenolic component and total phenol as well as TAL and PAL activity elevated significantly. Similar findings were observed in the drought-tolerant barley cultivars under drought and salinity stress which confirms the role of antioxidant activity in this components and enzymes (Ahmed et al., 2015). Generally, our results showed that combination of SA with PEG has improved phenolic compounds production and two key enzymes of phenolic biosynthesis pathway. Kabiri et al (2014) reported that SA increased total phenol, anthocyanin, and flavonoid content under drought stress and drought damages were reduced. In fact SA could induce activity of PAL and TAL activity and consequently phenolic compounds were increased (Bandurska and Cie´slak, 2013).

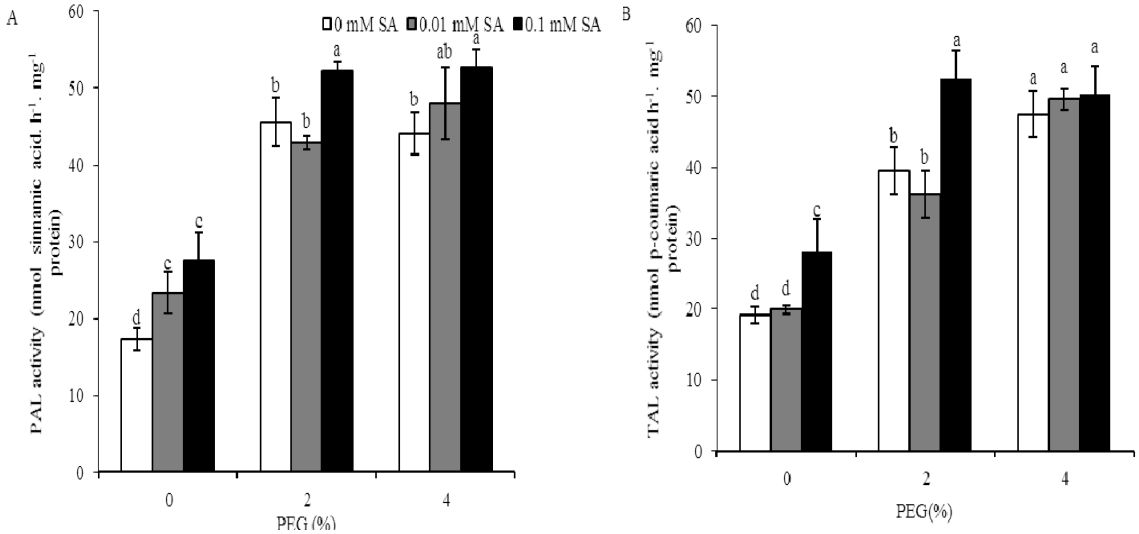


**Figure 6:** Effects of SA and PEG on (A) PAL and (B) TAL activity of *Artemisia aucheri*. Data are means ± SD. Different letters indicate significant differences ($P < 0.05$) based on Duncan's test.

Plants subjected to drought stress demonstrated higher total antioxidant capacity when exposed to higher PEG concentration. Total antioxidant capacity increased significantly in 0.01 mM SA treated controls and 0.01mM SA + 2 % PEG treated plants, the last showing a maximum level of antioxidant capacity. Moreover, plants subjected to drought and 0.1mM SA showed higher total antioxidant capacity compared to the same PEG level without SA (Fig. 7). It seemed that antioxidant property of phenolic compounds increased drought tolerance of *A. aucheri* as previously reported in barley (Saleh and Madany, 2015) and olive (Hashempour et al., 2014).

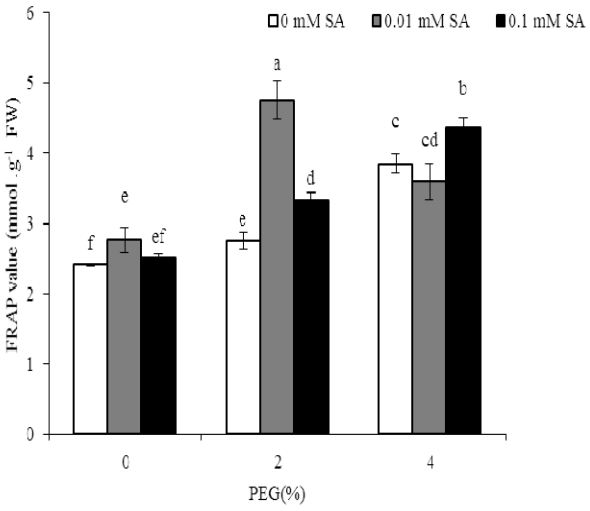


**Figure 7:** Effects of SA and PEG on total antioxidant capacity of *Artemisia aucheri*. Data are means ± SD. Different letters indicate significant differences ($P < 0.05$) based on Duncan's test.

## 4 CONCLUSION

Based on the present data, SA treatment alleviates drought stress induced by PEG in *A. aucheri* plants. This enhanced tolerance could be related to the improvement of antioxidant capacity via increase in PAL and TAL activities and the subsequent increase in anthocyanin, flavonoid and total phenol content. Furthermore, SA improves osmotic adjustment by conversion of storage polysaccharide to soluble carbohydrate.

## 5 ACKNOWLEDGEMENTS

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**Agris category code**: H01, H10

# Insecticidal effects of zinc oxide nanoparticles and *Beauveria bassiana* TS11 on *Trialeurodes vaporariorum* (Westwood, 1856) (Hemiptera: Aleyrodidae)

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**ABSTRACT**

Greenhouse whitefly, *Trialeurodes vaporariorum* is a major pest of horticultural and ornamental plants and is usually controlled with insecticides or biological control agents. In the current study, we examined the effects of synthesized zinc oxide nanoparticles (ZnO NPs) and *Beauveria bassiana* TS11 on *T. vaporariorum* adults. ZnO NPs were synthesized by precipitation method. Field emission scanning electron microscope images indicated that ZnO NPs were non-compacted uniformly. X-ray diffraction results confirmed the hexagonal wurtzite structure of ZnO NPs. Fourier transform infrared analysis showed an intense absorption peak at a range of 434-555 $cm^{-1}$ related to Zn-O bond. In bioassays, adults were exposed to different concentrations of ZnO NPs (3, 5, 10, 15, 20 mg $l^{-1}$) and fungi ($10^4$, $10^5$, $10^6$, $10^7$, $10^8$ spores $ml^{-1}$). $LC_{50}$ values for ZnO NPs and fungi were 7.35 mg $l^{-1}$and $3.28\times10^5$ spores $ml^{-1}$, respectively. Mortality rates obtained with ZnO NPs and fungi at the highest concentration were 91.6 % and 88.8 %, respectively. The results indicate a positive effect of ZnO NPs and *B. bassiana* TS11on adults. The current study was conducted under laboratory conditions, therefore, more studies are needed in field.

**Key words:** entomopathogenic fungus; nanoparticle; metal oxide; insecticide; bioassay

**IZVLEČEK**

**INSEKTICIDNI UČINKI NANO DELCEV CINKOVEGA OKSIDA IN TROSOV GLIVE *Beauveria bassiana* TS11 NA RASTLINJAKOVEGA ŠČITKARJA (*Trialeurodes vaporariorum* (Westwood, 1856) (Hemiptera: Aleyrodidae))**

Rastlinjakov ščitkar (*Trialeurodes vaporariorum*) je eden izmed glavnih škodljivcev hortikulturnih rastlin in se ga navadno zatira z insekticidi ali biološkimi agensi. V tej raziskavi smo preučevali učinke nano delcev cinkovega oksida (ZnO NP) in glive *Beauveria bassiana* TS11 na njegove odrasle osebke. ZnO NP je bil pripravljen z metodo usedanja. Analiza nano delcev ZnO z vrstičnim elektronskim mikroskopom je pokazala njihovo neizenačenost. Njihova nadaljna analiza z rentgenskimi žarki je potrdila njihovo heksagonalno strukturo. Analiza s Fourierjevo prosevno infrardečo spektrometrijo je pokazala močan absorpcijski vrh v območju 434-555 $cm^{-1}$, ki se je nanašal na Zn-O vez. V preiskusu smrtnosti so bili odrasli osebki ščitkarja izpostavljeni različnim koncentracijam nano delcev ZnO (3, 5, 10, 15, 20 mg $l^{-1}$) in trosov glive ($10^4$, $10^5$, $10^6$, $10^7$, $10^8$ spor $ml^{-1}$). Vrednosti $LC_{50}$ so bile za nano delce ZnO 7.35 mg $l^{-1}$ in $3.28\times10^5$ $ml^{-1}$ za trose glive. Smrtnost, ki je bila dosežena pri največjih koncentracijah nano delcev ZnO in trosov glive je znašala 91.6 % in 88.8 %. Ti izsledki kažejo pozitivni učinek obeh pripravkov na smrtnost odraslih osebkov rastlinjakovega ščitkarja. Glede na to, da je bila raziskava opravljena v laboratoriju je potrebno v bodoče opraviti še več raziskav v realnih razmerah.

**Ključne besede:** entomopatogene glive; nano delci; kovinski oksid; insekticid; biotest

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## 1 INTRODUCTION

The greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood, 1856) (Hemiptera, Aleyrodidae), is a globally distributed pest because of its quick multiplication, virus transmission, sap puncture, secretion of honeydew and promotion of sooty molds on the host leaves (Guzman et al., 1997). Excessive use of chemical pesticides and increased resistance to insecticides (Whalon et al., 2008), environmental pollutions, impacts on human health and other organisms and finally pesticides residues in nature and agricultural products have provided great impetus to the development of alternative techniques of pest control (Van Lenteren et al., 1996; Laznik et al., 2011; Sandhu et al., 2012).

Recent progress in nano-technology has provided new opportunities in the fields of science such as agriculture (Chaudhry et al., 2008). Application of nanomaterials can revolutionize agriculture by developing potential and effective methods for pest management (Rai and Ingle, 2012). Some studies have reported the toxic effects of metal nanoparticles (such as silver, zinc, aluminum, and titanium oxide) on plants, crustaceans, bacteria, fungi, pathogens and pests (Elchiguerra et al., 2005; Goswami et al., 2010; Kairyte et al., 2013; Kirthi et al., 2011; Manzo et al., 2011; Morones et al., 2005; Reddy et al., 2007; Rouhani et al., 2012; Samuel and Guggenbichler 2004). Among them, zinc oxide (ZnO) nanoparticle is best-known compound (Mitra et al., 2012) commonly used as agricultural fungicide (Waxman 1998). Furthermore, cytotoxicity in eukaryotic cells (Gupta and Gupta, 2005; Magrez et al., 2006), preventing growth in prokaryotic cells (Brayner et al., 2006), bactericidal and fungicidal activity and low effects of ZnO NPs on human cells have also been reported (Kairyte et al., 2013). Low cost synthesis, protective effect against ultraviolet (UV) rays, biocompatibility and non-toxicity has attracted the attention of many researchers (Brayner et al., 2006; Zhang et al., 2007; Ahmad Umar et al., 2009). In spite of the studies performed on NPs to control limiting factors in ecosystems, little research has been carried out to investigate the toxicity effect of ZnO nanoparticles on agricultural pests.

In addition to nanotechnology, biological control is another alternative method to chemical pesticides due to its non-toxicity for human and other non-target organisms, decreasing toxic residue in nature and food (Lacey et al., 2001). Aleyrodidae entomopathogens are limited to fungi since they are capable to penetrate the insect cuticle of sucking insects such as *Aphis gossypii* (Glover, 1877) (Gurulingappa et al., 2011), *Rhynchophorus ferrugineus* (Olivier, 1790) (Sewify et al., 2009), *Laniifera cyclades* (Druce, 1895) (Lozano-Gutierrz and Espana-Luna, 2008) and *Galleria melonella* (Linnaeus, 1758) (El-Sinary and Rizk, 2007). Among entomopathogenic fungi, *Beauveria bassiana* (Bals.-Criv.) Vuill. with broad host range is known as an effective organism to control medical and agricultural pests (Inglis et al., 2001). The aim of this research was to investigate the insecticidal activity of synthesized ZnO nanoparticles and *B. bassiana* TS11 on *Trialeurodes vaporariorum* under greenhouse conditions.

## 2 MATERIALS AND METHODS

### 2.1 Preparation of ZnO nanoparticles by precipitation

To prepare ZnO nanoparticles, first, 1 g of zinc oxide powder was dissolved in 100 ml of 1 % acetic acid oxidizing zinc-to-zinc cations. Then, the mixture was sonicated for 30 minutes. After 5 minutes, sodium hydroxide solution (1 M) was added drop wise to the above solution until the pH of solution reached to 10. The solution was heated in a water bath at 40-80 °C for 3 hours. Then, it was filtered through a filter paper and the precipitate was washed twice with distilled water. Finally, it was placed in an oven at 50 °C for 1 hour to form ZnO nanoparticles (Abdelhady, 2012).

### 2.2 Characterization of the synthesized ZnO nanoparticles

An analysis of nanoparticles was performed using Fourier transform infrared (FTIR) spectrometer (Bruker Optics Ft Tensor 27, Germany) by KBr (potassium bromide). The XRD analyses were performed using Bruker D8 X-ray diffractometer. FESEM (Hitachi S4160) analysis was carried out to observe the morphology of ZnO nanoparticles.

### 2.3 Entomopathogenic fungus culture and suspension preparation

Isolate TS11 of fungus *B. bassiana isolate* TS11 was obtained from Department of Plant Protection, University of Tehran, Iran. The fungus was inoculated on Sabouraud dextrose agar (SDA) medium in 8 cm Petri dishes and grown at 27 °C. After 12 days, to prepare inoculum, 10 ml of sterile distilled water containing 0.2 % of aqueous Tween 20 solution (Sigma Alrdich, Spain) was spread over the petri dishes using a suitable tool. The suspension was filtered through cheesecloth to separate conidia from remnants of the mycelium. The suspension was vortexed to separate spores from each other and to prevent mass formation during counting. Haemocytometer was used to count spores and prepare various concentrations of spores per unit volume.

### 2.4 Insect rearing

Adults of *T. vaporariorum* were collected from the surface of cucumber leaves (*Cucumis sativus* L) attacked with this pest from Sistan region (31.0256 °N, 61.5011 °E, and average of 480 meters above the sea level) located in the east of Iran. The adults were reared on young green bean (*Phaseolus vulgaris* L.) plants in a laboratory greenhouse (University of Zabol, Zabol, Iran) under controlled conditions (27±2 °C, 60±10 % RH and a photoperiod of 16:8 (L: D) h). In order to perform bioassays, Muniz and Nombela`s method (2001) was followed.

### 2.5 Bioassay and determination of lethal concentration of ZnO nanoparticles

To find concentrations with 10-90 % mortality, primary tests were done with concentration ranges between 1-30 mg $l^{-1}$ of the synthesized ZnO NPs. 20 adults of *T. vaporariorum* with same age were considered for each concentration. Following method was carried out in both primary and final tests. To diminish the activity of greenhouse whiteflies, the numbers of insects per concentration were released into the plastic tubes using an aspirator. The tubes were then put in the incubator at 5 °C for 2 minutes (personal observations). Then, the specific numbers of insects were transferred to glass petri dishes already with filter paper-covered floor, but before getting started, the desired concentrations of 5 ml were sprayed on them using a 5 ml handy sprayer. To avoid precipitation of nanoparticles in solutions, before spraying, all the prepared concentrations were sonicated for 10 minutes (Velayutham et al., 2013). After finishing, the insects were transferred to leaf cages installed over green bean leaves. The numbers of dead insects were counted after 24 hours and mortality was computed after three replications. In this experiment, distilled water was used for control.

### 2.6 Bioassays of *B. bassiana* TS11

Preliminary bioassays were conducted on adult insects with $10^2$-$10^{10}$ spores $ml^{-1}$ concentrations of *B. bassiana* TS11. Concentrations of $10^4$ and $10^8$ spores/ml caused 30 and 80 % mortality were selected as minimum and maximum concentrations, respectively and between them three logarithmic concentrations of $10^5$, $10^6$ and $10^7$ spores/ml were selected for final bioassays. In each treatment, 0.2 % Tween 20 (Sigma Aldrich, Spain) as an emulsifying agent was added to suspension of fungal conidia. Distilled water containing 0.2 % Tween 20 was used as control treatment. The experiments were conducted in a completely randomized design with three replicates of treatments.

Bioassays in these experiments started with control treatment; next, they were continued from lowest level of concentration to the highest level. In order to infect the insects, similar as in bioassay procedure with ZnO nanoparticles, the insects were first deactivated at 5 °C (2 min). 30 adult whiteflies were considered for each concentration. The specific numbers of the insects were individually placed in glass petri dishes with filter paper-covered floor; the desired spore concentrations were then sprayed on insects (5 ml of each concentration using a 5 ml handy sprayer). After application of fungal suspension, whiteflies were placed inside glasses containing water-agar and

green bean leaf. In order to provide moisture for primary germination of spores, the glasses were put in the germinator (27 °C, 80 % RH) for 24 hours. After 24 hours, whiteflies were released into leaf cages installed over young green bean leaves outside germinator and mortality of insects was recorded under controlled condition (27±2 °C, 60±10 % RH, a photoperiod of 16: 8 ([L: D] h) for 10 days.

### 2.7 Statistical analysis

To determine $LC_{50}$ and $LC_{25}$, SPSS 21 software (IBM, New York, US) with confidence limits of 95 % and Probit analysis were used.

## 3 RESULTS AND DISCUSSION

The current study presents the results of insecticidal activity of the synthesized ZnO nanoparticles after 24 hours and pathogenicity of entomopathogenic *B.bassiana* TS11 after 10 days. Mortality rate of insects was evaluated using Probit analysis. Table 1 illustrates values of $LC_{25}$ and $LC_{50}$, confidence limits (CL), slope and Chi-square ($\chi^2$) for ZnO nanoparticles and *B.bassiana* TS11.

**Table 1:** Mean values of *T. vaporariorum* mortality by lethal concentrations of ZnO nanoparticles and *B. bassiana* TS11

| Test Samples | Intercept ± $s_e$ | $LC_{25}$ (95 % CL) | $LC_{50}$ (95 % CL) | Slope ± $s_e$ | $\chi^2$ (df) |
|---|---|---|---|---|---|
| ZnO nanoparticles | -2.007 ± 0.264 | 3.76 mg $l^{-1}$ (2.80 - 4.61) | 7.35 mg $l^{-1}$ (6.21 - 8.58) | 2.32 ± 0.27 | 5.18 (3) |
| *B. bassiana* TS11 | -2.499 ± 0.288 | $0.106 \times 10^5$ spores $ml^{-1}$ ($0.28 \times 10^4$ - $0.26 \times 10^5$) | $3.28 \times 10^5$ spores $ml^{-1}$ ($1.62 \times 10^5$ - $6.2 \times 10^5$) | 0.453 ± 0.048 | 1.37 (3) |

$LC_{25}$ and $LC_{50}$: lethal concentration that kills 25 and 50 % of *T. vaporariorum* after exposure to nanoparticle concentration (mg $l^{-1}$) and *B.bassiana* TS11 (spores $ml^{-1}$), respectively. The estimated lethal concentration values (mg/l for ZnO NPs and spores $ml^{-1}$ for *B. bassiana* TS11 for treatment was given using Probit analysis. Values in parentheses indicate 95 % confidence limits (CL). Df and $s_e$ refers to degrees of freedom and standard error, respectively.

### 3.1 ZnO nanoparticles

Insecticidal activity of the synthesized ZnO nanoparticles revealed that values of $LC_{50}$ and $LC_{25}$ (7.35 and 3.76 mg $l^{-1}$, respectively) had significant lethal effects on *T. vaporariorum* adults. Statistical results showed a significant difference between concentrations of ZnO nanoparticles at 5 % level. Moreover, mortality rate depended on concentration, in other words, as concentration increased, lethality also significantly increased. Mortality rates of concentrations of 3, 5, 10 15 and 20 mg $l^{-1}$ were 21.6 %, 35 %, 53.3 %, 73.3 % and 91.6 %, respectively (Figure 1).

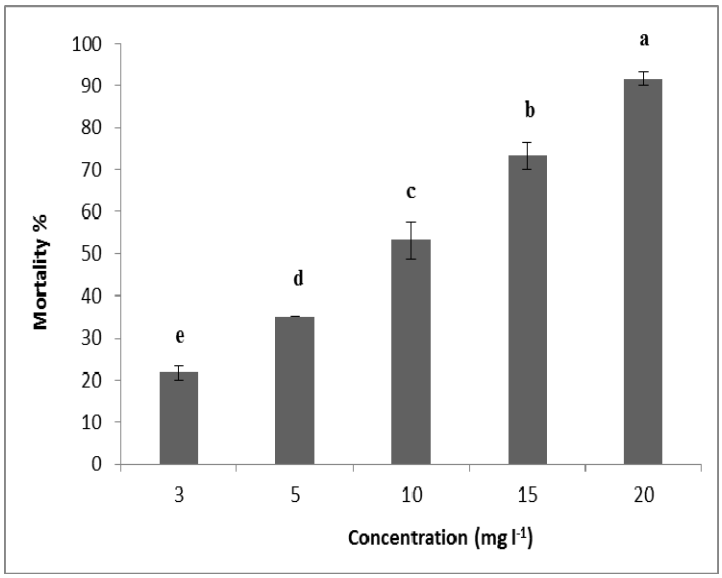


**Figure 1:** Mortality of *T. vaporariorum* adults, 24 hours after application of ZnO nanoparticles. Values followed by same letter indicate there is no overlap in 95 % confidence interval.

The FESEM images taken from ZnO nanoparticles sample indicated that ZnO nanoparticles are well-distributed with the lowest agglomeration of nanoparticles (Figure 2).

The peaks at 2$\theta$ values including 31.85, 34.5, 36.3, 47.65, 56.7, 62.95 with (100), (002), (101), (102), (103) and (110) diffraction, respectively are shown in the XRD patterns of ZnO nanoparticles (Figure 3). Furthermore, the results of XRD show the presence of ZnO crystals with hexagonal wurtzite structure. Average size of the synthesized ZnO nanoparticles was found to be 23.34 nm using Debye-Scherrer equation (1): $D=k\lambda/\beta \cos \theta$.

Where k is equal to 0.89; $\lambda$ is X-ray wavelength (1.54 A), $\beta$ is peak width at half maximum height in radian and $\theta$ is bragg diffraction angle of the maximum peak (Zhu et al., 2005; Suwanboon et al., 2013).

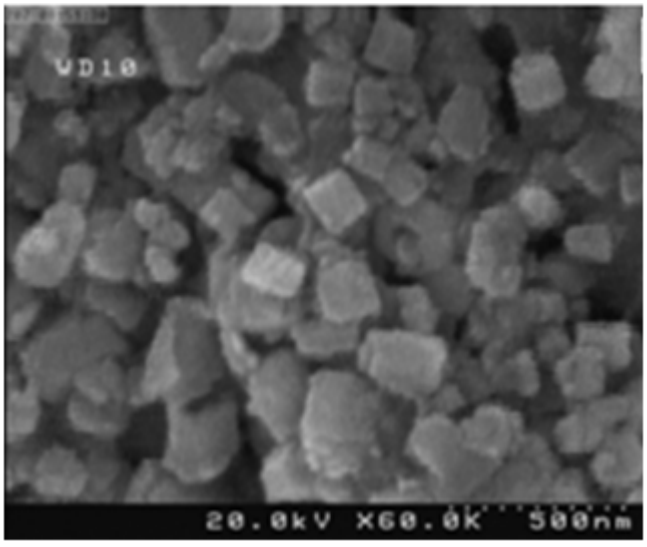


**Figure 2**: FESEM images of synthesized ZnO nanoparticles. Scale bar is 500 nm.

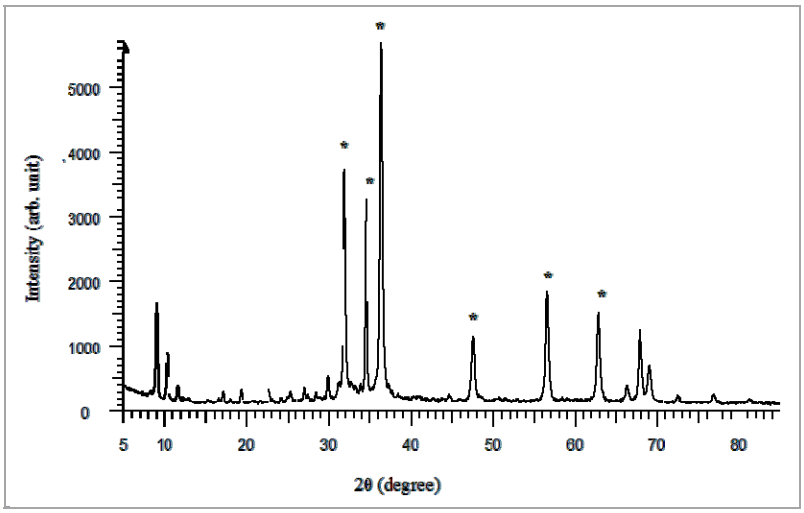


**Figure 3:** XRD spectrum of synthesized ZnO nanoparticles (Star symbols indicated the presence of ZnO NPs).

In FTIR spectrum of ZnO nanoparticles sample, two strong absorptions are seen at 503 $cm^{-1}$ and 432 $cm^{-1}$ in which 432 $cm^{-1}$ peak represents tensile bond of ZnO and 503 $cm^{-1}$ peak represents oxygen vacancies in ZnO (Figure 4).

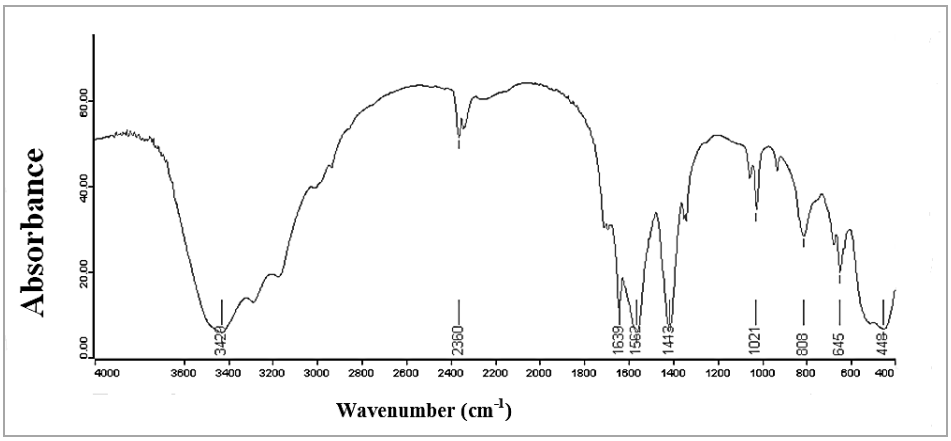


**Figure 4:** FTIR of synthesized ZnO nanoparticles

The current study revealed the positive effect of insecticidal activity of ZnO NPs on *T. vaporariorum* adults. Acaricidal, licicidal and larvicidal activities of the synthesized ZnO nanoparticles on blood - feeding parasites - *Rhipicephalus* (*Boophilus*) *microplus* (Canestrini, 1888), *Pediculus humanus capitis* (De Geer,1767), *Anopheles subpictus* (Grassi, 1899) and *Culex quinquefasciatus* (Say, 1823) revealed an increase in mortality when concentration increased; moreover, considering optimal time for lethal effects of ZnO nanoparticles after 24 hours, it was 100 % proved. In the comparison made between acaricidal, licicidal and larvicidal activities of zinc oxides and the synthesized ZnO nanoparticles, the mortality effect of ZnO nanoparticles was significant (Kirthi et al., 2011).

Rouhani et al. (2012) studied the insecticidal effects of imidacloprid, Ag and Ag-Zn nanoparticles on *Aphis nerii* (Boyer de Fonscolombe, 1841) and $LC_{50}$ values after 24 hours were seen to be 0.13 µl $ml^{-1}$, 424.67 and 539.46 mg $ml^{-1}$, respectively. In a similar study, Samih et al. (2011) investigated the insecticidal effect of Amitraz, ZnO nanoparticles and $ZnAl_2O_3$ against Pistachio psylly (*Agonoscena pistaciae* (Burckhardt and Lauterer, 1989)) and found out a

greater insecticidal effect of Amitraz than the above nanoparticles. It should be taken into consideration that nanoparticles, especially the synthesized zinc nanopartilces, besides their insecticidal activity and also their slower effect than imidacloprid and Amitraz insecticides, have a lower risk of resistance to these nanoparticles in comparison to commercial insecticides (Rouhani et al., 2011).

Clausen et al., (2011) investigated the efficiency of $ZnSO_4$ and ZnO nanoparticles on mortality of *Reticulitermes flavipes* (Kollar, 1837) (Isoptera, Rhinotermitidae). Their results confirmed that *R. flavipes* feeding on wood impregnated with ZnO nanoparticles, decreased to less than 4 % in comparison to control treatment. Despite low usage of wood impregnated with ZnO nanoparticles, 94-99 % of mortality rate was seen in termites. In contrast, using wood impregnated with $ZnSO_4$, 10-12 % was consumed and low mortality rate (10-29 %) was seen in these termites. Thus, ZnO nanoparticles have the necessary potential for application in wood preservatives for protecting woods against termites.

$LC_{50}$ value of the synthesized ZnO nanoparticles on *T. vaporariorum* were consistent with the results of the above researches. Therefore, it can be said that ZnO nanoparticles have the necessary potential for insecticidal activity on *T. vaporariorum* and causes maximum lethality (91.6 %) at the highest concentration.

Nowadays, the only successful control strategy for the greenhouse whitefly is the combined use of pesticides and natural enemies (Hayashi, 1996); therefore, applications of some concentrations of pesticides with minimal impacts on natural enemies seem quite necessary (Laznik and Trdan, 2014). In the current study, through calculation of $LC_{25}$, we can obtain a concentration of the synthesized ZnO nanoparticles to control *T. vaporariorum,* which is likely to distort its physiology. Since no research has been done on the impact of ZnO nanoparticles on natural enemies of the greenhouse whitefly, therefore, we conclude that using $LC_{25}$, survival rate of *T. vaporariorum* decreases; however, this concentration would have minimal impact on natural enemies. With application of $LC_{25}$ (3.76 mg $l^{-1}$), which is quite less compared to $LC_{50}$ (7.35 mg $l^{-1}$), we can prevent the occurrence of adverse effects on natural enemies of whiteflies.

Based on new and significant properties of nanoparticles, these materials are widely used in industrial and agricultural sectors; therefore, assessment of their potential toxic effects on human health and environment seems quite necessary (Auffan et al., 2009). As a discussion, the best approach to avoid adverse effects on the environment and ecotoxicological effects of nanoparticles on beneficial insects such as parasitoids wasps is using low concentrations of nanoparticles to control pest insects such as *T. vaporariorum*.

### 3.2 Bioassay of *B. bassiana* TS11

The results obtained from statistical analysis of bioassays on the greenhouse whitefly revealed the susceptibility of *T. vaporariorum* adults to *B. bassiana* TS11 isolate; however, the amount of mortality was different based on determined spore concentrations. More than 50 % of mortality was observed in concentrations of $10^6$, $10^7$and $10^8$ spores $ml^{-1}$ during the experimental period (10 days). Generally, the least amount of mortality after 10 days was obtained with $10^4$ spore $ml^{-1}$ concentration with an average of 33.3 %. Mortality rate of whiteflies depended on concentration and mortality rate increased along with the increase in concentration. Maximum mortality (88.8 %) rate was obtained with $10^8$ spore $ml^{-1}$. In concentrations of $10^5$, $10^6$ and $10^7$ spore $ml^{-1}$, the mortality rates were seen to be 42.2 %, 58.8 % and 78.8 %, respectively (Figure 5). Statistically, a significant difference was seen between concentrations of $10^5$ and $10^6$ spore $ml^{-1}$. However, in terms of mortality rate, there were no significant differences between concentrations of $10^7$ and $10^8$ and concentrations of $10^4$ and $10^5$ spore $ml^{-1}$.

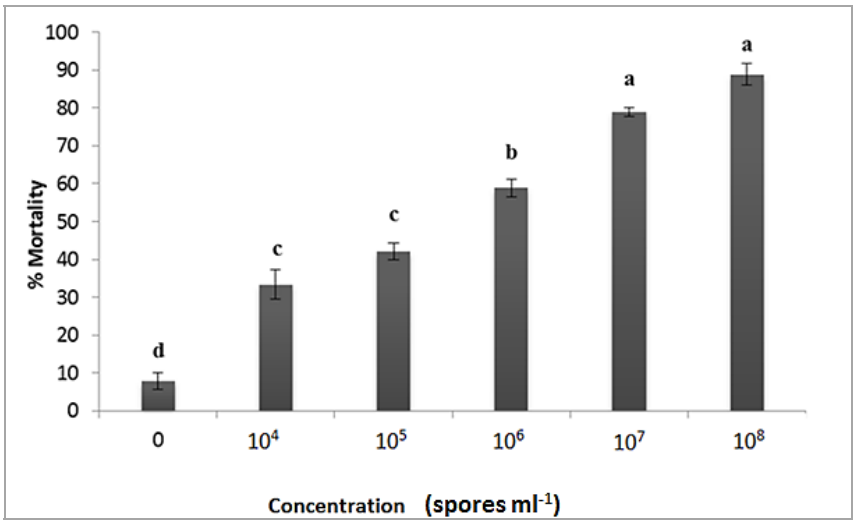


**Figure 5:** Mean of mortality of *T.vaporariorum* adults 10 days after treatment by *B.bassiana*

Among all antimicrobial agents, entomopathogenic fungi due to frequency in pathogenic races, broad host range and methods of pathogenicity can control a broad range of pests (Fan et al., 2007). The current study revealed that *B. bassiana* TS11 isolate can lead to infection and mortality of *T. vaporariorum*. Several studies have reported *B. bassiana* to control agricultural pests such as *Tetranychus cinnabarinus* (Boisduval, 1867) (Shi and Feng, 2004), *Chilo partellus* (Swinhoe, 1885) (Tefera and Pringle, 2004), whiteflies (Wraight et al., 1998, 2000; Ramos et al., 2000; Mascarin et al., 2013), *Helicoverpa zea* (Boddie, 1850), *Spodoptera exigua* (Hubner,1808), *Spodoptera frugiperda* (J.E. Smith, 1797) (Wraight et al., 2010), and *Aelia rostrata* (Boheman, 1852) (Mustu et al., 2011). Khosravi et al., (2014) studied the pathogenicity of four isolates, IRAN 403C, SP 566, SPT 22 and IR-K-40 of *B. bassiana* on *Arge rosae* (Linnaeus 1970). The results showed the great effect of these isolates on this hymenopteran species; however, IRAN 403C isolate with $LC_{50}$ value of $5.54\times10^5$ spore $ml^{-1}$ and mortality rate of 82.5 % was highly more effective.

Mascarin et al., (2013) reported that *B.bassiana* CG1229 isolate in a concentration of $1\times10^7$ spore $ml^{-1}$ led to more than 80 % of mortality rate in silver-leaf whitefly (*Bemisia argentifolii* (Gennadius, 1889)) populations. Therefore, *B.bassiana* has a high pathogenicity potential for this insect while it can significantly control this whitefly (Mascarin et al., 2013).

In the present study, *B. bassiana* TS11 had a significant pathogenic effect on *T. vaporariorum* adults, because it had a low $LC_{50}$ value and led to high mortality rate (88.8 %) in concentration of $10^8$ spore $ml^{-1}$. Mortality in control treatment was so low and no fungal growth was seen on dead adult whiteflies. In terms of pathogenicity of *B. bassiana* for *T. vaporariorum*, similar to the effects of synthesized ZnO nanoparticles, calculation of lethal concentrations such as $LC_{25}$ ($0.106\times10^5$ spore $ml^{-1}$) is in turn particularly important.

We should pay attention to the interactions of the entomopathogenic fungi and whiteflies natural enemies (i.e. *Encarsia* spp., *Eretmocerus* spp., etc.) to minimize adverse effects on them. Therefore, despite the best effect of *B. bassiana* on pest insects such as *T. vaporariorum*, we should also take into account the low lethal effects of this entomopathogenic fungus so that they have minimal impact on natural enemies.

## 4 CONCLUSION

The current study was performed to demonstrate the insecticidal effects of ZnO nanoparticles and *B. bassiana* on the greenhouse whitefly. The obtained results proved the efficiency of synthetic

nanoparticles and entomopathogenic fungi as effective control agents, which can lead also to the delay in pest resistance mechanisms to chemical insecticides. It is possible that by adding nanoparticles and entomopathogenic fungi to formulations of insecticides, toxicity of chemical insecticides for humans and other non-target organisms would be mitigated. Further study will need to focus on methods to increase stability and on physiological mechanisms of nanoparticles and entomopathogenic fungi interactions to increase their effects in integrated pest management programs at large greenhouse and field levels.

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**Agrovoc descriptors:** cereals, maize, Zea mays, varieties, drought stress, crop yield, chemical composition, lipid content, protein content

**Agris category code**: F06, f60, f62, q04

# Changes in seed oil and protein contents of maize cultivars at different positions on the ear in response to water limitation

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**ABSTRACT**

A field experiment was carried out as split-split plot in 2014 to assess the effects of four irrigation treatments (irrigations after 60, 80, 100 and 120 mm evaporation, respectively) on oil and protein changes of maize cultivars (SC704, NS640 and DC303: Late, mid and early maturing cultivars, respectively) at different seed positions on the ear (upper, middle and lower positions on the ear). Overall, the highest seed yield was obtained from SC704, followed by NS640 and DC303 cultivars. Seed yield of all cultivars was higher at lower seed position on ear than at middle and upper parts of the ear under different irrigation treatments. The highest oil and protein yields were also recorded for seeds at lower position on the ear. Seed yield of all maize cultivars at various seed positions decreased with increasing irrigation intervals. Oil percentage decreased, but protein percentage increased with decreasing water availability. Water limitation decreased oil and protein yields of maize cultivars. Changes in protein and oil yields of maize cultivars at different seed positions and irrigation treatments were attributed to changes in seed yield.

**Key words:** maize cultivars, oil, protein, seed position, seed yield, water deficit

**IZVLEČEK**

**SPREMEMBE V VSEBNOSTI OLJ IN BELJAKOVIN V ZRNJU RAZLIČNIH SORT KORUZE V ODVISNOSTI OD NJIHOVEGA POLOŽAJA NA STORŽU KOT ODZIV NA POMANJKANJE VODE**

V letu 2014 je bil izveden poljski poskus z deljenkami za ovrednotenje učinkov namakanja (namakanje po 60, 80, 100 in 120 mm evaporacije) na spremembe v vsebnosti olj in beljakovin v zrnju treh sort koruze (SC704, NS640 in DC303: pozno, srednje in zgodaj dozorevajoče sorte) v odvisnosti od položaja zrnja na storža v času namakanja. Celokupno je dala največji pridelek sorta SC704, ki sta ji sledili sorti NS640 in DC303. Pridelek zrnja vseh sort je bil večji pri nižjem kot pri srednjem ali višjem položaju zrnja na storžu pri vseh načinih namakanja. Tudi največje vsebnosti olja in beljakovin so bile ugotovljene v zrnju iz spodnjega dela storža. Pridelek zrnja se je pri vseh obravnavanih sortah koruze in položajih zrnja na storžih ob namakanju zmanjševal s povečanjem presledka med namakanji. Z zmanjševanjem razpoložljivosti vode se je odstotek olja zmanjševal, beljakovin pa povečeval. Omejitev oskrbe z vodo je pri vseh sortah zmajšala pridelek olja in beljakovin. Spremembe v pridelku olja in beljakovin obravnavanih sort koruze pri različnih položajih zrnja na storžu ob času obravnavanj pripisujemo spremembam v pridelku zrnja.

**Ključne besede:** sorte koruze, olje, beljakovine, položaj zrnja na storžu, pridelek zrnja, sušni stres

## 1 INTRODUCTION

Maize (*Zea mays* L.) is one of the major cereals in the world with broad possibilities of use in fresh or processed form. However, the development of increasingly productive maize hybrid varieties resulted in a loss of nutritional value, especially with decreases in protein and oil, due to the negative correlation with yield (Uribelarrea et al. 2004; Bueno et al. 2009).

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Starch, protein and oil contents are the most important chemical storage components of maize. Maize seeds contain 70-75 % starch, 8-10 % protein and 4-5 % oil (Boyer and Hannah, 2001). The maize proteins can be divided into classes according to their solubility. In maize, the concentration of α-zein protein is the highest, representing 50-60 % of the total protein. Although the α-zein is poor in essential amino acids such as lysine and tryptophan, the protein fractions in seeds are not constant and can vary depending on genotype (Prasanna et al. 2001).

Maize has its origin in a semi-arid area, but water deficit can limit the production of this crop (Ghassemi-Golezani et al., 1997). Drought stress inhibits the growth and development of all cultivars and hybrids of maize at different growth stages (Dai et al., 1990). Coincidence of drought stress with reproductive stages reduces duration of flowering and seed filling and consequently lowers the number of seeds per plant, mean seed mass and seed yield per unit area of common bean cultivars (Ghassemi-Golezani and Mardfar, 2008).

The final composition of the seed is known to vary by genotype and in response to the environmental conditions during seed development (Brummer et al., 1997; Westgate et al., 1999; Vollmann et al., 2000; Yaklich et al., 2002; Fehr et al., 2003; Wilson, 2004). As the increase in the seed oil content is usually accompanied by a decrease in seed mass, the reduction in seed yield is a limiting factor to a hybrid maize with high oil content (Mišević et al. 1989). Esmailian et al. (2012) reported that water deficit at seed filling stage of sunflower caused a decrease in oil content. Drought stress imposed during seed filling decreases oil and residual contents more than the protein content (Rotundo and Westgate, 2009). This generally resulted in an increase in final protein content (Rotundo and Westgate, 2009). It is well established that water deficit shortens the seed filling duration and apparently has little impact on rate of filling (Westgate et al., 1999; Egli and Bruening, 2004).

Seed position on the ear and plant is one of the components of within-plant variation that may account for part of the variation in physical (such as mass, shape) or physiological (such as germination and vigor) seed attributes (Illipronti, 2000). However, the effect of seed position on oil and protein contents of maize is not clear, so this research was carried out to investigate the impact of irrigation levels on seed yield and composition of maize cultivars at different positions of ear.

## 2 MATERIALS AND METHODS

A split-split plot experiment (using RCB design) with three replications was conducted in 2014 at the Research Farm of the Faculty of Agriculture, University of Tabriz, Iran (latitude 38.05°N, longitude 46.17 °E, altitude 1364 m sea level), in order to determine the influence of water deficit on oil and protein contents of maize seeds at different ear positions. The climate is characterized by mean annual precipitation of 245.75 mm per year, mean annual temperature of 10 °C. Irrigation treatments ($I_1$, $I_2$, $I_3$ and $I_4$: irrigation after 60, 80, 100 and 120 mm evaporation from class A pan, respectively) were located in main plots, cultivars ('S.C704, 'N.S640' and 'D.C303': late, mid and early maturing, respectively) in sub plots and seed positions ($P_1$, $P_2$, $P_3$: upper, middle and lower positions of ear, respectively) in sub-sub plots.

Seeds of maize cultivars were treated with 2 $g.kg^{-1}$ Mancozeb and then were sown by hand on 3rd May 2014 in 5 cm depth of a sandy loam soil. At the same time, plots were fertilized with 200 kg/ha urea (46 % N). Each plot consisted of nine rows of 2.5 m length, spaced 50 cm apart. All plots were irrigated immediately after sowing. Irrigation treatments were applied after seedling establishment. Hand weeding of the experimental area was carried out as required.

Ears of the maize plants from 1 $m^2$ of the middle part of each plot were harvested when seed moisture content was 16-18 %. Seed moisture content was determined in accordance with ISTA rules (2010). Subsequently, seeds (fruitlets) were separately detached from the upper, middle and lower parts of the ears, one third each. Then, seed yield of each plot was recorded. Percentages of oil

and protein for each sample were estimated by a seed analyzer (model: Zeltex ZX-50) and thereafter, oil and protein yields per unit area were calculated. All the data were analyzed on the basis of the experimental design, using MSTATC software. The means of each trait were compared according to Duncan multiple range test at $P \leq 0.05$. Excel software was used to draw figures.

## 3 RESULTS AND DISCUSSION

### 3.1 Seed yield

Seed yield per unit area was significantly influenced by irrigation, cultivar, position on the ear and interaction of these factors ($p \leq 0.01$). Therefore, regression curves were fitted on mean interaction data. In general, the highest seed yield under different irrigation treatments was recorded for lower position of seeds on the ear and decreased at middle and upper parts of the ear. Seed yield of SC704 and NS640 cultivars at upper parts of ear was much lower than that of other positions. SC704 had the highest seed yield under all irrigation treatments, followed by NS640 and DC303 cultivars. Seed yield of all maize cultivars at different positions on the ear decreased with increasing water limitations. This reduction in seed yield was higher for late (SC704) and mid (NS640) maturing cultivars, compared with early maturing cultivar (DC303). These differences among cultivars were greater under well-watering and mild stress, but considerably decreased with further decrease in water availability (Fig. 1).

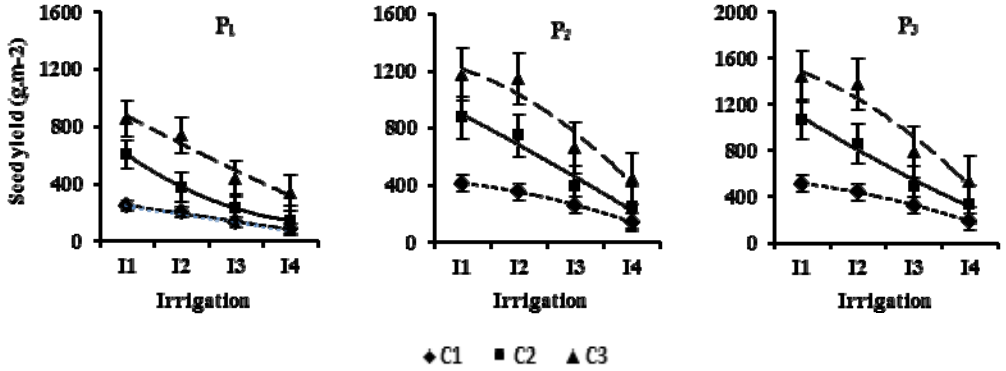


**Figure 1:** Changes in seed yield of maize cultivars at different positions on the ear and irrigation treatments (Means ± SE).
$C_1$, $C_2$, $C_3$: cultivars DC303, NS640 and SC704, respectively.
$P_1$, $P_2$, $P_3$: upper, middle and lower positions of seeds on the ear, respectively.
$I_1$, $I_2$, $I_3$, $I_4$: irrigation after 60, 80, 100 and 120 mm evaporation, respectively

### 3.2 Oil percentage and yield

Seed oil percentage and yield were significantly affected by cultivar, seed position, irrigation × cultivar and seed position × cultivar ($p \leq 0.01$). Seed oil yield also influenced by irrigation ($p \leq 0.01$) and seed position × irrigation ($p \leq 0.05$). Oil percentage of late (SC704) and mid (NS640) maturing cultivars decreased with decreasing water availability, while there was no tangible change in oil percentage of early maturing cultivar (DC303) under various irrigation treatments. Mid maturing cultivar (NS640) had the highest oil percentage under all irrigation treatments, but this superiority decreased with increasing water limitations (Fig. 2a).

Increasing water deficit resulted in reduction of oil yield per unit area of all maize cultivars, but this reduction was more pronounced for late and mid maturing cultivars. Nevertheless, SC704 had the highest oil yield under all irrigation treatments, followed by NS640 and DC303 (Fig. 2b).

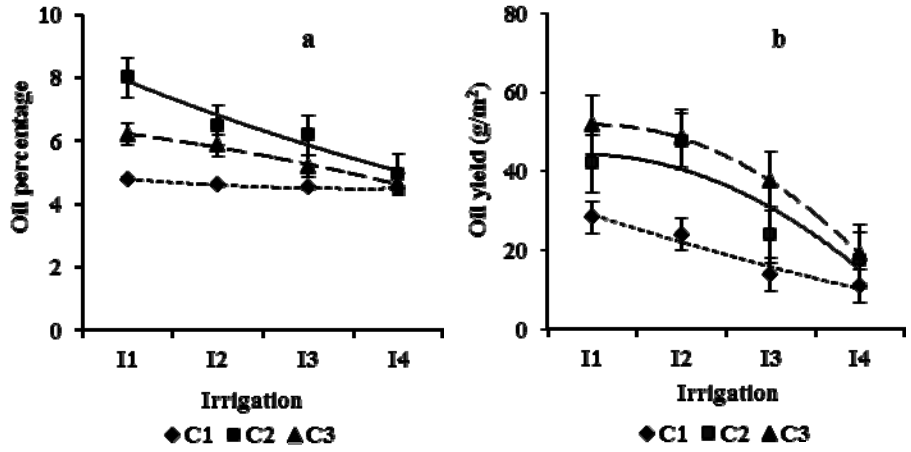


**Figure 2:** Changes in oil percentage (a) and yield (b) of maize cultivars at different irrigation treatments (Means ± SE).
$C_1$, $C_2$, $C_3$: cultivars DC303, NS640 and SC704, respectively.
$I_1$, $I_2$, $I_3$, $I_4$: irrigation after 60, 80, 100 and 120 mm evaporation, respectively

The highest oil percentage for all cultivars was obtained in seeds from lower parts of maize ear. Oil percentage of NS640 at all seed positions on the ear was higher than that of other cultivars, but this advantage was more evident at lower position on the ear ($P_3$). Differences in seed oil percentage of maize cultivars at middle and upper parts of the ear were minimized (Fig. 3a).

The lowest oil yield per unit area for all cultivars was recorded at upper position of seeds on the ear, which largely increased at middle and particularly at lower positions of the ear. The greatest seed oil yield at all parts of the ear was produced by SC704, followed by mid and early maturing cultivars. These differences among maize cultivars were enhanced at lower position of seeds on the ear (Fig. 3b).

Oil yield at all seed positions decreased as water deficit increased, but the reduction rate for seeds of upper position was less than those of other positions. The highest and the lowest oil yields under all irrigation treatments were recorded for seeds of lower and upper positions of the ear, respectively. This difference decreased with decreasing water supply (Fig. 4).

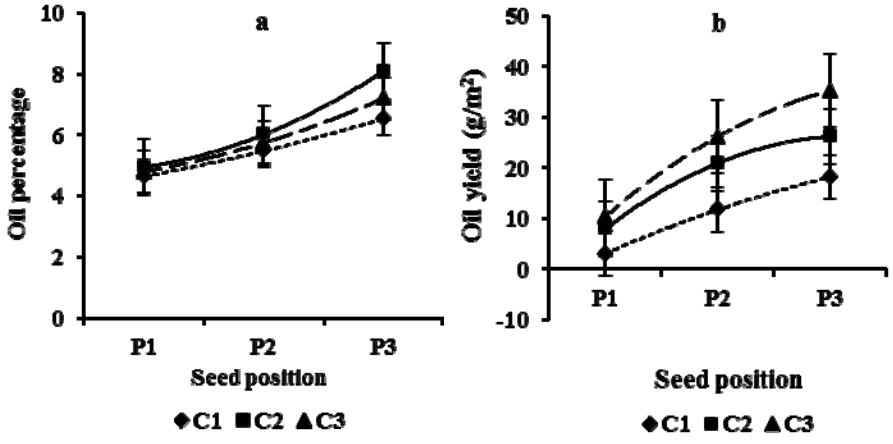


**Figure 3:** Changes in oil percentage (a) and yield (b) of maize cultivars at different seed positions on the ear (Means ± SE).
$C_1$, $C_2$, $C_3$: cultivars DC303, NS640 and SC704, respectively.
$P_1$, $P_2$, $P_3$: upper, middle and lower position of seeds on the ear, respectively.

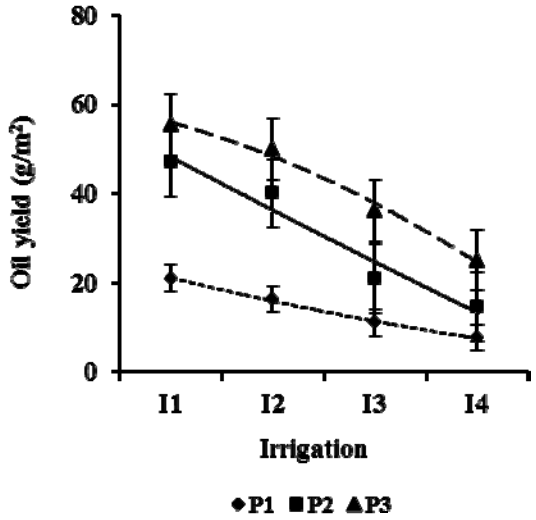


**Figure 4:** Seed oil yield of maize at different positions on the ear and irrigation treatments (Means ± SE).
$P_1$, $P_2$, $P_3$: upper, middle and lower position of seeds on the ear, respectively.
$I_1$, $I_2$, $I_3$, $I_4$: irrigation after 60, 80, 100 and 120 mm evaporation, respectively

### 3.3 Protein percentage and yield

Seed protein percentage was significantly influenced by the interactions of irrigation × cultivar ($p \leq 0.05$), seed position × cultivar and seed position × irrigation × cultivar ($p \leq 0.01$). In contrast, seed protein yield was only affected by irrigation, cultivar, seed position and irrigation × cultivar ($p \leq 0.01$).

Mean protein percentage of maize cultivars at all seed positions on the ear increased with increasing water stress. Protein percentage of mid maturing cultivar (NS640) at all seed positions was generally higher than that of other cultivars. However, there was little difference in protein percentage of maize cultivars under severe water deficit. Seeds of upper position of all cultivars showed comparatively more protein percentage under $I_1$ and $I_2$, but this difference decreased under $I_3$ and $I_4$ treatments (Fig. 5).

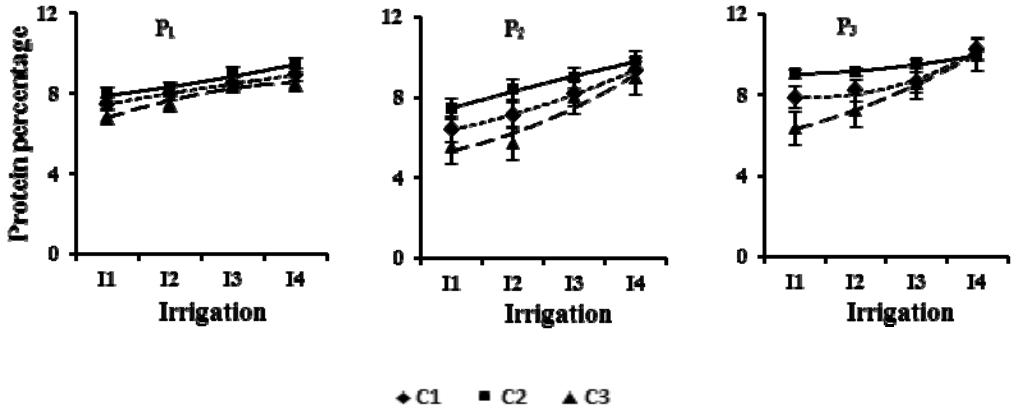


**Figure 5:** Changes in protein percentage of maize cultivars at different seed positions on the ear and irrigation treatments (Means ± SE).
$C_1$, $C_2$, $C_3$: cultivars DC303, NS640 and SC704, respectively.
$P_1$, $P_2$, $P_3$: upper, middle and lower position of seeds on the ear, respectively.
$I_1$, $I_2$, $I_3$, $I_4$: irrigation after 60, 80, 100 and 120 mm evaporation, respectively.

Protein yield of all maize cultivars decreased as a result of drought stress. This reduction in protein yield was higher for late maturing cultivar (SC704), compared with mid (NS640) and early (DC303) maturing cultivars. SC704 had the highest protein yield under all irrigation treatments. This advantage decreased with decreasing water supply (Fig. 6a). All maize cultivars produced considerably higher protein yield at lower position of seeds on the ear, followed by mid position seeds (Fig. 6b).

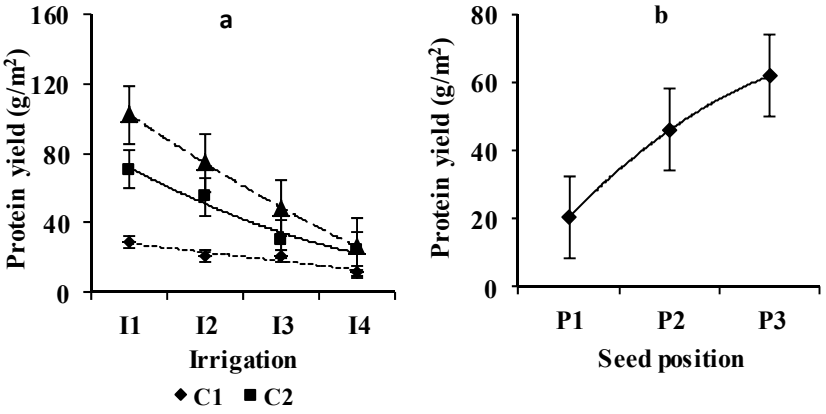


**Figure 6:** Seed protein yield of maize cultivars at different irrigation treatments (a) and seed positions (b) (Means ± SE).
$C_1$, $C_2$, $C_3$: cultivars DC303, NS640 and SC704, respectively.
$I_1$, $I_2$, $I_3$, $I_4$: irrigation after 60, 80, 100 and 120 mm evaporation, respectively
$P_1$, $P_2$, $P_3$: upper, middle and lower position of seeds on the ear, respectively.

## 4 DISCUSSION

Decreasing seed yield with increasing drought stress duration (Fig. 1) may be resulted from the short seed filling duration (Ghassemi-Golezani and Lotfi, 2013), as shown for chickpea (Davis et al., 1999), lentil (Erskine, and Ashkar, 1993), wheat (Li et al., 2000), barley (Samarah, 2005), maize (Aparicio-Tejo and Boyer, 1983), soybean (Berevedan and Egli, 2003) and sunflower (Whitfield et al., 1989). Reduced grain filling occurs due to decreased assimilate segregation and activities of sucrose and starch biosynthesis enzymes (Srivastava and Suprasanna, 2015). Drought stress occurring during seed development curtails the seed sink potential by reducing the number of endosperm cells and amyloplasts formed (Saini and Westgate, 2000), thus reducing seed mass as a result of a reduction in the capacity of the endosperm to accumulate starch (Nicolas et al., 1985). Higher seed yield at lower position of seeds on the ear (Fig. 1) was related with early formation and longer filling period of these seeds. The greater seed yield in late maturing cultivar could be attributed to longer period of radiation use and energy store of this cultivar, compared with other cultivars (Sangoi, 2000). These differences may be also influenced by the number of seeds per plant (Dalil and Ghassemi-Golezani, 2012; Ghassemi-Golezani et al., 2015).

Changes in oil and protein percentages of maize cultivars (Figs. 2a and 5) suggest that oil percentage decreases as protein percentage increases in response to water deficit. Similar results were reported for soybean under drought stress (Ghassemi-Golezani and Farshbaf-Jafari, 2012; Ghassemi-Golezani and Lotfi, 2013) and salt stress (Ghassemi-Golezani and Taifeh-Noori, 2011). It has been reported that the increase in

protein percentage is due to reduced carbohydrate accumulation under drought stress (Mahalakshmi et al., 2006). The inverse relationship between seed protein and oil contents makes it difficult to improve both traits simultaneously (Panthee et al., 2006).

Differences in oil and protein percentages among maize cultivars indicate that these parameters can be influenced by differences in genetic constitution (Ghassemi-Golezani and Lotfi, 2013). Reductions in oil (Figs. 2b and 4) and protein (Fig. 6a) yields due to water deficit were closely related with seed yield under different irrigation treatments (Fig. 1). Variations in oil and protein production of cultivars and seeds of different positions on the ear (Figs. 3b and 6) were also largely associated with seed yield per unit area. Therefore, differences in oil and protein yields among irrigations, cultivars and seed positions on the ear could be mainly attributed to the effects of these treatments on seed yield per unit area (Fig. 1). Similarly, Ghassemi-Golezani and Farshbaf-Jafari, (2012) found a significant positive correlation of oil and protein yields with seed yield of soybean.

Decreasing protein yield with increasing water deficit duration (Fig. 6a) could be attributed to inhibition of nitrate absorption (Ghassemi-Golezani and Lotfi, 2013). It has been stated that the reduction in nitrogen uptake under drought stress conditions might be due to the reduction of absorbed water and a decrease in root permeability (Strogonov et al., 1970). However, the negative effect of water limitation on nitrogen accumulation into seeds was less than that on dry matter accumulation. Therefore, the protein percentage of seeds increased (Fig. 5), but protein yield decreased (Fig. 6a) as a result of decreasing water availability.

## 5 CONCLUSION

Late maturing maize cultivar produced the highest seed yield, followed by mid and early maturing cultivars. Seed yield of all cultivars was higher at lower position of seeds on the ear than at middle and upper parts of the ear. The highest oil and protein yields of seeds were also obtained from lower position of ears. Seed yield of all maize cultivars at various seed positions on the ear decreased with decreasing water supply. Oil percentage decreased, but protein percentage increased as water deficit severed. Drought stress decreased oil and protein yields of maize cultivars at different seed positions on the ear, mainly due to reduction in seed yield per unit area.

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**Agris category code**: F30

# High-efficient transgenic hairy roots induction in chicory: re-dawn of a traditional herb

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**ABSTRACT**

Plant roots can be manipulated by *Agrobacterium rhizogenes* to stimulate the production of heterologous proteins for pharmaceutical applications as green cell-factories. During the present study, four bacterial strains (A4, ATCC15834, ATCC11325 and A13) in combination with three co-cultivation media (MS, B5, LS) were examined to establish an efficient and reliable transformation system for chicory (*Cichorium intybus* L.) using *A. rhizogenes*. The maximum chicory hairy roots induction was achieved using A13 strain. The observation confirmed that MS medium was more effective on hairy root growth. Dried biomass accumulation of hairy roots infected by A13 strain was 1.10 g $l^{-1}$ in MS medium which was significantly higher than those grown in LS and B5 medium (0.88 and 0.72 g $l^{-1}$, respectively). Beta-glucuronidase (GUS) gene was introduced by A13 strain carrying the pCAMBIA1304 binary vector. The results showed that the highest frequency of transformation (63.15 %) was achieved using A13 strain and MS cultivation medium. Detection of GUS and *hpt*II genes by PCR and GUS histochemical localization confirmed the integrative transformation in hairy roots. In conclusion, the whole process was successfully optimized as a pre-step to manipulate the chicory hairy root cells to improve the unique potential of secondary metabolite production.

**Key words:** Chicory, *A. rhizogenes*, hairy root, GUS, A13

**IZVLEČEK**

**UČINKOVITA INDUKCIJA TRANSGENIH LASASTIH KORENIN PRI NAVADNEM POTROŠNIKU: NOVA UPORABA TRADICIONALNEGA ZELIŠČA**

Korenine lahko z bakterijo *Agrobacterium rhizogenes* spremenimo v "zelene celične tovarne", ki proizvajajo heterologne proteine, uporabne v farmaciji. V tej raziskavi je bila preučevana uporaba štirih sevov bakterije *A. rhizogenes* (A4, ATCC15834, ATCC11325 in A13) v kombinaciji s tremi ko-kultivacijskimi gojišči (MS, B5, LS) za vzpostavitev učunkovitega transformacijskega sistema za navadni potrošnik (*Cichorium intybus* L.). Največja indukcija lasastih korenin je bila dosežena z uporabo seva A13. Opazovanja so potrdila, da je bilo za rast lasastih korenin učinkovitejše MS gojišče. Biomasa lasastih korenin, okuženih s sevom A13 je bila 1.10 g $l^{-1}$ na MS gojišču, kar je bilo značilno več kot pri rasti korenin na gojiščih LS in B5 (0,88 in 0,72 g $l^{-1}$). Gen za beta-glukuronidazo (GUS) je bil vnešen z A13 sevom, ki je vseboval pCAMBIA1304 binarni vektor. Izsledki so pokazali, da je bila največja frekvenca transformacije (63,15 %) dosežena z uporabo A13 seva in MS gojišča. Detekcija GUS in *hpt*II genov s PCR in GUS histokemično lokalizacijo je potrdila njuno vključitev v lasaste korenine. Celoten proces je bil uspešno optimiziran kot predstopnja v obdelavi celic lasastih korenin navadnega potrošnika za izboljšanje sposobnosti tvorbe sekundarnih metabolitov.

**Ključne besede:** navadni potrošnik, *A. rhizogenes*, lasaste korenine, GUS, A13

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## 1 INTRODUCTION

Induction of hairy roots in herbal plants by soil-borne bacterium *Agrobacterium rhizogenes* provides a useful systems for synthesis of valuable pharmaceutical compounds, among them also secondary metabolites. The induced hairy roots exhibit several superior features such as rapid growth, genetic and biochemical stability, ability to growth in hormone-free media, ease of maintenance and the ability to synthesize a variety of chemical compounds (Giri and Narasu, 2000). *A. rhizogenes* can also co-transfer the T-DNA of binary vectors which allows production of transgenic plants possessing foreign genes after regeneration from hairy roots (Lee et al., 2004; Tomilov et al., 2007). Transformation of several plant species including carrot (Srinivasan et al., 2014), cauliflower (Puddephat et al., 2001), mustard (Kastell et al., 2013), and potato (Otani et al., 1993) upon *A. rhizogenes*-mediated co-transformation has been reported. Typically, the regenerated plants are genetically stable and often have morphological and physiological changes like wrinkled leaves, extremely abundant and plagiotropic root system and also reduced apical dominance, internode length and leaf size (Tepfer, 1984). Chicory (*Cichorium intybus* L.), a member of Asteraceae family is traditionally used to cure various ailments and has also other beneficial properties. It has the ability to prevent liver damages, anti-ulcerogenic (ulcer healing effects) and anti-inflammatory effects, appetizer, digestive, stomachic (improving stomach function and increasing appetite), liver tonic, cholago, cardiotonic (acts as a stimulant of the heart). The root, leaf and seeds of chicory contain a number of medicinally important compounds such as inulin, sesquiterpene lactones, coumarins, flavonoids and vitamins (Nandagopal and Kumari, 2007). Recent pharmacological investigation of the root extract of this plant revealed immunomodulation and anticancer properties (Karimi et al., 2014). Sesquiterpene lactones which have high anti-cancer property were isolated from *A. rhizogenes* LBA 9402 transformed hairy roots of *C. intybus* in 2002 (Malarz et al., 2002). Moreover, in an investigation done by Bais et al. (2000) the possible benefits of coumarins production in hairy root cultures of *C. intybus* was proven; 4.06 and 3.71 fold increase in esculin and esculetin production as derivative of coumarin were achieved in induced hairy roots using fungal elicitors.

Several attempts have also been made to enhance hairy root induction, and to regulate pathways leading to production of important bioactive compounds. It was previously confirmed that optimizing the composition of organic/inorganic nutrients of the media for hairy root cultures is essential to gain high production of secondary metabolites (Sivakumar, et al., 2005). Different concentrations of salts and elicitors in culture media have a major role in hairy root growth and induction of secondary metabolites production (Wang and Wu, 2013). It is worth to mention that hairy roots growth drastically increase by change in some major mineral components (Pakdin et al., 2014).

In the present study the effect of different co-cultivation media and various *A. rhizogenes* strains on the induction and growth of hairy root cultures of chicory were studied. Moreover, *A. rhizogenes* strain A13 (MAFF02-10266) (Daimon et al., 1990) harboring a wild mikimopine-type Ri plasmid and pCAMBIA1304 vector was used for GUS transformation in hairy root of cotyledon explants. Introduction of GUS reporter gene was demonstrated by histochemical staining with X-glucuronide. The aim of the work presented here was improving a genetic transformation protocol for *C. intybus* and investigating the potential for genetic manipulation of the important secondary metabolite pathways.

## 2 MATERIALS AND METHODS

### 2.1 Plant material

Chicory's seeds (*Cichorium intybus* L., Asteraceae) were delivered by the Botanical Garden of Sari University in Iran. The seeds were surface-sterilized by immersion in a sodium hypochlorite solution (5 %) containing a wetting agent (Tween-20) for 30 min. Seeds were then

rinsed three times with sterile water and were cultured on MS medium containing 3 % sucrose and 0.8 % plant agar (pH=5.7) (Murashige and Skoog, 1962). Cultures were grown at 24±2 °C and 40±2 % relative humidity under a 16/8 h photoperiod supplied by cool white fluorescent lighting at an intensity of 68 µmol $m^2$ per second.

### 2.2 Hairy root induction

Hairy root induction in *C. intybus* was studied after treatment with various *A. rhizogenes* strains. To determine the best strain, *A. rhizogenes* A13 (MAFF02-10266), A4, ATCC 15834 and ATCC 11325 were co-cultivated with cotyledon explants of *C. intybus*. Single colonies of *A.rhizogenes* were inoculated in 10 ml of liquid LB (Luria-Bertani) medium containing 50 mg $l^{-1}$ kanamycin, and 40 mg $ml^{-1}$ riphampicin. The cultures were incubated overnight in a rotary shaker at 28±2 $^{o}$ C with shaking at 180 rpm in the dark. Three ml of the overnight culture was used to inoculate 50 ml of LB medium and was grown at the same condition, until an optical density (OD) of 0.3–0.6 at 600 nm. Then, the bacterial suspension was precipitated at 4500 rpm at 4 ˚C. The pellet was washed with inoculation medium (half-strength MS medium containing 100µM acetosyringon and 15 g $l^{-1}$ sucrose, pH 5.7) to a final density of OD600 = 0.6. Seven day-old and well established cotyledon segments of *C. intybus* grown in *in-vitro* condition were selected and immerged into the bacteria suspension for 2 min. The explants were then dried on sterile filter paper and were inoculated on three different co-culture media at pH 5.7: MS, LS (Linsmaier and Skoog) and B5 (Gamborg et al., 1968). Co-cultivation was prolonged for 48-72 hours to complete T-DNA insertion. The incubation condition was set on 25±2 ˚C in the dark. Following co-cultivation period, the explants were sub cultured on the same media supplemented with 500 mg $l^{-1}$ cefotaxime. This step was repeated to eliminate bacterial contamination thoroughly. The samples were screened to find the transformed ones. In order to throw light on the potential of different *A. rhizogenes* strains in hairy root induction, percentages of induced hairy root were counted out of 100 in each treatment group according to the observation of hairy roots development in cotyledon segments.

### 2.3 Hairy root growth

To survey the effect of different culture media on hairy root development, one of the established hairy root systems was cultured in three different media (MS, LS and B5). Ten mm long root tips were transferred to the new media. The cultures were grown at 25 ˚C in the dark and constant shaking at 90 rpm. Finally hairy root dry mass was recorded after 30 days to determine hair root growth capacity.

### 2.4 Bacterial strain and binary vector

Mikimopine-type *A. rhizogenes* strain A13 (MAFF02-10266) harboring a wild mikimopine-type Ri plasmid was used to optimize heterogene introduction into the induced hairy roots of *C. intybus*. To investigate the transformation efficiency, pCAMBIA1304 (CAMBIA, Canberra, Australia) binary vector was transferred to A13 strain by the method of Alkaline lysis (Birnboim and Doly, 1979). The pCAMBIA1304 harbor a GUS-*mgfp5* fusion reporter under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter, a selectable marker gene *hpt*II (responsible for hygromycin resistance) and bacterial selectable gene *npt*II (responsible for kanamycin resistance). The neomycin phosphotransferase II (*npt*II) was located out of T-DNA borders, allowing the kanamycin screening to identify positive transformants of *A. rhizogenes* A13 strain and for elimination of those which lacks the binary vector. Therefore, a concentration of 100 mg $l^{-1}$ kanamycin was used to select bacterial transformants. Finally, The PCR analysis was conducted using specific primers of the GUS reporter gene to confirm the transgenic bacteria. Forward (5'-ACGTCCTGTAGAAACCCCAA-3' and reverse (5'-CCCGCTTCGAAACCAATGCC-3') primers were synthesized by BIOMATIK (BIOMATIK, Canada). PCR amplification of GUS gene was conducted in a Bio-Rad thermocycler through 94 $^{o}$C (5 min), 35 cycles of: 94 $^{o}$C (1 min), 59 $^{o}$C (1 min) and 72 $^{o}$C (1 min) and final extension 72 $^{o}$C for 7 min. Amplification products were resolved and visualized on agarose gels. Amplification products were resolved and visualized on agarose gels.

### 2.5 Optimal antibiotic concentration for explant selection

The efficiency of six different concentrations of hygromycin (Sigma Aldrich, USA) (0, 2.5, 5, 10, 20 and 30 mg $l^{-1}$) were studied in order to determine the optimum antibiotic concentration to screen putative explant transformants. The hairy root segments were cultured on MS medium containing the antibiotic for 5 weeks. The hygromycin inhibition test was performed in triplicates for each antibiotic concentration and hairy roots color was monitored to assess the value of mortality i.e., browny roots considered as dead explants.

### 2.6 DNA analysis of chicory clones transformed by pCAMBIA1304 Vector

The survived clones of *C. intybus* on the antibiotic enriched medium were further analyzed using PCR. The aim was done to detect *rol*B and *rol*C genes in transformed lines. Total DNA was isolated according to Dellaporta et al. (1983) from the hairy root clones. The following sets of oligonucleotide primers were used to amplify the two genes of interest: *rol*B: 5-GCTCTTGCAGTGCTAGATTT-3 and 5-GAAGGTGCAAGCTACCTCTC-3; *rol*C: 5-CTCCTGACATCAAACTCGTC-3 and 5-TGCTTCGAGTTATGGGTACA-3. The absence of residual *A. rhizogenes* was confirmed by PCR detection of *vir*D gene which is outside the T-DNA of Ri plasmid with specific primers 5-ATGTCGCAAGGCAGTAAG-3 and 5-CAAGGAGTCTTTCAGCATG-3. DNA amplification reactions were performed under the following thermo cycle conditions: 94 ˚C (5 min), 35 cycles of 95 ˚C (45 s), 58 ˚C (30 s) for *rol*B and *rol*C and 50 ˚C for *vir*D genes and 72 ˚C (1 min), with a final extension step at 72 ˚C for 7 min. In order to select the GUS transformed clones, multiplex-PCR using GUS and *hpt*II primers was done on hairy root clones which amplified *rol* genes. The designed primers to amplify *hpt*II were 5'-CAGTCAATGACCGCTGTTATG-3' and 5'-AGACCTGCCTGAAACCGAACT-3'. Fifty nanograms of template DNA and 1µl of 10 pmol $\mu l^{-1}$ primer were mixed with 2.5 µl of 10X PCR buffer, 0.5 µl of 10mM dNTP mixture (equimolar dATP, dCTP, dGTP, dTTP), 1 µl of 50 mM $MgCl_2$ and 0.2 µl of *taq* DNA polymerase (Fermentas, Vietnam) (5 U $\mu l^{-1}$) in a total volume of 25 µl. Plasmid DNA from *A. rhizogenes* strain A13 was used as a positive control and natural roots of chicory were used as negative control. The expected PCR products were 430 bp for *rol*B, 612 bp for *rol*C and 430 bp for *hpt*II genes. The amplification products were separated by 1.5 % agarose gel electrophoresis, stained with ethidium bromide and photographed.

### 2.7 GUS histochemical assay

Hairy roots were subjected to X-glucuron treatment according to the method by Jefferson et al. (1987). Ten days after co-cultivation, roots were immersed in sodium phosphate buffer (50 mM and pH 7.0) containing 2 mM 5- bromo-4-chloro-3-indolyl-β-glucoronic acid (X-Gluc). The reaction was allowed to proceed for 20 h in the dark at 37 ºC. GUS-expressing cells were detected microscopically by the distinct blue color that developed as a result of enzymatic cleavage of X-glucuronide.

### 2.8 Callus induction and regeneration capability

Transgenic hairy roots harboring the GUS gene were cultured on solid MS medium containing different plant growth regulators (PGRs); combinations of benzyladenine (BA) (0.2, 0.5, 1 mg $l^{-1}$) and naphthalene acetic acid (NAA) (0.2, 0.5 mg $l^{-1}$) and also BA (0.2, 0.5 mg $l^{-1}$) and 2,4-D (0.5, 1, 2 mg $l^{-1}$) were supplemented into the callus-inducer medium. The samples were incubated at 25±2 in the dark for 4 weeks for callus induction.

Hairy root calli derived from different PGRs were subcultured in regeneration medium containing various PGRs, i.e. BA (0.2, 0.5, 1 mg $l^{-1}$) and IBA (0.2, 1 mg $l^{-1}$) and also BA (0.2, 0.5, 1 mg $l^{-1}$) and NAA (0.1, 0.5 mg $l^{-1}$). The effect of callus inducer medium on regeneration capability was lately studied.

### 2.9 Statistical analysis

All the experiments were set up in a completely randomized design (CRD) with three replicates per treatment. Data expressed as mean ± SD and the means were compared using one-way ANOVA and statistical significance of result measured by using Duncan's multiple range, Posthoc test (P = 0.05).

The statistical analyses were performed using the statistical package SPSS (Statistical Package for Social Science; version 17). Further analyses and also design graphs were done using Microsoft Excel 2010.

## 3 RESULTS AND DISCUSSION

### 3.1 Comparison of different bacterial strains and medium effect on transformation efficiency

Selection of the most effective bacterial strains by desired growth and virulence phenotypes have a significant impact on final explant transformation efficiency (Lee et al., 2010). Moreover, it has been previously confirmed that selection of appropriate medium before clone propagation stage has a significant impact upon the final efficiency of a commercial hairy root system (Pakdin and Farsi, 2013). Our results showed that the rates of transformation (were 63.15 %, 36.47 %, 18.2 % and 0 % for A13, A4, 15834 and 11325 strains respectively (Fig. 1). Transformation rates were calculated based on hairy roots' emergence and accordingly, 11325 strain did not induce any hairy root in the studied media. In all studied media, results showed that MS was the most suitable cultivation medium for hairy root induction and B5 was the least suitable. Finally we realized that the combination of A13 strain and MS medium is the best transformation for *C. intybus* hairy roots. Hairy root induction frequencies in different plant species by different *A. rhizogenes* strains is various and the ability of *A. rhizogenes* to infect plant species are strain dependent (Porter R, 1991; Sharafi et al., 2013). Similar studies were made on comparison of hairy root induction by different strains of *A. rhizogenes*. A4 and A13 strains showed highest efficiency in hairy root induction in *Solanum mammosum* L. which were 21.41 ± 10.60 % and 21.43 ± 8.13 % respectively (Ooi et al., 2013). In another study, hairy root induction frequencies in *Dracocephalum kotschyi* Boiss. was 52.3 %, 69.6 %, 48.6 %, 89.0 %, and 80.0 % using A4, A13, LBA9402, MSU440, and ATCC15834 strains, respectively (Sharafi et al., 2014).

However, a range of parameters including explant type, OD600 value of the *A. rhizogenes* cell culture, duration of co-cultivation, pH of co-cultivation medium and temperature during co-cultivation were previously evaluated in *A. rhizogenes*-mediated transformation (Henzi et al, 2000; Cao et al, 2009), but there are only few reports on the effect of the medium on the initiation of hairy roots after co-cultivation (Pakdin and Farsi, 2013; Bivadi et al., 2014). Our results, as shown in the Figure 1, show that induction medium had a significant effect on the ability of different *A. rhizogenes* strains for hairy root induction. MS medium with 45.34 % of root induction was the best combination for inducing of chicory hairy roots in all strains, LS and B5 medium with 35.94 % and 22.22 % respectively, were less effective. The induction frequencies of A13 and A4 in the LS and MS medium were not significantly different nonetheless, A13 showed the highest ability in MS medium. The results indicated that the effects of the induction media, *A. rhizogenes* strain and their interaction, were significant ($p$<0.05). The presence of interaction between culture media and *A. rhizogenes* strains illustrates that the hairy root induction ability of different *A. rhizogenes* strains is related to the induction medium. Pakdin et al. (2013), investigated hairy root induction in *Valeriana officinalis* L. using various *A. rhizogenes* strains, A4, ATCC 15834, ATCC 11325 and A13 along with three different induction media, half strength MS, Gamborg's B5 and LS Data showed that A4 had the greatest transformation frequency (54 %) in LS medium, while the best medium for ATCC 15834 was ½ MS with 40.67 % efficiency. ATCC 11325 showed a similar transformation frequency (9.67 %) in both ½ MS and LS media, but did not induce any hairy root in B5 medium and A13 did not induce hairy root at all (Pakdin and Farsi, 2013). Bivadi et al. (2014) reported that in full strength MS and ¼ MS medium, the transformation rate in *Hypericum perforatum* L. were 64.66 % and 47.30 %, whereas in ½ MS and B5 the transformation rate were 78 % and 86.33 % respectively. In another study, maximum transformation frequency of tested bacterial strain K599 on *Glycyrrhiza glabra* L. was 47 % obtained in 3 weeks old explants on MS basal semi solid medium (Mehrotra et al., 2008). During this study,

NB and B5 media showed only 20 % for transformation frequency. Moreover, WP medium did not support any induction of hairy roots in cultured leaf explants infected even after 50 days of incubation. These results indicate that selection of *Agrobacterium* strain and media conditions for co-cultivation is plant-species dependent and should be examined before transformation.

Given the pathogenicity severity of A13 strain compared to the other strains (more than 27 % more successful than the A4 strain), and also regarding to the growth rate of induced hairy root clones, A13 strain was selected for gene cassette transformation. Fortunately, interaction of A13 strain and MS medium successfully enhanced hairy root induction efficiency. Sensitivity of A13 strain to rifampicin and kanamycin had made easier the screening of plasmid-harboring bacteria in comparison with other agrobacterium strains.

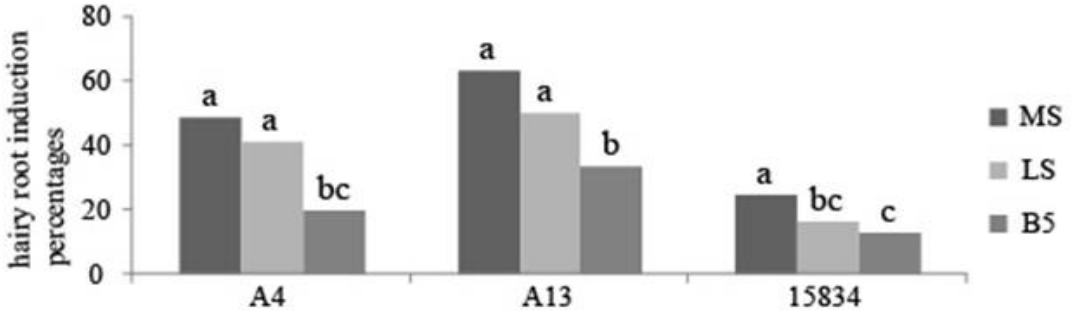


**Figure 1:** Comparison of different combinations of plant tissue culture media and *A. rhizogenes* strains on hairy root induction of chicory. Results are the mean of three replicates ± SD for percentages of induced hairy roots. Means with the same letter are not significantly different ($p > 0.05$).

### 3.2 Comparison of different media and bacterial strain on hairy root growth

The influence of nutrient supplementation through different culture media on the dry mass of hairy roots was determined. Based on the obtained results, MS medium affected the hairy root growth and dry mass positively; on the contrary, B5 medium provided the poorest condition for the hairy root development and consequently brought the least dry mass. In more details and according to the Table 1, accumulation of dried hairy roots biomass in MS medium which was infected by A13 strain was 1.10 g $l^{-1}$ which was significantly higher than those grown in LS and B5 medium 0.88 and 0.72 g $l^{-1}$, respectively (P = 0.05). Hence, MS medium was considered as the best medium for transgenic hairy root growth in following steps.

Maximum accumulation of biomass was recorded for hairy roots induced by A13 strain (1.10 g $l^{-1}$) in MS medium. In contrary, minimum dry mass of hairy roots was induced by A4 strain (0.05 g $l^{-1}$) in B5 medium (Table 1). Moreover, the interaction of variant hairy root-inducing bacterial strains with different culture media has been studied. Generally, the hairy roots induced by A13 strain present the highest dry mass of produced hairy roots, regardless of the cultivation medium. On the other hand, the effect of medium type on growth of hairy root induced by A4 stain was statistically significant in all different media. In brief, hairy roots growth was at least in B5 medium and the best records were observed in MS medium (Fig. 2).

These results were in line with previously published studies, e.g. B5 and ½ B5 media were the best basal media for hairy root growth of *V. officinalis* (Pakdin et al., 2014) and the NB medium composition supported best growth of hairy roots in *Glycyrrhiza glabra* followed by MS, B5 and WP media (Mehrotra et al., 2008). In *G. glabra* 20 times increase in root biomass on fresh mass basis was recorded after 45 days of culture in NB medium. Thus, it is clear that transformation frequency, hairy root induction and biomass accumulation are strain specific characteristics and are strongly affected by explants age and media ingredient (Sarma et al., 1997).

**Table 1:** Comparison of different combinations of plant tissue culture media and *A. rhizogenes* strains on hairy root dry mass. Results are the mean of three replicates for dry mass of induced hairy roots in *Cichorium intybus*. Means with the same letter are not significantly different ($p > 0.05$).

| Medium | Strain | Dry mass (g $l^{-1}$) |
|---|---|---|
| | A13 | $1.10^{A}$ |
| MS | A4 | $0.73^{B}$ |
| | 15834 | $0.34^{C}$ |
| | A13 | $0.88^{AB}$ |
| LS | A4 | $0.48^{C}$ |
| | 15834 | $0.43^{C}$ |
| | A13 | $0.72^{B}$ |
| B5 | A4 | $0.05^{E}$ |
| | 15834 | $0.16^{D}$ |

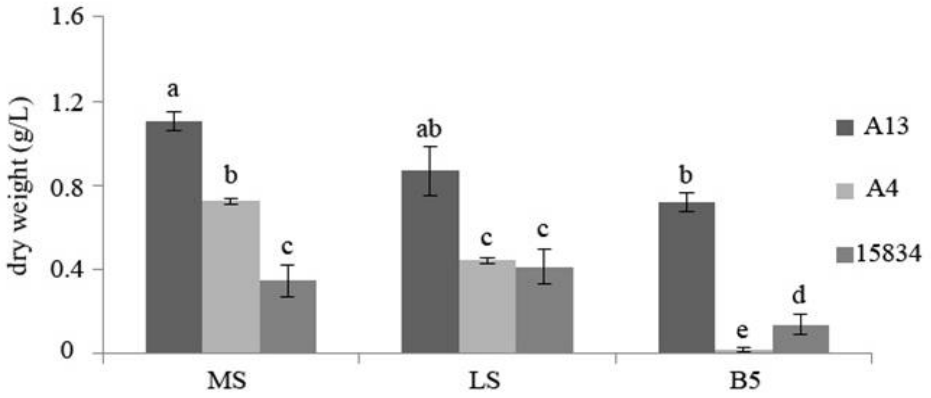


**Figure 2:** A comparison of the effects of different combinations of *Agrobacterium* strains and plant tissue culture media on dry weight of chicory hairy roots. The mean of three replicates ± SD for percentages of emerged hairy roots were calculated and those with the same letter are not significantly different ($p > 0.05$).

### 3.3 Molecular confirmation of transformed bacteria

Amplification of a 1098 bp fragment by PCR using specific GUS primers indicates the presence of the GUS sequence and also desired plasmid in selected colonies appeared on the selective medium (Fig. 3). Lack of GUS amplification in non-transgenic bacteria as a negative control and its amplification in extracted plasmid as a positive control had confirmed the plasmid transformation. Beside, in order to examine the expression of transferred reporter gene, GUS expression assay was performed. Transformed *Escherichia coli* T. Escherich, 1885 harboring pCAMBIA1304 vectors were stained for GUS activity with GUS staining solution. Appearance of blue color via x-Gluc hydrolysis approved the successful transformation and expression of GUS gene in T-DNA fragment of 1304 vector (Fig. 6.a).

Bacterial *uid* A gene encoded GUS, regarded as the most widely used reporter gene for gene expression in plants (Resmi et al., 2005). GUS consumes and brakes glucuronide as substrate, leading to a color reaction so that its presence is visible. As expected, culture medium containing bacteria, turned blue after 18 hours in staining solution that indicated GUS expression. Moreover, PCR with specific primers of *rol* B and C genes was conducted on extracted DNA from the selected colonies. Amplification of the fragments with approximate length of 430 bp for *rol* B and 612 bp for *rol*C genes confirmed the hairy root inducing characteristic of the suspected colonies (Fig. 4).

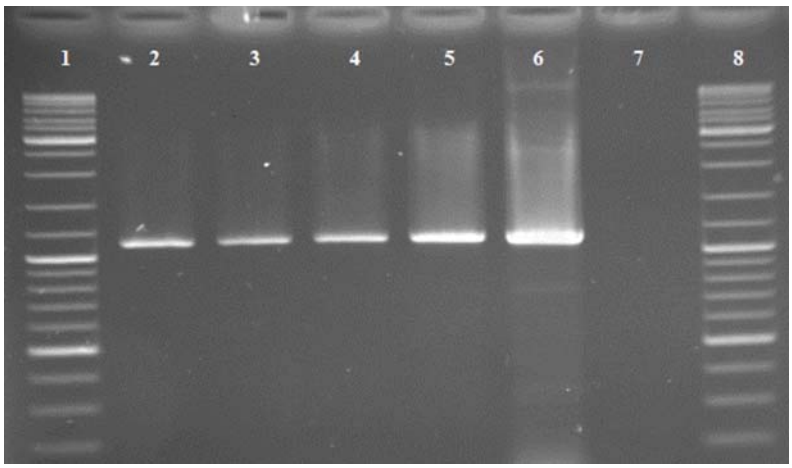


**Figure 3:** PCR analysis results of GUS gene in colonies of A13 strain: 1-Size marker (Fermentas SM0331), 2-4: Fragments amplified from *Agrobacterium* A13 strain single colonies 1 to 3, 5-Amplified fragment from *E. coli* harboring 1304 plasmid, 6-Amplified fragment from isolated plasmid pCAMBIA 1304 used as a positive control, 7- Absence of amplification fragment in empty A13 strain without the plasmid pCAMBIA 1304, 8-Size marker (Fermentas SM0331)

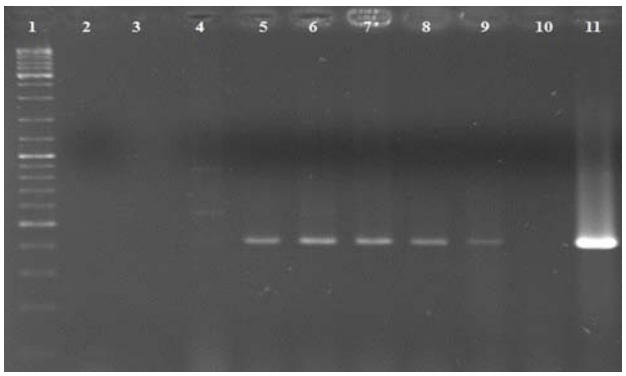


**Figure 4:** PCR analysis results of *rol* B gene: 1- Size marker (Fermentas SM0331), 2-4- Absence of fragment from non-transformed root clone derived DNA, 5-9- Amplified fragments from DNA isolated from hairy roots treated with A13,10- Absence of fragment from DNA isolated from un-treated root, 11- Amplified fragment from DNA isolated from *A. rhizogenes* strain A13.

### 3.4 Molecular confirmation of *C. intybus* transformed lines

#### 3.4.1 Selection of putative clones using hygromycin

In order to screen transgenic explants and determine minimum selective concentration, both transfected and control explants were grown in solid MS medium containing different concentrations of hygromycin (0, 2.5, 5, 10, 20, 30 mg $l^{-1}$). Non-transgenic roots grown only at concentrations lower than 5 mg $l^{-1}$. Roots on increased the concentrations of hygromycin led roots to turn black and finally died, therefore 5 mg $l^{-1}$ was chosen as the selective concentration. Thus, roots were selected after growing in selective medium and further were subcultured in liquid MS medium. In a study on *Lotus corniculatus* L., the authors reported 4 mg $l^{-1}$ of hygromycin as the best concentration for transformed line screening (Bo et al., 2009). As another example, transformed *Prunus domestica* L. was selected in 5 mg $l^{-1}$ of hygromycin (Lining et al., 2009).

#### 3.4.2 DNA analysis of hairy roots

To survey the stable transformation of hairy roots, amplification of *rol* genes with two specific primers was performed using genomic DNA

extracted from survived hairy root clones. The hairy roots were induced by *A. rhizogenes* harboring Ri plasmid and binary vector, pCAMBIA1304. The explants were further screened in medium enriched with 5 mg $l^{-1}$ hygromycin. The results of the separation of PCR amplified products and fragments detection confirmed the presence of two *rol* B (430 bp) (Fig. 4) and *rol* C (612 bp) genes in the genome of the hairy root cells (Krolicka et al., 2001). To verify the absence of residual *A. rhizogenes* infection on hairy roots, the *vir* D gene outside the T-DNA region of Ri plasmid was also studied. A putative size of PCR product for *vir* D was only amplified from the colony of *A. rhizogenes* and no product was obtained using DNA from any of the studied hairy root samples. The findings clearly confirmed that the hairy roots were not contaminated by *A. rhizogenes.*

Similar investigation was done using multiplex-PCR reactions with specific primers of GUS and *hpt*II genes. Among 20 putative transgenic hairy root clones, from 13 clones we positively amplified a fragment with 1098 bp length with the GUS primers. Four clones amplified the hygromycin gene segment with 430 bp length and a total of four clones were positive for both reporter genes by PCR. The electrophoresis pattern is shown in Figure 5. It indicates the introduction of binary vector T-DNA and *Agrobacterium* Ri plasmid T-DNA into genome of studied roots.

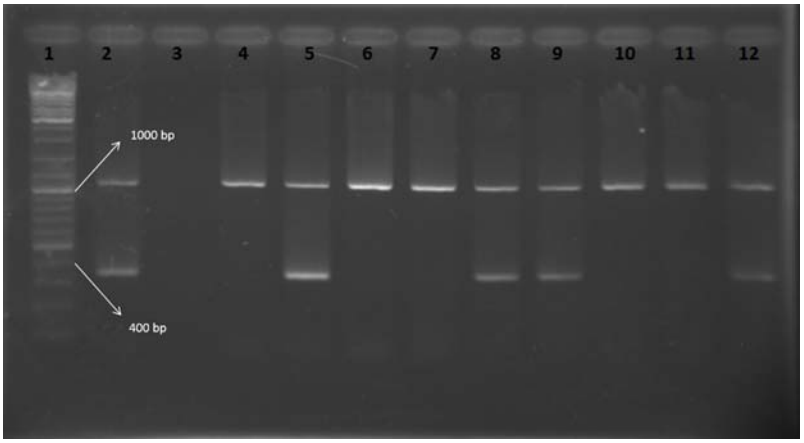


**Figure 5:** Hygromycin and GUS gene duplex PCR analysis results: 1-Size marker (Fermentas SM0331), 2-Amplified fragment from isolated plasmid pCAMBIA 1304 as positive control, 3- Absence of amplified fragment from non-transformed hairy roots, 4-6-7-10-11-Amplified fragments from transgenic hairy roots derived DNA that received only GUS gene, 5-8-9-12-Amplified fragments from transgenic hairy roots derived DNA that received both GUS and hygromycin genes.

### 3.5 GUS staining assay

To investigate *Uid* A gene expression, the samples which present positive amplification for both reporter genes were further analyzed using GUS histochemical assay. Blue color development induced by beta-glucuronidase reporter gene expression was observed in two transgenic hairy root clones, after histochemical staining. Only the tip of hairy roots became blue in clone 8 (Fig. 6 d) and in clone 21, the entire root was in blue color after histochemical staining (Fig. 6 f and g). Explanation for this pattern may be a response to the powerful activity of the CaMV 35S promoter in the vascular tissue which in turn result in accumulation of blue dots in root tips where are mitotically active sites. The reason of the GUS expression only in tip of the clone 8 is that is the most active in, as was found with bell pepper leaf like structures and also in the mitotic root tip (Yamakawa et al., 1998). This pattern of expression is consistent with previous results for GUS gene expression directed by the CaMV 35S promoter (Jefferson et al., 1987). The color after staining indicates successful transformation and expression of GUS reporter gene from *A. rhizogenes* in hairy roots of the chicory plant. Interestingly the growth rate of the clone No. 21 was lower than other clones. This observation was in line with the results of a study conducted in

transformation of phenylalanine ammonia-lyase (PAL) using A13 strain of *A. rhizogenes* to *Capsicum frutescens* L. (Yamakawa et al., 1998). In this study the morphology and growth rate of transgenic roots harboring *pal* gene varied from non-transgenic roots, growth rate of root was lower than in non-transgenic roots and the diameter was 3 times higher. Results showed that the GUS transformation and expression by A13 strain was highly stable because GUS expression was observed even a year after transformation and this indicates the stability of GUS transformation and expression and also insertion of the gene in to the plant genome and non-occurrence of methylation, deletion or mutation. The efficiency of transformation was estimated about 10 % in *C. frutescens* (Tamakawa et al., 1998) and 5 to 10 % in gene transformation using *A. rhizogenes* strain A13 in *Vaccaria pyramidata* Medik. *(*Masaaki et al., 2000).

### 3.6 Transgenic hairy root regeneration

Transgenic hairy roots cultured in callus induction medium were able to produce callus under the influence of all hormonal components. Production, development and friability of callus were significantly higher in medium containing 2, 4-D hormone and clearly increased with increased concentrations of 2, 4-D up to 2 mg $l^{-1}$ . After 8 weeks of growth in callus induction medium, calluses were transferred to regeneration medium. The produced calluses in all culture medium containing 2, 4-D were not able to develop green color in any of the regeneration medium and no color changes were observed after two months (Fig. 6jk). In other treatments containing (BA) (0.2, 0.5, 1 mg $l^{-1}$) and NAA (0.2, 0.5 mg $l^{-1}$), callus turned green as a sign of regeneration. The synergistic effect of NAA 0.5 mg $l^{-1}$ and BA 0.5 mg $l^{-1}$ showed a greenish white callus whereas higher concentrations of BA along with NAA 0.5 mg $l^{-1}$ produced a green colored callus. Whitish green and green color calli were developed under the influence of higher concentrations of BA. Growth of callus originated from hairy roots and also root pieces were reduced with increasing concentrations of applied cytokinin BA. At lower concentrations of BA (lower than 1 mg $l^{-1)}$ hairy roots and callus continued their rapid growth and concentration 1mg $l^{-1}$ of BA prevented callus growth. Although some transgenic hairy root calluses showed early stages of regeneration, such as the formation of a hard, compact and green callus, after two months chicory shoots development was not observed in any of the treatments (Fig. 6l).

According to the obtained results and previously published reports describing hairy root regeneration, lack of hairy roots regeneration in chicory were attributed to insufficient concentrations of growth regulators in the medium (especially cytokinin hormone). Therefore to achieve a desirable rate of regeneration, green callus could be sub-cultured into MS medium containing high concentrations of cytokinin (2-4 mg $l^{-1}$ BA or kinetin) in combination with low concentrations of auxin (0.5-1 mg $l^{-1}$ NAA or IAA). Based on our observations, after 8 weeks, the green color of callus tissue developed and the growth of hairy roots almost stopped. However, although the early stages of regeneration were observed in cultures, none of the applied treatments induced regeneration in transgenic chicory.

Several reports have provided details of plant regeneration from hairy roots of various species (Han et al., 1993; Choi et al., 2004). Plantlets regenerate directly from transgenic hairy roots after transferring to hormone-containing medium. For example shoot regeneration was promoted from hairy roots of *Robinia pseudoacacia* L. cultured in medium containing 10 μmol $l^{-1}$ NAA and 5 μmol $l^{-1}$ BAP (Han et al., 1993).

Moreover, transgenic roots can produce somatic embryos by adding appropriate plant growth regulators, e.g., Cho and Wildholm (2002) reported that plant hairy roots of *Astragalus sinicus* L. were developed to somatic embryos in media containing 7.5-10 mg $l^{-1}$ 2, 4-D. **C**hicory plants regeneration from hairy roots have been reported by Harsh et al. (2001). According to this study, supplementation of (4 BA and 1 NAA mg $l^{-1}$) was considered as the best motive agent for regeneration. In our study despite applying the before mentioned concentrations, no regeneration was observed, this may be caused by difference in the type of the strain used for induction of hairy roots or due to the location of T-DNA insertion into the plant genome. Unsuccessful shoot regeneration from hairy root has also been reported; Guellec et al.

(1990) failed to induce plant regeneration from transformed roots of *Vitis vinifera* L. obtained by *A. rhizogenes-mediated* transformation.

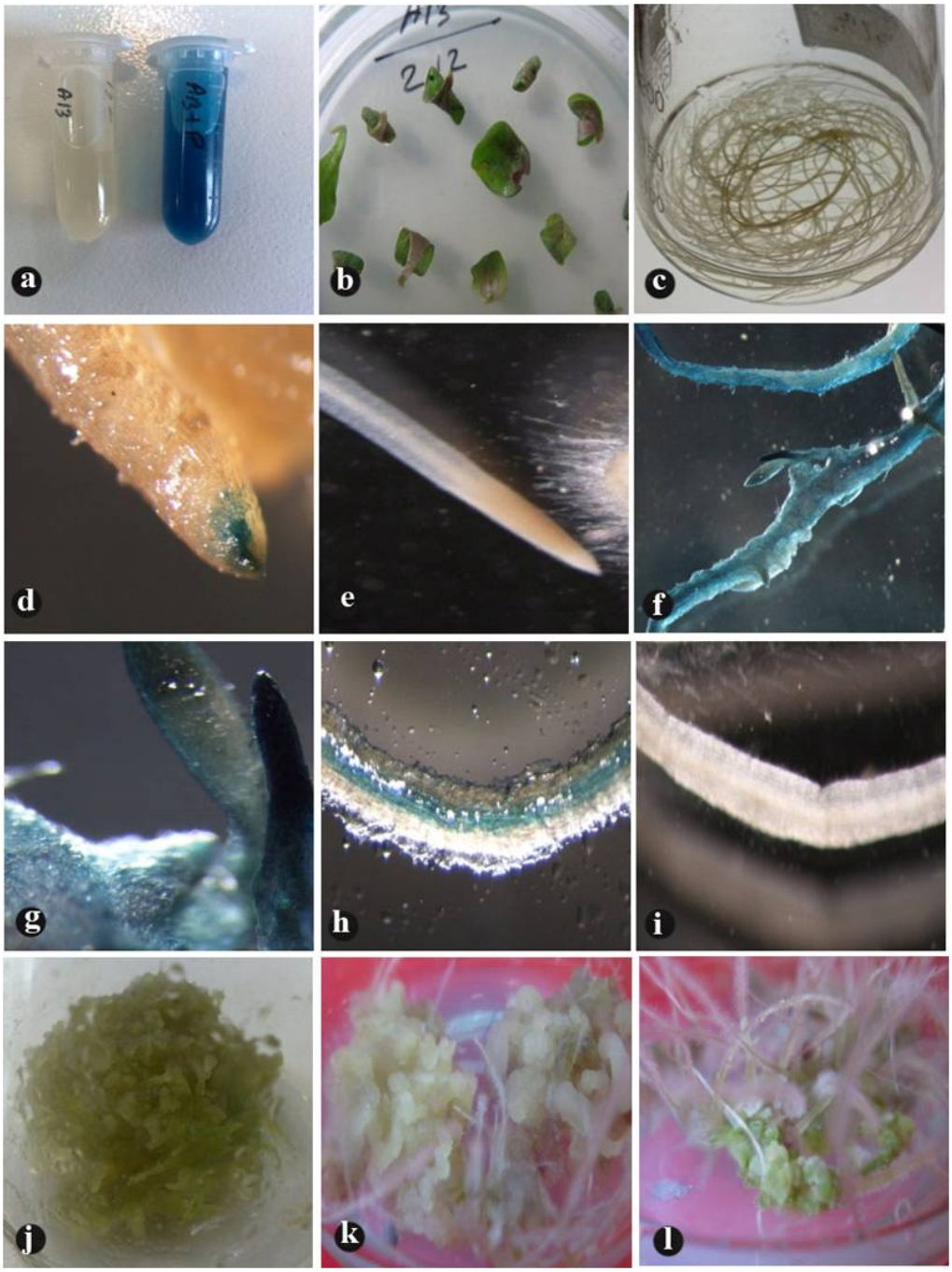


**Figure 6:** GUS expression in chicory hairy root. (a) Histochemical GUS staining of transgenic bacteria (right) and lack of GUS expression in non-transgenic bacteria (left), (b) Chicory cotyledon in MS co-cultivation medium, (c) Hairy root induced by A13 strain in liquid MS medium, (d) Transient GUS expression in tip of the root clone 8, (e) Lack of GUS expression in non-transgenic roots, (f, g) GUS expression in clone 21, (h) GUS expression in hairy root vascular tissue, (i) Lack of expression in non-transgenic root vascular tissue, (j) Hairy root callus developed on MS regeneration medium supplemented with 1 mg $l^{-1}$ IBAand 0.5 mg $l^{-1}$BA, (k) Hairy root callus developed on MS regeneration medium supplemented with 0.1 NAA and 0.5 BA mg $l^{-1}$ (l) Hairy root callus developed on MS regeneration medium supplemented with 1NAA and 4 BA mg $l^{-1}$.

## 4 CONCLUSION

As described in the introduction, chicory (*Cichorium intybus*) has received much attention in Persian/Chinese traditional medicine. We propose that an efficient transformation techniques could contribute not only to basic studies but also to the molecular breeding of chicory using various genetic resources. Moreover, secondary metabolite secretion using chicory hairy root platform could serve pharmacological application in which an optimized pre-step cultivation would end to a successful process.

## 5 ACKNOWLEDGMENTS

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# *In vitro* application of integrated selection index for screening drought tolerant genotypes in common wheat

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**ABSTRACT**

This experiment was conducted on 20 wheat genotypes during 2010-2011 growing season at the Razi University, Kermanshah, Iran. A completely randomized design with six replications was used for callus induction and a 20 × 2 factorial experiment with three replications was used for response of genotypes to *in vitro* drought stress. ANOVA exhibited highly significant differences among the genotypes for callus growth rate, relative fresh mass growth, relative growth rate, callus water content, percent of callus chlorosis and proline content under stress condition (15 % PEG). PCA showed that the integrated selection index was correlated with callus growth index, relative fresh mass growth, relative growth rate and proline content indicating that these screening techniques can be useful for selecting drought tolerant genotypes. Screening drought tolerant genotypes and *in vitro* indicators of drought tolerance using mean rank, standard deviation of ranks and biplot analysis, discriminated genotypes 2, 18 and 10 as the most drought tolerant. Therefore they are recommended to be used as parents for genetic analysis, gene mapping and improvement of drought tolerance.

**Key words:** biplot analysis, mature embryo culture, drought stress, physiological indicators, principal component analysis, *Triticum aestivum*

**IZVLEČEK**

***IN VITRO* UPORABA INTEGRALNEGA SELEKCIJSKEGA INDEKSA ZA IZBOR NA SUŠO ODPORNIH GENOTIPOV NAVADNE PŠENICE**

Poskus je bil izveden na 20 genotipih navadne pšenice v rastni sezoni 2010-2011 na Razi University, Kermanshah, Iran. Popolno naključni načrt poskusa s šestimi ponovitvami je bil uporabljen za indukcijo kalusa, z 20 × 2 faktorskim poskusom s tremi ponovitvami pa se je ugotavljal odziv genotipov pšenice na sušni stres v razmerah *in vitro*. ANOVA je pokazala visoko značilne razlike med genotipi v rasti kalusa, prirastku sveže mase, hitrosti njene prirasti, vsebnosti vode v kalusu, odstotku kloroze kalusa in v vsebnosti prolina v stresnih razmerah (15 % PEG). PCA analiza je pokazala, da je integralni selekcijski indeks koreliral z indeksom rasti kalusa, s prirastkom sveže mase, hitrostjo njene prirasti in vsebnostjo prolina, kar kaže, da so te presevne metode uporabne za izbor na sušo odpornih genotipov. Pri izboru na sušo odpornih genotipov s kazalniki ospornosti na sušo v *in vitro* poskusu kot osnovnim merilom, sta standardna deviacija vrednosti analiziranih znakov in "biplot" analiza izločili genotipe 2, 18 in 10 kot najbolj odporne na sušo. Zaradi tega so bili ti priporočeni za uporabo kot starševske rastline za genske analize, gensko mapiranje in izboljševanje odpornosti na sušo.

**Ključne besede:** biplot analiza, kultura zrelega embrija, sušni stres, fiziološki indikatorji, analiza glavnih komponent, *Triticum aestivum*

## 1 INTRODUCTION

Common wheat (*Triticum aestivum* L.) as one of the most widely adapted and strategic crop, plays an important role in food security and poverty alleviation and has an important role in economy

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(Khan et al., 2011). It is nutritious, easy to store and transport and can be processed into various types of food. Wheat production will have to be doubled to 1200 MT by the 2025 in order to meet increasing world demands and future needs (Vasil, 2003). But, wheat production is restricted by drought exposed areas and this loss led to considerable economic and social problems due to its great importance on human nutrition (Ilker et al., 2011).

Drought is one of the major causes of crop loss worldwide commonly reduces average yield for many crop plants by more than 50 % (Bayoumi et al., 2008; Pan et al., 2002). Reducing the losses of crop production due to drought stress is a major area of concern to ensure food security (Anjum et al., 2011). So, it is an urgent need to develop new genotypes with traits that could not only tolerate serious drought stress at various stages of growth but can also produce higher grain yield under drought stress conditions (Amiri et al., 2013). The ability of improving wheat genotypes that are able to maximum use of existing water and drought tolerant is one of the main aims of increasing grain yield potential in semi-arid and dry areas (Ghasemali et al., 2011). Therefore, developing high-yielding wheat genotypes under drought conditions in arid and semi-arid regions is an important aim of breeding programs (Leilah and AL-Khateeb, 2005). But success has been limited by inadequate screening techniques. Therefore, wheat breeders are always looking for new means to improve grain yield and other agronomic traits (Khakwani et al., 2011). The adoption of new criteria such as Integrated Selection Index (ISI) through a physiological approach may help in achieving some of the aims to increase wheat yield. A physiological approach would be the most attractive way to develop new varieties (Araus et al., 2008). Understanding the physiological processes associated with yield's trait relationships in modern wheat genotypes is essential to further increase grain yield and improve management strategies (Yea et al., 2011). Measurements of different physiological processes of plant response to drought is an important information on the reactions of the plant intended to remove or to reduce the harmful effects of water deficit in the soil or plant tissues.

An another view, breeding for drought tolerance by selecting solely for grain yield is difficult due to its low heritability under drought conditions (Farshadfar et al., 2012). Much attention is shifted towards crop improvement biotechnological technique. Tissue culture techniques are becoming increasingly popular as an alternative means of plant vegetative propagation, mass production of chemicals, and genetic engineering (Shah et al., 2009). Adoption of novel techniques such as exploitation of *In vitro* tissue culture may facilitate to increase food production and nutritional values of crops (Mahmood et al., 2012). *In vitro* selection technique has been used to improve abiotic environmental stresses such as cold hardiness, salt tolerance and drought tolerance (Zair et al., 2003; Bajji et al., 2004; Gawande et al., 2005). *In vitro* culture of plant cells and tissues such as mature embryos and immature embryos has attracted considerable interest over recent years because it provides a means to study plant physiological and genetic processes and offers a potential to improve cultivars by increasing genetic variability and are considered to be an important complement to classical plant breeding methods (Binott et al., 2009; Sorkheh et al., 2011).

The main aims of the present study were therefore to (1) screen bread wheat genotypes for drought tolerance under *in vitro* conditions and (2) introduce an integrated selection index for callus physiological indicators of drought tolerance.

## 2 MATERIALS AND METHODS

Twenty bread wheat genotypes including a cultivar and 19 landraces of Kermanshah province, listed in Table 1, were provided from Seed and Plant Improvement Institute of Karaj, Iran. A Completely Randomized Design (CRD) with six replications was used for callus induction and a $20 \times 2$ factorial experiment based on CRD design with three replications was carried out for response of genotypes to *in vitro* drought stress during 2010-2011 growing season at the Campus of Agriculture and Natural Resources, Razi University, Kermanshah, Iran (latitude 34˚ 21' North,

longitude 47˚ 9' East, altitude 1319 m above sea level). The genotypes were exposed to 15 % concentration of PEG 6000 (Merck, Germany) for 14 days, beside 0 % concentration as control. The growing morphogenic calli derived from mature embryos were also exposed to Murashige and Skoog (1962) medium without PEG and medium containing 15 % concentration of PEG. Spikes were harvested from main tillers at the physiological maturity stage of growing cycle. Seeds of them were rinsed with water then were surface-sterilized in 70 % (v/v) ethanol for 5 min, rinsed thrice with sterile distilled water, incubated further in commercial bleach (5 % sodium hypochlorite) for 10-15 min and rinsed thrice with sterile distilled water again. In order to easy separating of embryos, seeds were incubated in sterile distilled water for 30 min. All the operations and inoculation were performed under strict aseptic conditions in a laminar airflow cabinet. The surface-sterilized seeds were incubated at 33 °C for 2 h in sterile distilled water for imbibitions to occur. The mature embryos were easily separated from the endosperm in imbibed seeds and placed scutellum up on MS medium supplemented with 30 $g.l^{-1}$ sucrose and was adjusted to pH = 5.7, solidified with 8 $g.l^{-1}$ agar and 2.5 $mg.l^{-1}$ 2,4-dichlorophenoxy acetic acid (2,4-D) (Merck, Germany). The medium was autoclaved at 121 °C for 20 min and incubated at 25 °C for 28 days in growth chamber and in the darkness. Callus was maintained by sub-culturing every 21-28 days on the same MS medium. In drought stress conditions the cultures were kept in an incubator without any light. The following callus characteristics were measured under drought stress conditions:

**Table 1:** Codes and names of genotypes used in presented study

| Code | Genotype | Code | Genotype |
|---|---|---|---|
| 1 | WC – 5047 | 11 | WC – 47636 |
| 2 | WC – 4530 | 12 | WC – 4584 |
| 3 | WC - 4780 | 13 | WC – 46697 – 11 |
| 4 | WC – 4566 | 14 | WC – 4823 |
| 5 | WC – 47360 | 15 | Pishtaz |
| 6 | WC – 4640 | 16 | WC– 47341 |
| 7 | WC – 47456 | 17 | WC – 47379 |
| 8 | WC - 47628 | 18 | WC – 4931 |
| 9 | WC – 47367 | 19 | WC – 47381 |
| 10 | WC – 47399 | 20 | WC - 5053 |

### 2.1 Callus Growth Rate (CGR)

CGR ($mm.day^{-1}$) of cultured embryos on MS medium were measured at 7, 14, 21 and 28 days, respectively after transferring calli to medium. CGR was calculated using the following formulas (Compton, 1994):

$CGR_1 = d_7 / 7$, $CGR_2 = d_{14} / 7$, $CGR_3 = d_{21} / 7$, $CGR_4 = d_{28} / 7$

$CGR = (CGR_1 + CGR_2 + CGR_3 + CGR_4) / 4$

Where $d_7$, $d_{14}$, $d_{21}$, $d_{28}$ were diameter of callus in days 7, 14, 21 and 28, respectively. Diameter of callus was calculated as:

Diameter of callus (DC) = $\sqrt{length \times width}$

### 2.2 Relative Fresh Mass Growth (RFWG)

$RFMG = [(M_2 - M_1)] / M_1$

Where $M_1$ and $M_2$ are the initial mass of callus before and after four weeks, respectively (Chen et al. 2006).

### 2.3 Relative Growth Rate (RGR)

$RGR = [LnM_2 - LnM_1] / GP$

Where $M_1$ and $M_2$ are the initial and final mass of callus and GP is the growth period, respectively (Birsin and Ozgen, 2004). The time interval between two consecutive measurements was 21 days.

### 2.4 Callus Growth Index (CGI)

CGI or increasing value of callus fresh mass was calculated as:

$CGI = (M_1 - M_0) / M_0$

Where $M_0$ is the mass of callus before treatment and $M_1$ the final mass of callus after two weeks of treatment (Abdelsamad et al., 2007). Callus growth index was calculated for two levels of PEG (0 and 15 %) and the average of two levels was used for calculation.

### 2.5 Relative tolerance (Rt %)

Percentage of Rt was calculated for each genotype using the following formula (Abdelsamad et al., 2007):

Rt % = (value under stress / value under non-stress) × 100

### 2.6 Reduction Percentage (RP)

RP was calculated for the both stress (15 %) and non-stress level (0) using the following formula (Abdelsamad et al., 2007):

RP = (value under 15 % stress level - value at 0 % stress level)

### 2.7 Callus Water Content (CWC)

Callus samples of known fresh mass were dried in an oven set at 70 °C for 24 h and RWC was calculated by following formula (Errabi et al., 2006):
RWC = [(FM - DM) / DM] × 100
Where, FM and DM are the callus fresh and dry mass, respectively.

### 2.8 Percentage of Callus Chlorosis (PCC)

PCC (%) was determined visually as percentage of necrotic callus, 16 days after moving callus to the PEG containing medium.

### 2.9 Proline Content (PC)

Extraction and estimation of free proline content was done according to the procedure described by Errabi et al. (2006).

### 2.10 Integrated Selection Index (ISI)

Based on statistical analysis of studied traits and the following three formulas ISI was calculated (Farshadfar, 2012):

(1) $S_{ij} = (X_{ij} - \mu_j) / \sigma_j$

(2) $MP_{ij} = (S_{ij}d + S_{ij}w) / 2$

(3) $ISI_i = b_1MP_{i1} + b_2MP_{i2} + \ldots + b_jMP_{ij}$

Where $S_{ij}$ = standardized physiologic value of trait j (j = 1 to 9, i.e. CGR, RFMG, RGR, CGI, Rt %, RP, CWC, PCC and PC) in genotype i under non-stress (w) and stress (d) conditions, $X_{ij}$ = callus characteristics value of genotype i on trait j, $\mu_j$ = mean value of trait j in all genotypes, $\sigma_j$ = the standard deviation of trait j, $MP_{ij}$ = the mean productivity of trait j on genotype i, $b_j$ the mass value of trait j, $b_j$ was populated from the average contribution to factor 1 and ISI = integrated selection index. Formula (1) standardizes the value of different traits to the same unit of measure; formula (2) evaluates the appearance of genotypes for each trait; and formula (3) integrates the appearance of genotypes for all traits.

### 2.11 Statistical analysis

Analysis of variance, mean comparison using Duncan's Multiple Range Test and biplot analysis using principal component analysis (PCA), based on the rank correlation matrix were performed by MSTAT-C, SPSS ver. 16 and STATISTICA version 8. Standard Deviation of Ranks (SDR) was measured as:

$$S_i^2 = \frac{\sum_{j=1}^{m}(Rij - \bar{R}i)^2}{l-1}$$

Where $R_{ij}$ is the rank of *in vitro* drought tolerance indicator and $\bar{R}i$ is the mean rank across all *in vitro* drought tolerance indicators for the ith genotype and SDR= $(S_i^2)^{0.5}$.

Rank sum (RS) = Rank mean ($\bar{R}i$) + Standard Deviation of Rank (SDR) (Farshadfar and Elyasi, 2012).

## 3 RESULTS

### 3.1 Analysis of variance and mean comparisons

Highly significant differences were observed among the genotypes for CGR, RFMG and RGR (Table 2). Analysis of variance for callus growth rate (CGR), relative fresh mass growth (RFMG), relative growth rate (RGR), callus water content (CWC), percentage of callus chlorosis (PCC) and proline content (PC) indicated highly significant differences among the genotypes in the stress conditions (15 %) (Table 3). The analysis of variance also showed significant effect of the 15 % concentration of PEG on the indicators of drought tolerance in comparison with untreated control. Moreover, the genotype × drought interaction for CWC and PC was significant. The results indicated that CGR, RFMG, RGR and CWC decreased in the stress condition (15 % PEG level) as compared with non-stress condition (0 % PEG Level) but, PC and PCC were increased in 15 % PEG level as compared with 0 % PEG level (Table 4).

The results obtained from Table 5 exhibited that the highest amount of RFMG, RGR, CWC, PC and ISI belonged to genotypes number 2, while genotype number 13 showed the highest amount of CGR. The lowest amount of CGR, RGR, and PC was attributed to genotypes number 4, 11 and 20, respectively. Moreover, the lowest amount of RFMG, CWC and ISI were belonging to genotypes number 17. An integrated selection index (ISI) for drought resistance was proposed and used to identify drought resistant wheat genotypes. In ISI, nine traits including callus growth rate (CGR), relative fresh mass growth (RFMG), relative growth rate (RGR), callus growth index (CGI), relative tolerance (Rt %), reduction percentage (RP), callus water content (CWC), percentage of callus chlorosis (PCC) and proline content (PC) were chosen as the most relevant factors related to drought resistance, as determined by statistical analysis. In our study, genotypes number 17, 4 and 20 displayed the lowest and genotypes number 2, 1 and 10 the highest values for ISI. The highest and the lowest PCC were related to genotypes 17 and 2, respectively.

**Table 2:** Analysis of variance for callus induction traits

| S.O.V | Df | Mean Square | | |
|---|---|---|---|---|
| | | CGR | RFMG | RGR |
| Genotype | 19 | 0.003** | 0.094** | 0.004** |
| Error | 100 | 0.00018 | 0.003 | 0.00018 |
| CV % | - | 6.14 | 8.57 | 9.47 |

**= Significant at the 1 % probability level; CGR=Callus Growth Rate; RFMG=Relative Fresh Mass Growth; RGR=Relative Growth Rate

**Table 3:** Analysis of variance for mature embryos callus characters under stress conditions

| S.O.V. | df | Mean Squares | | | | | |
|---|---|---|---|---|---|---|---|
| | | CGR | RFMG | RGR | CWC | PCC | PC |
| Genotype (G) | 19 | 0.121** | 0.071** | 0.004** | 140.131** | 0.055** | 0.684** |
| Drought (D) | 1 | 1.126** | 0.421** | 0.014** | 3190.971** | 5.607** | 11.102** |
| D×G | 19 | 0.010ns | 0.018ns | 0.002ns | 81.198** | 0.013ns | 0.242** |
| Error | 80 | 0.011 | 0.011 | 0.001 | 3.811 | 0.015 | 0.004 |
| CV % | - | 7.92 | 9.77 | 3.37 | 2.29 | 8.87 | 3.87 |

ns and **= Non-significant and significant at the 1 % probability level, respectively; CGR=Callus Growth Rate; RFMG=Relative Fresh Mass Growth; RGR=Relative Growth Rate; CWC=Callus Water Content; PCC=Percentage of Callus Chlorosis; PC=Proline Content.

**Table 4:** Mean comparison of *in vitro* indicators of drought tolerance under stress (15 % PEG) and non-stress (0 % PEG) using mature embryo culture

| Drought (%) | CGR | RFMG | RGR | CWC | PCC | PC |
|---|---|---|---|---|---|---|
| 0 | 1.4385[a] | 0.3882[a] | 0.0185[a] | 90.3970[a] | 15.6033[a] | 2.0909[a] |
| 15 | 1.2448[b] | 0.1534[b] | -0.0027[b] | 80.0836[b] | 42.7461[b] | 4.3008[b] |

Means, in each column, followed by at least one letter in common are not significantly different at the 1 % probability level. CGR=Callus Growth Rate; RFMG=Relative Fresh Mass Growth; RGR=Relative Growth Rate; CWC=Callus Water Content; PCC=Percentage of Callus Chlorosis; PC=Proline Content.

## 3.2 Screening drought tolerance indicators and drought tolerant genotypes

### 3.2.2 *In vitro indicators of drought tolerance*

Callus growth index (CGI) exhibited remarkable differences among the genotypes in the means of increasing value of selected calli. Genotypes number 2, 10, 18, 19 and 13 showed the highest callus increasing value, respectively (Table 5). The highest amount of relative tolerance (Rt %) in the induced drought stress condition was attributed to genotypes number 5, 11, 3, 9 and 14, respectively. The lowest amount of reduction percentage (RP) from 0.0 to 15 % PEG belonged to genotypes number 17, 7, 3, 4 and 5 and the highest amount of RP was shown by genotype number 20 (Table 5). With regard to callus (resulted from mature embryos) increasing value, percentage of relative tolerance (Rt %) and the amount of reduction percentage (RP) genotypes number 2 and 5 were selected as the most drought tolerant at *in vitro* conditions.

**Table 5:** Ranks (R), ranks mean ($\bar{R}$), standard deviation of ranks (SDR), rank sum (RS) of *in vitro* indicators of drought tolerance using mature embryo culture and integrated selection index (ISI) of investigated genotypes

| Genotype | CGR | R | RFWG | R | RGR | R | CGI | R | Rt % | R | RP | R |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1.29 | 14 | 0.775 | 2 | 0.035 | 2 | 0.270 | 6 | 48.64 | 16 | 7.75 | 12 |
| 2 | 1.40 | 7 | 1.234 | 1 | 0.048 | 1 | 0.536 | 1 | 41.56 | 19 | 11.08 | 19 |
| 3 | 1.22 | 18 | -0.054 | 16 | -0.004 | 14 | -0.072 | 15 | 81.96 | 3 | 2.00 | 3 |
| 4 | 1.01 | 20 | -0.097 | 18 | -0.007 | 17 | -0.114 | 19 | 74.13 | 6 | 2.15 | 4 |
| 5 | 1.05 | 19 | 0.099 | 13 | 0.001 | 13 | -0.005 | 13 | 92.73 | 1 | 3.00 | 5 |
| 6 | 1.25 | 16 | -0.064 | 17 | -0.005 | 15 | -0.078 | 17 | 67.11 | 9 | 8.29 | 14 |
| 7 | 1.28 | 15 | 0.687 | 3 | 0.030 | 3 | 0.218 | 7 | 35.98 | 20 | 1.67 | 2 |
| 8 | 1.51 | 3 | -0.113 | 19 | -0.012 | 18 | -0.127 | 18 | 48.53 | 17 | 6.15 | 11 |
| 9 | 1.40 | 6 | 0.132 | 12 | 0.006 | 12 | 0.059 | 10 | 78.12 | 4 | 8.83 | 17 |
| 10 | 1.37 | 12 | 0.601 | 4 | 0.029 | 4 | 0.338 | 2 | 56.85 | 13 | 4.84 | 9 |
| 11 | 1.40 | 8 | 0.076 | 14 | -0.024 | 20 | -0.024 | 14 | 87.64 | 2 | 3.70 | 6 |
| 12 | 1.43 | 5 | 0.151 | 11 | 0.008 | 10 | 0.003 | 12 | 63.87 | 10 | 4.32 | 8 |
| 13 | 1.55 | 1 | 0.533 | 6 | 0.023 | 6 | 0.278 | 5 | 59.72 | 11 | 5.30 | 10 |
| 14 | 1.38 | 11 | 0.165 | 10 | 0.007 | 11 | 0.035 | 11 | 76.36 | 5 | 8.47 | 16 |
| 15 | 1.54 | 2 | 0.307 | 9 | 0.014 | 9 | 0.134 | 8 | 72.29 | 7 | 8.15 | 13 |
| 16 | 1.38 | 10 | 0.374 | 8 | 0.018 | 8 | 0.096 | 9 | 67.22 | 8 | 8.40 | 15 |
| 17 | 1.39 | 9 | -0.223 | 20 | -0.020 | 19 | -0.231 | 20 | 49.76 | 15 | 0.50 | 1 |
| 18 | 1.44 | 4 | 0.557 | 5 | 0.025 | 5 | 0.293 | 3 | 57.73 | 12 | 4.22 | 7 |
| 19 | 1.32 | 13 | 0.401 | 7 | 0.019 | 7 | 0.280 | 4 | 50.30 | 14 | 9.82 | 18 |
| 20 | 1.25 | 17 | -0.030 | 15 | -0.007 | 16 | -0.075 | 16 | 48.24 | 18 | 14.54 | 20 |

**Table 5:** Continued

| Genotype | CWC % | R | PCC % | R | PC | R | ISI | R | $\bar{R}$ | SDR | RS |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 87.04 | 6 | 19.58 | 2 | 2.75 | 13 | 4.92 | 2 | 7.5 | 5.68 | 13.18 |
| 2 | 89.07 | 1 | 19.11 | 1 | 6.93 | 1 | 11.30 | 1 | 5.2 | 7.51 | 12.71 |
| 3 | 86.47 | 9 | 32.52 | 17 | 2.64 | 14 | -3.91 | 17 | 12.6 | 5.64 | 18.24 |
| 4 | 88.01 | 3 | 39.87 | 19 | 1.53 | 18 | -5.90 | 19 | 14.3 | 6.96 | 21.26 |
| 5 | 85.60 | 13 | 20.00 | 3 | 3.64 | 7 | -1.43 | 13 | 10.0 | 5.68 | 15.68 |
| 6 | 85.22 | 17 | 30.07 | 12 | 4.10 | 5 | -2.01 | 14 | 13.6 | 3.95 | 17.55 |
| 7 | 85.80 | 12 | 24.32 | 4 | 3.90 | 6 | 3.75 | 4 | 7.6 | 6.06 | 13.66 |
| 8 | 87.24 | 4 | 33.85 | 18 | 4.43 | 3 | -2.02 | 15 | 12.6 | 6.79 | 19.39 |
| 9 | 85.45 | 15 | 30.78 | 13 | 2.64 | 15 | -1.07 | 11 | 11.5 | 4.03 | 15.53 |
| 10 | 86.47 | 10 | 26.31 | 6 | 4.70 | 2 | 4.35 | 3 | 6.5 | 4.17 | 10.67 |
| 11 | 86.70 | 8 | 28.33 | 10 | 2.99 | 9 | -3.15 | 16 | 10.7 | 5.29 | 15.99 |
| 12 | 86.42 | 11 | 31.82 | 16 | 2.81 | 11 | -1.20 | 12 | 10.6 | 2.84 | 13.44 |
| 13 | 85.10 | 18 | 31.66 | 14 | 2.80 | 12 | 2.08 | 7 | 9.0 | 4.97 | 13.97 |
| 14 | 85.49 | 14 | 26.93 | 9 | 2.56 | 16 | -0.65 | 10 | 11.3 | 3.33 | 14.63 |
| 15 | 85.22 | 16 | 26.66 | 8 | 3.21 | 8 | 1.25 | 9 | 8.9 | 3.67 | 12.57 |
| 16 | 87.09 | 5 | 26.40 | 7 | 4.13 | 4 | 1.99 | 8 | 8.2 | 2.97 | 11.17 |
| 17 | 69.58 | 20 | 49.46 | 20 | 1.46 | 19 | -9.74 | 20 | 16.3 | 6.43 | 22.73 |
| 18 | 88.91 | 2 | 25.14 | 5 | 2.39 | 17 | 3.04 | 5 | 6.5 | 4.58 | 11.08 |
| 19 | 86.77 | 7 | 28.75 | 11 | 2.89 | 10 | 2.43 | 6 | 9.7 | 4.32 | 14.02 |
| 20 | 73.74 | 19 | 31.79 | 15 | 1.32 | 20 | -4.03 | 18 | 17.4 | 1.90 | 19.30 |

CGR=Callus Growth Rate; RFMG=Relative Fresh Mass Growth; RGR=Relative Growth Rate; CGI=Callus Growth Index; Rt %=Relative tolerance; RP=Reduction Percentage; CWC=Callus Water Content; PCC=Percentage of Callus Chlorosis; PC=Proline Content; ISI=Integrated Selection Index.

### 3.2.2 *Biplot analysis method*

The relationships among different physiological indices of drought tolerance are graphically displayed in a biplot of $PCA_1$ and $PCA_2$ (Figure 1). The $PCA_1$ and $PCA_2$ axes which justify 71.46 % of total variation, mainly distinguish the indices in different groups. CGR groups RP and we refer to group 1 = G1 indices which introduce genotypes number 13, 15 and 16 as mild drought tolerant. The PCs axes separated ISI, CGI, RFMG, RGR, PC and CWC in a single group (G2) that identify genotype number 2, 1, 7, 10 and 18 as the most drought tolerant. PCC and Rt % were separated as groups 3 (G3) and 4 (G4) that distinguished genotypes 17, 20, 8 and 3, 4, 5, 11 as drought susceptible genotypes, respectively (Figure 1).

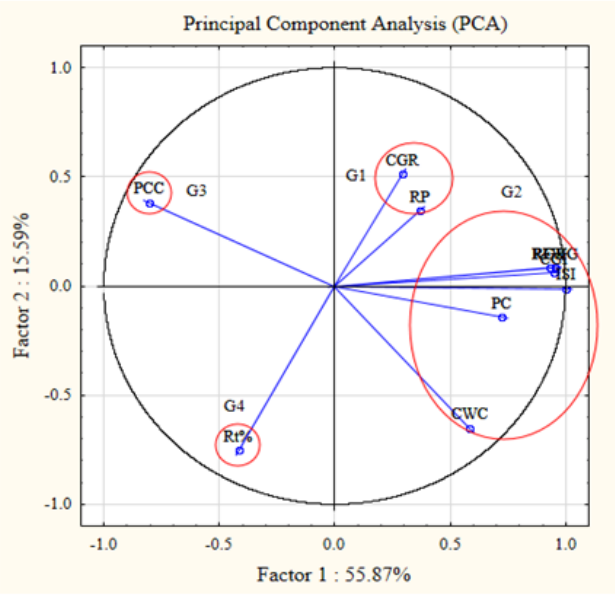


**Figure 1:** Biplot analysis of *in vitro* indicators of drought tolerance using mature embryo culture

3.2.3 *Ranking method*

In consideration to all indices, genotypes number 10, 18, 16, 15 and 2 exhibited the lowest RS respectively; hence they were identified as the most drought tolerant genotypes, while genotypes number 17, 4, 8, and 20 as the most sensitive (Table 5).

## 4 DISCUSSION

Observation of highly significant differences among the genotypes for CGR, RFMG and RGR indicated the presence of genetic variability, different responses of genotypes to callus induction and possible selection of callus induction in bread wheat genotypes at *in vitro* level using mature embryos of wheat. Abdulaziz et al. (2002) studied the callus to varying degree of polyethylene glycol (PEG)-induced water stress. They studied callus growth, water content and proline accumulation, and their results revealed that increasing water stress induced by increasing concentration of PEG caused a progressive reduction in callus fresh mass. In the present work, the sharp increase in proline content might theoretically, attribute to the genes for synthesis and degradation of proline which are up-regulated strongly under drought stress. It might be an adaptation to the purpose of which is to overcome the stress condition and it could supply energy for growth and survival and thereby help the plant to tolerate stress (Sankar et al., 2007). Abdelsamad et al. (2007) reported that significant differences of genetic responses were observed for the four wheat genotypes at 10 and 20 % PEG for callus induction, callus fresh mass, growth index, relative water content and relative tolerance percentage.

Principal component analysis (PCA), based on the rank correlation matrix was used to better understand the relationships, similarities and dissimilarities among the *in vitro* indicators of drought tolerance. One interesting interpretation of biplot is that the cosine of the angle between the vectors of two indices approximates the correlation coefficient between them. The cosine of the angles does not precisely translate into correlation coefficients, since the biplot does not explain all of the variation in a data set. Nevertheless, the angles are informative enough to allow a whole picture about the interrelationships among the *in vitro* indices (Yan and Kang, 2003). This procedure was also employed in chickpea (*Cicer arietinum* L.) (Zali et al., 2011) for clustering stability statistics, in barley (Khalili et al., 2013) and in bread wheat (Farshadfar et al., 2012; Farshadfar et al., 2013a,b) for screening selection criteria of drought tolerance.

The estimates of different indicators of drought tolerance indicated that the identification of drought-tolerant genotypes based on a single criterion was contradictory. Therefore, the ranking method can be used to have an overall judgment. In this method to determine the most desirable drought tolerant genotype according to the all indices mean rank, standard deviation of ranks and rank sum (RS) of all criteria is calculated. Results of the ranking method showed that genotypes number 10, 18, 16, 15 and 2 exhibited the lowest RS respectively; hence they were identified as the most drought tolerant genotypes, while genotypes number 17, 4, 8, and 20 as the most sensitive. These results are in agreement with the results of our new index (ISI). Therefore they can be used as parental materials for crossing, genetic analysis, mapping quantitative trait loci (QTLs) and marker assisted selection. The same procedures have been used for screening quantitative indicators of drought tolerance in barely (Khalili et al., 2013), in wheat (Mohammadi et al., 2011) and in bread wheat (Farshadfar, 2012; Farshadfar and Elyasi, 2012; Farshadfar et al., 2012; Farshadfar et al., 2013a,b).

## 5 CONCLUSION

Screening drought tolerant genotypes and indicators of drought tolerance using mean rank, standard deviation of ranks and biplot analysis under *in vitro* condition, which led to save time and money, discriminated genotypes "WC–4530", "WC–4931" and "WC–47399" as the most drought tolerant. These genotypes should be tested in a field trial and then looking for the association or correlation between *in vitro* and *in vivo* conditions. Therefore, these genotypes can be recommended to be used as parents for genetic analysis, gene mapping and improvement of drought tolerance in bread wheat when the results of *in vitro* and *in vivo* conditions are certified.

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**Agris category code**: F01

# Effects of tuber size, soaking hours and sprouting media on sprouting of tiger nut (*Cyperus esculentus* L. var. *sativa*) tubers

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**ABSTRACT**

Tiger nut, *Cyperus esculentus* L., is one of the underutilized and neglected food crops in most parts of the world leading to limited information on its production technology. A trial arranged in a 3×4×2 factorial of a completely randomized design was conducted in a green house of the Federal University of Agriculture, Abeokuta, Nigeria, to evaluate the effects of tuber size (large, medium and small), soaking duration (0, 24, 48 and 72 hours) and sprouting media (cotton wool and polythene bag) on the sprouting of tiger nut. Significant interactions were observed between growth media and soaking hours as well as between tuber sizes and soaking hours on the sprouting of tiger nut. Soaking beyond 24 hours before placement in sprouting medium led to a significant reduction in number of sprouted tubers in polythene bags. Small sized tubers had reduced ($p$<0.05) number of sprouts when soaked beyond 24 hours. Sprouting of tiger nut was better using medium size tubers soaked for 24 hours and placed between layers of cotton wool.

**Key words:** soaking hours, sprouting media, tiger nut, tuber sizes

**IZVLEČEK**

**UČINEK VELIKOSTI GOMOLJEV, UR NAMAKANJA IN NAKALITVENEGA MEDIJA NA NAKALITEV GOMOLJEV UŽITNE OSTRICE (*Cyperus esculentus* L. var. *sativa*)**

Užitna ostrica (*Cyperus esculentus)* je ena izmed premalo uporabljenih in spregledanih vrtnin v večjem delu sveta zaradi pomanjkanja informacij o tehnologiji njene pridelave. V ta namem je bil v rastlinjaku Federal University of Agriculture, Abeokuta, Nigeria izveden naključni 3×4×2 faktorski poskus za ovrednotenje učinkov velikosti gomoljev (veliki, srednji in drobni), časa namakanja (0, 24, 48 in 72 ur) in nakalitvenega medija (bombažna volna in polietilenske vrečke) na nakalitev užitne ostrice. Značilni medsebojni učinki so bili opaženi med rastnim medijem in časom namakanja in velikostjo gomoljev. Namakanje več kot 24 ur pred namestitvijo gomoljev v nakalitveni medij je vodilo k značilnemu zmanjšanju nakalitve v polietilenskih vrečkah. Majhni gomolji so imeli manjše ($p$<0.05) število poganjkov, če so bili prej namakani več kot 24 ur. Nakalitev gomoljev užitne ostrice je bila boljša, če so bili uporabljeni srednje veliki gomolji, namakani 24 ur in položeni med plasti bombažne volne.

**Ključne besede:** ure namakanja, nakalitveni medij, užitna ostrica, velikost gomoljev

## 1 INTRODUCTION

Tiger nut, *Cyperus esculentus* is one of the underutilized food crops grown in many parts of the world, with little climatic challenges. It is still considered a mere weed in many quarters: for example it was one of the plants listed as weeds with some food value in Africa (Hillocks, 1998). More recently, this crop has also been recognized as one of the 32 uncultivated plants with agricultural potentials in Nigeria (NACGRAB, 2008). Tiger nut though not usually cultivated in

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the southwestern part of Nigeria, it is not only consumed but it is a source of income to certain groups of people. Tiger nut is sold commercially as either fresh tubers (achieved after hours of soaking) or dried tubers especially by the Hausas who are seen displaying tiger nut and selling a kilogram of its fresh tubers for about $1.

According to Sanchez-Zapata et al. (2012), tiger nut has attracted very little scientific and technological attention. This is in spite of its numerous food, health and economic benefits. Tiger nut is popular in Spain where it is grown primarily for the production of "horchata de chufa" (wikipedia encyclopedia ), it contains appreciable amount of essential elements like Na, K, Mg, Mn, Ca, Fe and Zn (Imam et al., 2013), it is also rich in dietary fiber, vitamins C and E, used in control of diabetes and blood pressure (www.tigernuts.com, Bamishaiye and Bamishaiye, 2011, www.naij.com, empowered sustenance.com, www.naij.com,).

Exploiting the potentials of such underutilized food crops will become more and more cardinal to achieving food security especially in the developing countries and ensuring ecological stability in the face of ever increasing human population and climate change.

Constraints identified to be restricting the production of tiger nut include the label of the crop as a mere weed by many developed countries, difficulties of harvesting the tubers, problems of pest like red ants, termites and rodents. Another important constraint is erratic field establishment due to lack of uniformity in tuber sizes and differential rate of sprouting when planted directly. Adequate stand establishment cannot be compromised in crop production as labor, time, solar radiation and other inputs will be wasted if the field is scanty. Efforts directed at ensuring uniform field include soaking of tubers intended for planting and sprouting the tubers in baskets, while picking sprouted tubers at intervals for field establishment. Teiteh and Ofori (1998) reported that in Ghana, the common practice among local growers of tiger nut is to soak the tubers in water for 3 to 4 days to facilitate germination while changing the water every day to prevent rot; and that the soaked nuts may be planted direct, or kept in a basket until they begin to sprout between 7 and 12 days after soaking. Sprouted nuts are picked every day and planted.

Repeated picking of sprouted tubers for field establishment will be cumbersome and boring as a single operation will be carried out several times before completion. Furthermore, since agriculture is time bound, loss of time could later lead to exposure of crops to shortage of growth resources especially water in rain-fed agriculture. Since the tubers also vary in size, the establishment of an appropriate soaking period for a range of tuber size is important. Preparation of an appropriate growth medium that could produce more synchronized sprouting can be very helpful for the production of tiger nut.

The aim of the study was to evaluate the sprouting of tiger nut as affected by tuber sizes, soaking hours and different growth media. The objectives of the trials are:

1. To evaluate the effect of soaking duration on the sprouting.
2. To determine appropriate soaking duration for different tuber sizes.
3. To evaluate the effect of sprouting media on the rate of sprouting of tiger nut.

## 2 MATERIALS AND METHODS

The trial was carried out in a screen house of the College of Plant Science and Crop Production of the Federal University of Agriculture, Abeokuta, between January and February 2015.

Dried tiger nut tubers used for both trials were sorted into small, medium and large by visual observation and then weighed. The average tuber weights based on 240 tubers were 0.14 g, 0.31 g and 0.52 g, for small, medium and large tubers respectively. The sorted tubers were soaked in water for 0, 24, 48 and 72 hours.

The soaking treatment commenced with the tubers that were soaked for 72 hours, then those soaked for 48 hours and finally those soaked for 24 hours,

in order to conclude all treatments at the same time.

This trial was a 3×4×2 factorial based on a completely randomized design involving three replicates. There were three tiger nut tuber sizes large, medium and small, as specified above, soaked in water at a room temperature for either 0, 24, 48 or 72 hours and sprouted in either polythene bag or cotton wool.

After the soaking treatments, twenty tubers each of small, medium and large tuber sizes which had been soaked for 24, 48, or 72 hours were then placed between two layers of cotton wool or black polythene in Petri dishes for the sprouting trial, and the control (0 hour or no soaking) was used in the analysis as the standard. The standard planted in the soil to represent direct planting. Both the cotton wool and polythene bag were sprinkled with water to prevent desiccation. The polythene bag was then loosely tied while for the cotton wool, its top layer was used to cover the tubers before water was sprinkled on them.

### 2.1 Data collection

The data collected involved cumulative number of sprouted tubers on daily basis from the 3$^{rd}$ to the 14$^{th}$ day after being kept in sprouting media. Temperatures of the growth media were also recorded. The collected data were subjected to analysis of variance (ANOVA), while means that were significantly different were separated using the least significant difference (LSD) at 5 % probability level using GENSTAT statistical package.

## 3 RESULTS AND DISCUSSION

### 3.1 Effects of tuber sizes, soaking hours and growth media on sprouting of tiger nuts

Table 1 presents the effect of tuber sizes, soaking hours and growth media on sprouting of tiger nuts. Sprouting was not significantly affected by both tuber sizes and soaking hours except on the 4$^{th}$ day after placement on growth media when the control (no priming) was characterized by a significantly lower number of sprouted tubers relative to other treatments.

The growth media significantly affected the sprouting. The tubers enclosed in cotton wool sprouted significantly faster than those in polythene bags ($P$<0.05) from the 3$^{rd}$ to the 14$^{th}$ day of observation. The interaction between the soaking hours and growth media on the sprouting of tiger nut was also significant from the fourth to fourteenth day after placement in the growth media.

Better sprouting in cotton wool when compared to the polythene bag, in this study, could be due to better aeration. Oxygen is needed for germination and might be limited in polythene bags, even though polythene bag could better conserve moisture and have higher temperature which are also factors required for germination. The superiority of these two factors in polythene bag did not lead to a faster rate of sprouting. The oxygen level has been shown to be an important environmental factor in seed germination, Data from germination of cauliflower (*Brassica oleracea* L. var. *botrytis*) showed that reducing $O_2$ percentage to 10 % slightly delayed germination, but further reductions to 5 or 3 % both delayed and reduced the final germination percentage (Power and Fonteyn, 1995). Similarly, the major impact of oxygen supply in seed germination can be seen in the fact that once germination begins with seed imbibitions, dry seeds resume metabolic activities including respiration (Bradford et al., 2007).

**Table 1**: Effects of tuber sizes, soaking hours and growth media on sprouting of tiger nut tubers

| | Cumulative number of sprouted tubers on different days after planting | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | **3rd** | **4th** | **5th** | **6th** | **7th** | **8th** | **9th** | **10th** | **11th** | **12th** | **13th** | **14th** |
| **Tuber Size** | | | | | | | | | | | | |
| **Small** | 3.4 | 5.8 | 7.9 | 9.3 | 9.6 | 10.5 | 11.2 | 11.7 | 12.5 | 12.9 | 13.3 | 13.6 |
| **Medium** | 6.0 | 7.3 | 9.2 | 11.0 | 11.7 | 13.4 | 13.7 | 14.0 | 14.7 | 15.1 | 15.3 | 15.3 |
| **Large** | 6.5 | 8.9 | 11.0 | 12.3 | 12.6 | 13.4 | 13.6 | 13.8 | 14.1 | 14.8 | 14.8 | 14.8 |
| **LSD** | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| **Soaking hours** | | | | | | | | | | | | |
| **0** | 3.0 | 2.8 | 7.9 | 10.8 | 11.8 | 13.0 | 13.4 | 13.4 | 13.7 | 14.9 | 15.2 | 15.2 |
| **24** | 5.5 | 9.3 | 10.8 | 11.6 | 12.3 | 14.1 | 14.9 | 15.5 | 16.3 | 16.6 | 16.8 | 16.8 |
| **48** | 7.2 | 8.8 | 9.5 | 10.8 | 10.8 | 11.8 | 12.1 | 12.6 | 13.3 | 13.6 | 13.6 | 13.6 |
| **72** | 5.4 | 8.4 | 9.3 | 10.3 | 10.4 | 10.7 | 10.7 | 11.1 | 11.6 | 12.1 | 12.2 | 12.7 |
| **LSD** | ns | 2.72 | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| **Growth Media** | | | | | | | | | | | | |
| **Cotton Wool** | 7.2 | 9.9 | 12.1 | 14.6 | 15.3 | 16.3 | 16.6 | 16.8 | 16.9 | 17.3 | 17.4 | 17.4 |
| **Poly Bag** | 3.4 | 4.8 | 6.7 | 7.1 | 7.3 | 8.5 | 9.0 | 9.5 | 10.6 | 11.3 | 11.5 | 11.8 |
| **LSD** | 1.64 | 1.92 | 1.74 | 1.73 | 0.84 | 1.76 | 1.79 | 1.82 | 1.85 | 1.85 | 1.84 | 1.84 |
| **TS×SH** | 4.01 | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| **TS× GM** | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| **GM× SH** | ns | 3.84 | 3.48 | 3.46 | 1.68 | 3.52 | 3.58 | 3.64 | 3.70 | 3.71 | 3.67 | 3.68 |
| **TS×SH×GM** | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |

**TS** – Tuber Sizes **SH** – Soaking Hours **GM** – Growth Media ns = not significant

### 3.2 Interaction of growth media and soaking hours on sprouting of tiger nut tubers

The interactions of growth media and soaking hours on the sprouting of tiger nut from the 4th to 14DAP (days after planting) is presented in Figures 1-11. A similar pattern was observed across all the days of observations; sprouting in cotton wool was best achieved by soaking the tubers for 48 hours. On the other hand apart from the observation made on the fourth day after placement, sprouting of tiger nut in the polythene bags declined as the duration of soaking increased beyond 24 hours, this cut across the 5th to the 14th day after placement in the polythene bag.

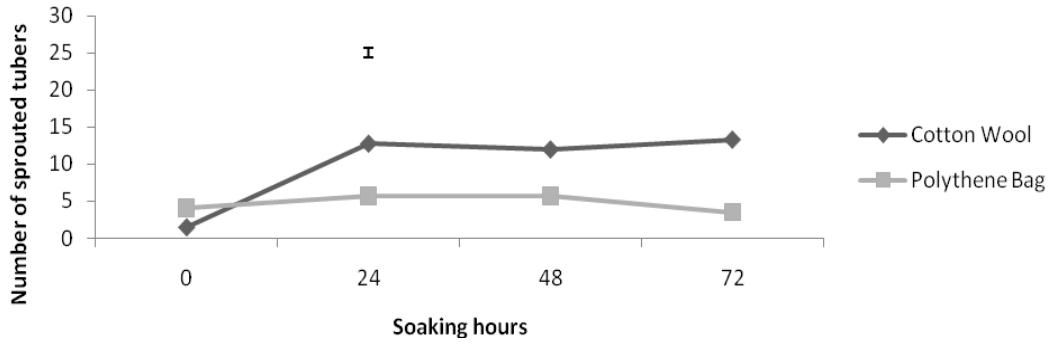


**Figure 1:** Interaction of growth media and soaking hours on sprouting of tiger nut tubers at 4DAP

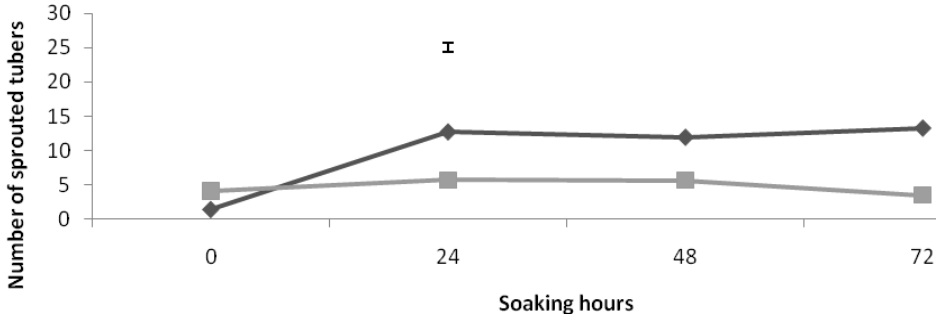


**Figure 2:** Interaction of growth media and soaking hours on sprouting of tiger nut tubers at 5DAP

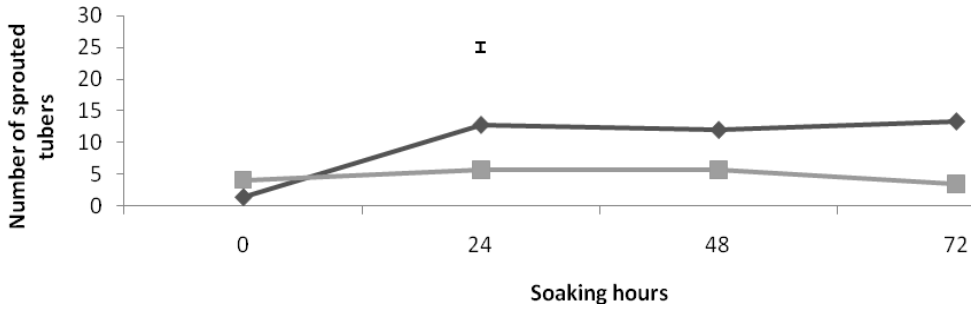


**Figure 3:** Interaction of growth media and soaking hours on sprouting of tiger nut tubers at 6DAP

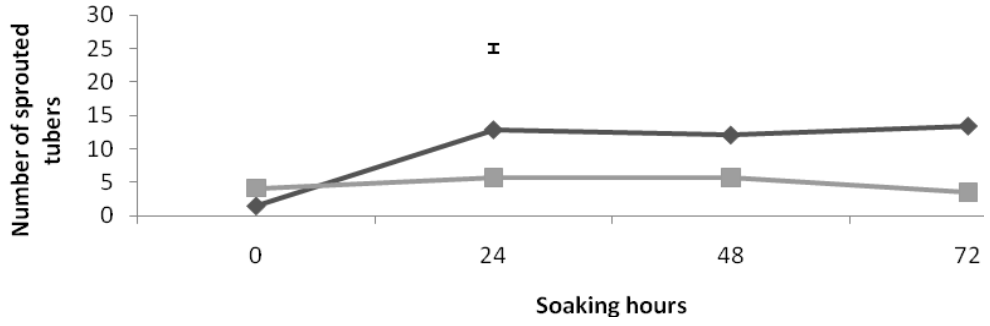


**Figure 4:** Interaction of growth media and soaking hours on sprouting of tiger nut tubers at 7DAP

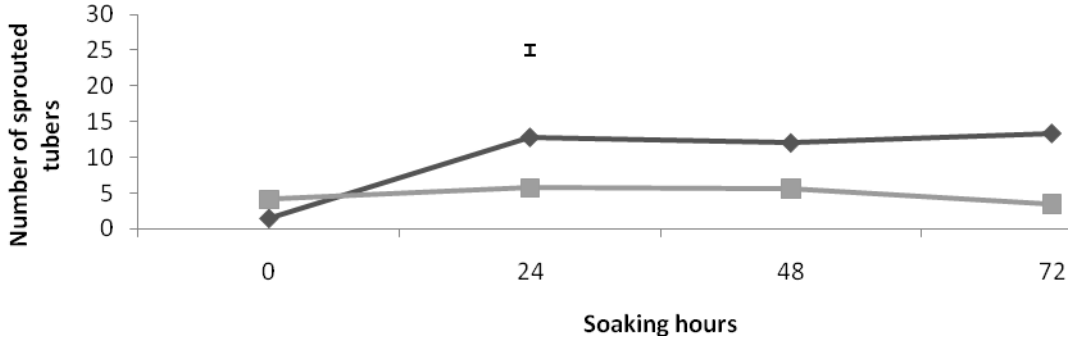


**Figure 5:** Interaction of growth media and soaking hours on sprouting of tiger nut tubers at 8DAP

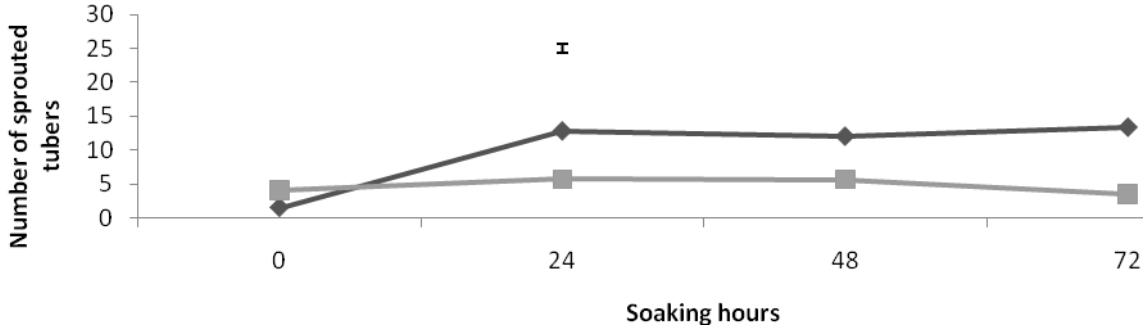


**Figure 6:** Interaction of growth media and soaking hours on sprouting of tiger nut tubers at 9DAP

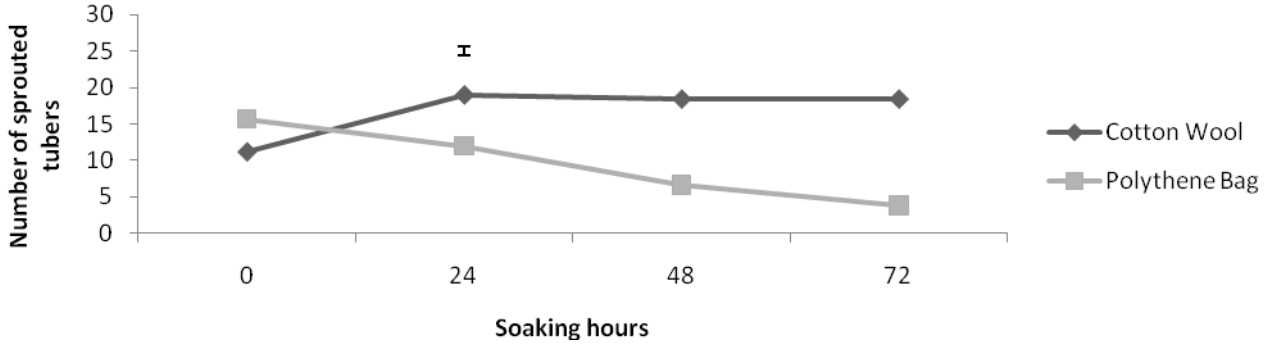


**Figure 7:** Interaction of growth media and soaking hours on sprouting of tiger nut tubers at 10DAP

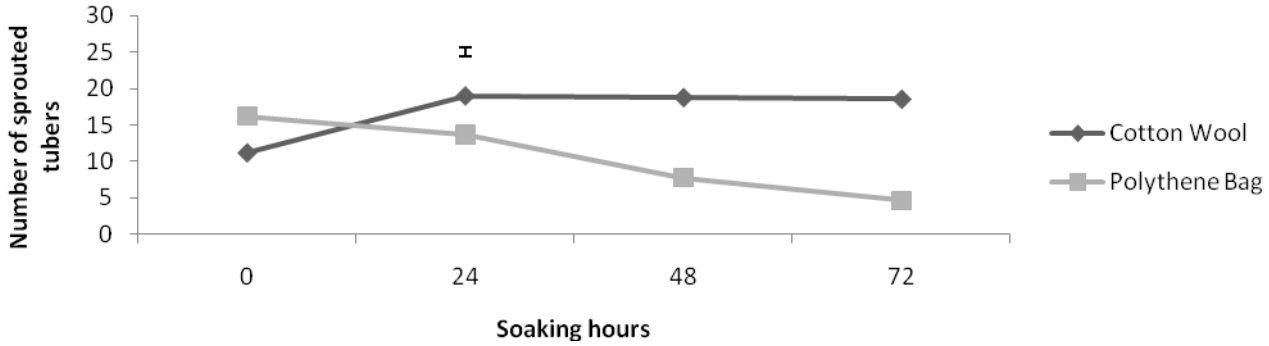


**Figure 8:** Interaction of growth media and soaking hours on sprouting of tiger nut tubers at 11DAP

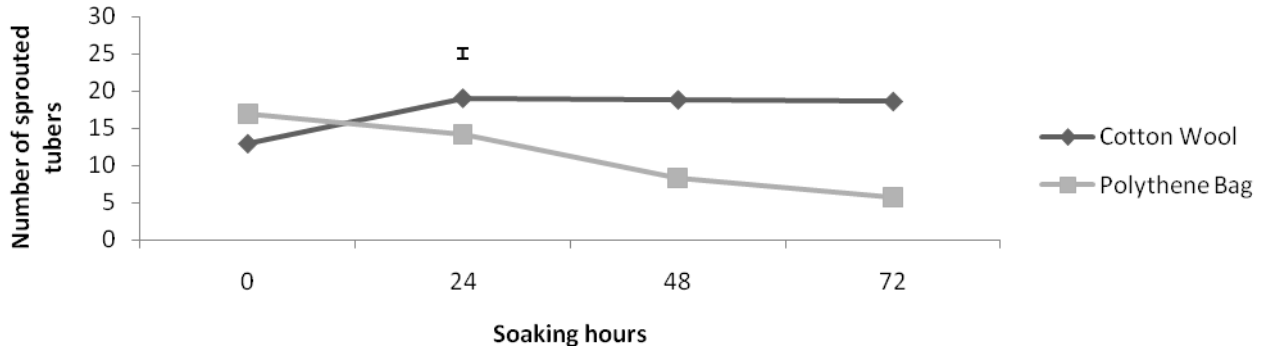


**Figure 9:** Interaction of growth media and soaking hours on sprouting of tiger nut tubers at 12DAP

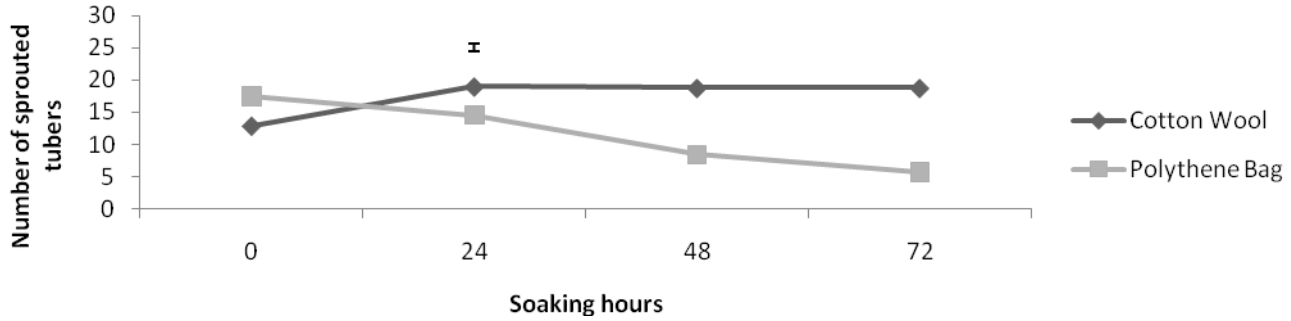


**Figure 10:** Interaction of growth media and soaking hours on sprouting of tiger nut tubers at 13DAP

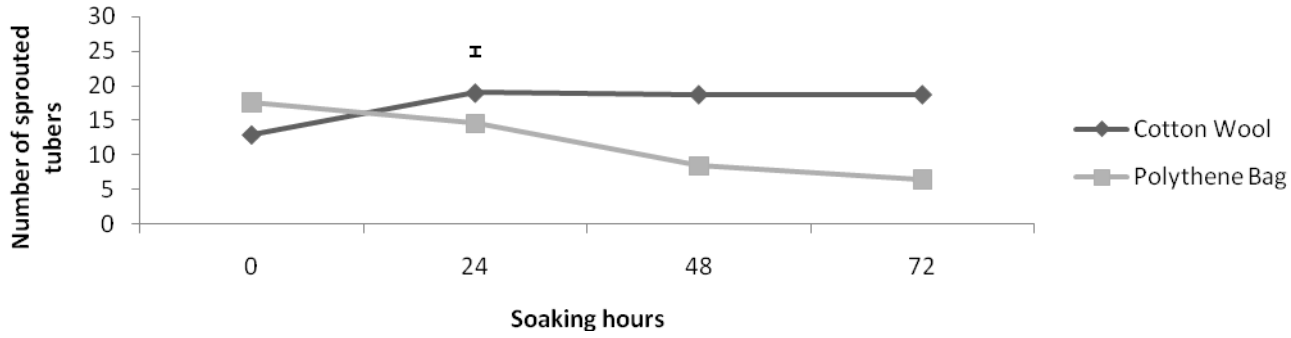


**Figure 11:** Interaction of growth media and soaking hours on sprouting of tiger nut tubers at 14DAP

The use of cotton wool resulted in a higher number of sprouted tubers across all experimental days. Lower number of sprouted tubers in polythene bags when soaking was beyond 24 hours could mean that tubers soaked beyond 24 hours already had imbibed water to their maximum capacity, bringing them into a condition of saturation with moisture, and relatively higher temperature could have led to injury and probably death of some tubers.

### 3.3 Interactions of priming hours and tuber sizes on sprouting of tiger nut

Figure 12 shows the interaction between priming duration and tuber sizes on the sprouting of tiger nut tubers. Both large and medium sized tubers produced significantly ($p$<0.05) higher number of sprouts at 48 hours of soaking. On the other hand, small sized tubers could not tolerate soaking beyond 24 hours; thus for small sized tubers, maximum number of sprouted tubers were obtained at 24 hours of soaking which was similar to the control. Beyond 24 hours of soaking the

number of sprouted tubers continuously and significantly declined for small sized tubers.

construct, with the *hptII* gene (Figure 1 - only 20 out of 139 regenerants are presented, Table 4).

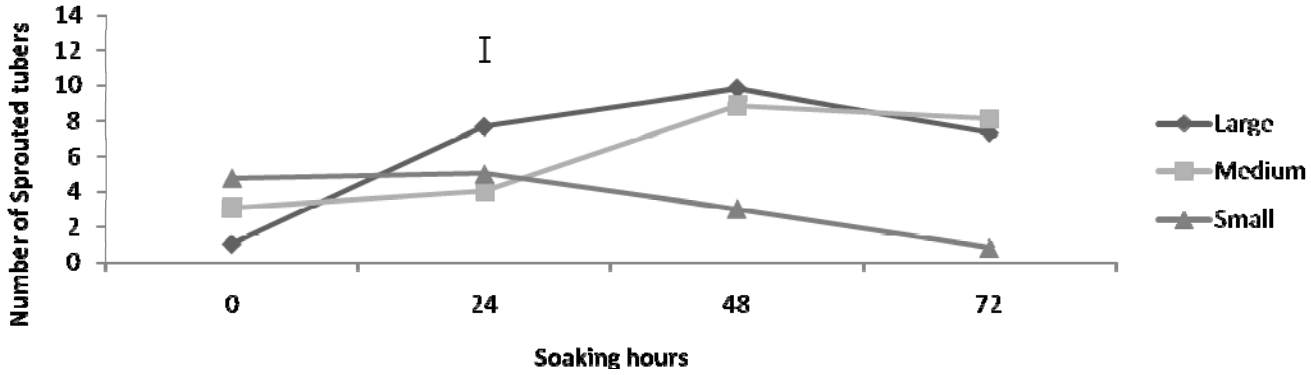


**Figure 12:** Interaction of soaking hours and tuber sizes on sprouting of tiger nut tubers at 3DAP

The results obtained in this trial in which interactions between tuber sizes and soaking hours significantly affected rate and total number of sprouted tubers can be attributed directly to variations in the mass of the tubers; even though imbibitions could have been at similar rate, saturation was faster in the small sized tubers relative to the medium and large tubers. Keeping small sized tubers beyond 24 hours in water would have made some of the tubers to be water-soaked and died. This finding is very crucial as tubers are seldom sorted before soaking; Teiteh and Ofori (1998) reported that in Ghana the common practice among local growers of tiger nut is to soak the tubers in water for 3 to 4 days to facilitate germination. This finding therefore suggests that tubers should be sorted and soaked for different hours based on their sizes before planting.

### 3.4 Effects of tuber sizes and soaking hours and growth media on temperature of the growth media during sprouting of tiger nut tubers

Tuber sizes and soaking duration had no significant effect on the temperature recorded in the sprouting tiger nuts, however the temperature recorded in the growth media differed significantly (Table 2). Higher significant temperatures were recorded in the polythene bags across the period of observation except at the $5^{th}$ and $11^{th}$ day. There were no significant differences in all the interactions.

**Table 2:** Effects of tuber sizes and soaking hours on temperature of growth media during sprouting of tiger nut tubers

| | Temperature ($^{O}$C) of growth media at different days after planting | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **3rd** | **4th** | **5th** | **6th** | **7th** | **8th** | **9th** | **10th** | **11th** | **12th** | **13th** | **14th** |
| **Tuber Size** | | | | | | | | | | | | |
| **Small** | 33.8 | 33.4 | 33.0 | 32.9 | 32.4 | 33.5 | 33.5 | 35.1 | 33.0 | 33.3 | 33.0 | 35.4 |
| **Medium** | 34.0 | 33.5 | 33.0 | 33.0 | 32.5 | 33.8 | 33.5 | 35.4 | 33.0 | 33.5 | 34.0 | 35.5 |
| **Large** | 34.0 | 33.5 | 33.0 | 33.0 | 32.5 | 33.7 | 33.5 | 35.3 | 33.0 | 33.5 | 34.0 | 35.5 |
| **LSD** | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| **Soaking hours** | | | | | | | | | | | | |
| **0** | 33.8 | 33.2 | 33.0 | 32.9 | 32.3 | 33.4 | 33.4 | 35.2 | 33.0 | 33.2 | 33.9 | 35.3 |
| **24** | 34.0 | 33.7 | 33.0 | 33.0 | 32.5 | 33.8 | 33.6 | 35.3 | 33.0 | 33.5 | 34.0 | 35.5 |
| **48** | 34.0 | 33.4 | 33.1 | 33.0 | 32.5 | 33.8 | 33.5 | 35.1 | 33.0 | 33.5 | 34.0 | 35.5 |
| **72** | 34.0 | 33.5 | 33.0 | 33.0 | 32.5 | 33.7 | 33.5 | 35.3 | 33.0 | 33.5 | 34.0 | 35.5 |
| **LSD** | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| **Growth Media** | | | | | | | | | | | | |
| **Cotton Wool** | 32.0 | 31.0 | 33.1 | 32.0 | 31.0 | 32.1 | 33.0 | 34.0 | 33.0 | 31.0 | 33.0 | 34.0 |
| **Poly Bag** | 35.9 | 35.9 | 33.0 | 33.9 | 33.9 | 35.3 | 34.0 | 36.5 | 33.0 | 35.9 | 34.9 | 36.9 |
| **LSD** | 0.22 | 0.28 | ns | 0.11 | 0.17 | 0.23 | 0.08 | 0.2 | ns | 0.28 | 0.11 | 0.08 |
| **TS×SH** | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| **TS× GM** | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| **GM× SH** | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
| **TS×SH×GM** | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |

**TS** – Tuber Sizes **SH** – Soaking Hours **GM** – Growth Media ns= not significant

Higher significant temperatures obtained in the polythene bag in this study can be attributed partly to the color of the material as black body tend to absorb more heat, and the heat could easily be trapped in the polythene bags relative to the cotton wool, dark colors also absorb heat more effectively than light colors (www.calwineries.com/learn/grape-growing/terrain/heat-retention). Black body absorbs all radiation incidents on its surface and they appear black because of complete absorption of all wavelengths (Bowden and Honsberg, 2015). Variation in temperature of the growth media could also have contributed to the variation observed in the rate of sprouting between the two media. Germination, speed of germination and average germination time were positively influence when seeds of *Diptychandra unrantiaca* (Mart.) Tul., were grown at the temperature of 25 $^{0}$C to 30 $^{0}$C (Morbeck de Oliveira et al., 2013). The effect of the black body was aggravated because of poor circulation of air in the polythene bags. This implies that even though there was restriction of oxygen during soaking, ample aeration should be allowed when the tubers are being sprouted. Since aeration could be a major militating factor in polythene bags, perforation of the bags could be tried to enhance aeration. Confirmation of this can be made in future studies by measuring the oxygen content in both growth media.

## 4 CONCLUSIONS

The results of the present study have shown that in tiger nut, cotton wool provided a better sprouting medium than polythene bag. Priming for 48 hours could be adequate when sprouting was done on cotton wool. Large and medium sized tubers, as used in this study, could tolerate up to 48 hours of soaking while soaking small tubers beyond 24 hours led to a reduction in number of sprouted tubers. Therefore, it can be concluded that in tiger nut production, the soaking duration can have a significant effect on the sprouting of the tubers. Smaller tubers require shorter period of soaking in order to achieve good sprouting, and cotton wool is a better sprouting medium relative to polythene bags. Sorting of tubers meant for planting is therefore recommended in tiger nut production and if sorting is not possible, soaking should not exceed 24 hours. The medium sized tubers are probably the most desirable as planting material. Cotton wool or cotton material could be considered the best sprouting medium.

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DOI: 10.14720/aas.2016.107.2.09
**Agrovoc descriptors:** Agaricus bisporus, Pleurotus ostreatus, edible fungi, growing media, agricultural wastes

**Agris category code**: F01,p35

# Agronomic quantitative assessment of substrates based on spents of *Agaricus bisporus* and *Pleurotus ostreatus*

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**ABSTRACT**

In this work the agronomic viability of substrates based on spents of *Agaricus bisporus* (J.E.Lange) Imbach) (SAS) and *Pleurotus ostreatus* (Jacq.) P. Kumm. (SPS) was studied. In addition to the commercial substrate used as reference, six different treatments were considered. In this experiment, SPS and SAS were mixed in different ratios (from 6,000 g and 0 g, respectively, up to 3,000 g and 3,000 g, respectively). SAS was subjected to a heat treatment in a growing room ("cook out") and then to a maturation treatment which consisted of a controlled recomposting process in growing rooms. SPS was subjected to a pasteurizing heat treatment (60 °C - 65 °C, 8 h) and progressive decrease for at least 15 h to a "spawning" temperature (25 °C).
SPS (5,400 g) + SAS (600 g) and SPS (4,800 g) + SAS (1,200 g) were prepared substrates with biological efficiencies (BE) of 35.98 % and 39.68 % respectively, lower than the control (46.18 %) and acceptable yields. The average unit mass of fruiting body harvested was low.

**Key words:** agricultural wastes, edible mushrooms, *Pleurotus ostreatus,* quantitative parameters, spent mushroom substrates

**IZVLEČEK**

**OVREDNOTENJE UPORABNOSTI SUBSTRATA, PRIPRAVLJENEGA IZ OSTANKOV GOJIŠČ GOJENJA DVOTROSNEGA KUKMAKA (*Agaricus bisporus* (J.E.Lange) Imbach) IN BUKOVEGA OSTRIGARJA (*Pleurotus ostreatus* (Jacq.) P. Kumm.) ZA GOJENJE BUKOVEGA OSTRIGARJA**

V raziskavi je bila preučevana primernost ponovne uporabe ostankov gojišč, na katerih so gojili dvotrosni kukmak (*Agaricus bisporus* (J.E.Lange) Imbach), (SAS) in bukov ostrigar (*Pleurotus ostreatus* (Jacq.) P. Kumm), (SPS) kot substratov za nadaljne gojenje gob v primerjavi s komercialnimi substrati. Iz ostankov gojišč zgoraj omenjenih gob je bilo pripravljenih šest mešanic, katerih primernost je bila ovrednotena glede na komercialni substrat, ki je služil kot kontrola.V poskusu so bile pripravljene mešanice SPS in SAS v različnih razmerjih, od 6000 g in 0 g, do 3000 g in 3000 g vsakega od substratov. SAS substrat je bil prekuhan v prostorih za gojenje in nato prepuščen zorjenju v procesu nadzorovanega ponovnega kompostiranja. SPS substrat je bil pasteriziran (60 °C - 65 °C, 8 h), nakar se je počasi ohlajal najmanj 15ur do inkubacijske temperature (25 °C).
Mešanice SPS (5,400 g) + SAS (600 g) in SPS (4,800 g) + SAS (1,200 g) so bili primerni substrati z biološko učinkovitostjo (BE) 35.98 % in 39.68 %, ki je manjša kot pri kontroli (46.18 %), a da še sprejemljiv pridelek. Povprečna masa pobranih gob je bila majhna.

**Ključne besede:** kmetijski odpadki, užitne gobe, *Pleurotus ostreatus,* kvantitativni parametri, izrabljena gojišča gob

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## 1 INTRODUCTION

The commercial production of mushrooms of the genus *Pleurotus* is, along with other species of edible mushroom (*Agaricus bisporus* (Lange) Imbach, and *Lentinula edodes* (Berkeley) Pegler), a modern and unique economic activity within the field of agronomy, with a remarkable presence in Spain and around the world. Approximately, 13,500 t of this fungus is produced in Castilla - La Mancha (67 % of the national total) (Pardo et al., 2009). The mushroom growing sector in Spain generates about $5\times10^5$ tons of spent compost, while the EU, as a whole, produces more than $3.5\times10^6$ tons (Pardo et al. 2009; Picornell et al., 2010). This lignocellulosic material called mushroom spent substrate, can be used in various fields of agriculture (animal feed (Zadrazil, 1980), amendments (Tajbakhsh et al., 2008), substrates of nurseries, nurseries, (Medina et al., 2009)), bioremediation (Faraco et al., 2009), aquaculture, vermiculture and biofuel (Pathak et al., 2009). But these uses are not enough to take advantage of the high volume generated annually, which accumulates in collection centres located in production areas of Spain. These spent substrates are potential contaminants, as well as, a waste of energy to recycling them. Bisaria et al. (1997) emphasized the importance of protein supplementation in substrates with low nitrogen content, in an organic or mineral form in small amounts. Excess nitrogen can reduce the degradability of the substrate, adversely interfering with production and biological efficiency. Shin et al. (1997) and Chang and Miles (2004) report that, possibly, supplementation with wheat bran (WB) is important to supply the needs of vitamins and other growth factors in the fungi nutrition.

The aim of this work is the agronomic quantitative assessment of substrates based on SAS and SPS. The use of the remaining spent mushroom substrate after the cultivation of *P. ostreatus* in new production cycles would be an agronomically viable alternative to using wheat straw (WS), which is currently used exclusively as a base material. If you consider the economic problems (associated with the use of this cereal farmer's by-product and the high market price of WS, especially in drought years) using the remaining spent mushroom substrate is more beneficial. The material could be integrated through new formulations and methodologies with the benefits of lowering production costs and reducing the environmental impact of wastes largely not reused for other farming purposes.

## 2 MATERIALS AND METHODS

### 2.1 Analytical methodology used for the characterization of materials

In this experiment, the chemical characterization (moisture content, total nitrogen, protein, ash, organic matter, C/N ratio, crude fiber, NFE, NDS, and cellulose) was studied using two different base materials in the made substrates. The characterization of raw materials and processed substrates was measured according to following parameters: moisture (MAPA, 1994), pH (Ansorena, 1994), total nitrogen (Tecator, 1987; MAPA, 1994), ash (MAPA, 1994), organic matter (Ansorena, 1994), C:N ratio, crude fiber (ANKOM, 2008), crude fat (ANKOM, 2009), nitrogen free extractives (NFE) (González et al., 1987), and cellulose and neutral detergent-soluble (NDS) (ANKOM, 2005; 2006a, 2006b). Furthermore the exploration of mites (Krantz, 1986) and nematodes (Nombela and Bello, 1983) was performed.

### 2.2 Preparation of substrates and experimental design

The only agronomic factor being studied in this experiment is the type of base substrate with four block replicates. In accordance with the experimental design, seven different treatments were generated in the process, as well as two reference commercial substrates. To all the treatments, except the controls, 50 g $kg^{-1}$ $CaSO_4$ (gypsum) was added to the base material. In varying amounts, $CaCO_3$ was also added (Table 1).

**Table 1:** Treatments tested (g/bag) in the Experiment

| TREATMENT | SPS (g) | SAS (g) | GYPSUM (g) |
|---|---|---|---|
| T1 | 6,000 | 0 | 300 |
| T2 | 5,400 | 600 | 300 |
| T3 | 4,800 | 1,200 | 300 |
| T4 | 4,200 | 1,800 | 300 |
| T5 | 3,600 | 2,400 | 300 |
| T6 | 3,000 | 3,000 | 300 |
| T7 | Commercially controlled based substrates (6.3 kg $bag^{-1}$) | | |

T, treatment; SPS, spent *Pleurotus ostreatus* substrate; SAS, spent *Agaricus bisporus* substrate; T7, commercial substrate

SPS (Champymar, Quintanar del Rey, Cuenca) was composed of wheat straw (fresh, maximum one week emptying rooms). Materials were mixed and the moisture content adjusted. Once this was done, a pasteurizing heat treatment (60 °C - 65 °C, 8 h) was performed. Afterwards, the temperature was decreased to the "spawning" temperature (25 °C) over 15 h. Finally, a supplementation and "spawning" (dose 30 g $kg^{-1}$ mycelium Fungisem K-15) were performed and the samples were bagged in CIES pilot plant.

All substrates were packed into transparent polyethylene bags of 29 cm in diameter and a height ranging from 25 to 35 cm, according to the substrate type, totalling 6.3 kg approximate of mass. Four holes 2.2 cm in diameter were uniformly punctured over the side of the bags.

A controlled recomposting process was carried out. This consisted of a heat treatment in the growing room. Since air heats faster than the substrate, it reaches the maximum temperature (71 °C) in 19 h, whereas the substrate reaches this temperature in 22 h. After the heat treatment, the temperature is decreased. The air temperature falls more rapidly than the substrate temperature causing the second to be above the first. Finally, the temperature equilibrates to 34 - 38 °C after 31 h.

### 2.3 Driving and monitoring of the crop cycle

The total research time was 70 days. The experiment was carried out at the Center for Research, Experimentation and Mushroom Services (CIES), (Cuenca, Spain) in an experimental greenhouse with controlled temperature, substrate temperature, relative humidity, and carbon dioxide concentration and followed the recommended ranges for the variety of selected mycelium (Funginsem K-15) in each stage of development (CIES, 2007).

During recomposting and maturation the moisture content of the mass was maintained at 500 g $kg^{-1}$ without leaching. In this process, the evolution of the moisture content in non-intervened composting had values ranging from 527 g $kg^{-1}$ (at the beginning of the process) to 504 g $kg^{-1}$ (at the end of the ripening process).

Substrate incubation was approximately 17 days (without external ventilation or lighting). During the incubation period, the relative humidity inside the greenhouse ranged between 90 % and 95 %, while the substrate temperature was between 16 °C and 24 ºC. The room temperature ranged between 18 ºC and 22 ºC to help control the temperature of the substrates made. After this, fruiting was induced by ventilation (to keep $CO_2$ levels regulated between 0.28 % to 0.10 %), reduction of room temperature (22 °C to 14 °C) and substrate temperature (24 °C to 15 °C), humidity (91 % to 94.50 %), and lighting. These values are similar to the microclimatic conditions recommended by other researches (Pardo et al., 2005a; García Rollán, 2007; Pardo et al., 2007; Gregori et al., 2008; López-Rodríguez et al., 2008; Gea et al., 2009; Kurt and Buyukalaca, 2010).

### 2.4 Evaluation of the quantitative parameters

Depending on the duration of spawn run time of the substrate by the mycelium (Funginsem K-15) and tested contaminations, a parameter designated as the germination index (GI) was established. The

GI was on a scale from 0 (no invasion) to 5 (full invasion). Mushrooms were harvested daily at their optimal commercial development. The quantity of fruiting bodies arising and mushrooms harvested were tracked throughout the whole mushroom growth cycle. The quantity was determined by the number of fruit bodies that simultaneously grew from the same hole in the substrate bag. To calculate the yield of mushrooms produced daily, each bag was weighed to the nearest gram. The estimated net yield was determined by weighing the fruiting bodies after cutting the unmarketable stipe and mass loss calculated. Once fruiting occurred, the BE was calculated and expressed as a percentage of the fresh fruiting bodies over the substrate dry mass. The BE was calculated from the yield provided by each packet, taking into consideration the charge density of the substrate in the bags and their moisture content. The unit mass of *Pleurotus ostreatus* (gross and net), expressed in grams, was determined from the yields obtained and the quantity of fruiting bodies harvested.

The earliness was established as the time in days since the "spawning" of the substrate to the first flush harvested (weighing the daily relative production of the substrate). Similarly, a second estimation of earliness was performed considering the total harvest.

Fruiting degree was defined as the ratio between the quantity of mushrooms produced and the quantity of holes made in the bags.

Growth and nutritional value of genus *Pleurotus* spp. mushrooms depend mainly on the type of substrate and growing conditions (Curvetto et al., 2002), but also the treatments applied on made substrates and the growing mycelium (Pardo et al., 2005b). These considerations are valid for other edible fungi, for example, *Lentinula edodes* (Berkeley) Pegler ("shiitake") (Philippoussis et al., 2002; Ozcelik and Peksen, 2007).

### 2.5 Statistical analysis

To carry out the statistical analysis, two software packages were used: Statgraphics® Plus version 5.1, and SPSS®. The techniques employed were descriptive statistics, principal component analysis, variance analysis, and correlation and regression method to evaluate the data.

Differences of $P < 0.05$ were considered significant.

**Table 2:** Physicochemical characterization of source materials and substrates used

| | | pH | Moisture | Total nitrogen | Protein | Ash | Organic matter | C/N ratio | Crude fiber | Crude fat | NFE | Cellulose | NDS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BASE MATERIALS | SPS | 5.51 | 776.00 | 6.10 | 38.10 | 158.30 | 841.70 | 80.00 | 298.40 | 3.50 | 501.70 | 310.40 | 308.10 |
| | SAS | 7.78 | 504.00 | 13.40 | 83.80 | 646.00 | 354.00 | 15.30 | 161.00 | 3.20 | 106.10 | 90.50 | 62.70 |
| SUBSTRATES MADE | T1 | 5.88 | 756.00 | 6.00 | 37.50 | 276.60 | 723.40 | 69.90 | 289.10 | 3.00 | 393.80 | 293.40 | 213.10 |
| | T2 | 6.96 | 742.00 | 7.40 | 46.30 | 384.30 | 615.70 | 48.30 | 271.60 | 3.40 | 294.50 | 215.10 | 176.30 |
| | T3 | 7.51 | 740.00 | 7.10 | 44.40 | 388.90 | 611.10 | 49.90 | 243.00 | 3.90 | 319.80 | 209.10 | 144.90 |
| | T4 | 7.94 | 719.00 | 7.60 | 47.50 | 474.10 | 525.90 | 40.10 | 239.60 | 4.20 | 234.60 | 188.40 | 125.50 |
| | T5 | 7.85 | 755.00 | 7.20 | 45.00 | 419.90 | 580.10 | 46.70 | 238.30 | 2.70 | 294.10 | 185.20 | 131.30 |
| | T6 | 8.07 | 725.00 | 8.60 | 53.80 | 508.80 | 491.20 | 33.10 | 231.00 | 2.50 | 204.00 | 153.30 | 111.80 |
| | T7 | 8.15 | 672.00 | 9.60 | 60.00 | 143.00 | 857.00 | 51.80 | 418.70 | 5.10 | 373.20 | 375.80 | 124.00 |
| | Average | 7.48 | 729.00 | 7.64 | 47.80 | 370.80 | 629.20 | 48.50 | 275.90 | 3.54 | 302.00 | 231.47 | 146.70 |
| | CV (%) | 10.88 | 3.98 | 15.11 | 15.10 | 33.60 | 19.80 | 23.50 | 24.00 | 26.02 | 22.65 | 33.25 | 24.43 |

T, treatment; SPS, spent *Pleurotus ostreatus* (Jacq.) P. Kumm. substrate; SAS, spent *Agaricus bisporus* (Lange) Imbach. substrate; T1, SPS 6,000 g; T2, SPS 5,400 g + SAS 600 g; T3, SPS 4,800 g + SAS 1,200 g; T4, SPS 4,200 g + SAS 1,800 g; T5, SPS 3,600 g + SAS 2,400 g; T6, SPS 3,000 g + SAS 3,000 g; T7, commercially controlled based substrates; CV, coefficient of variation; NFE, nitrogen free extractives; NDS, neutral detergent-soluble. Results expressed in g $kg^{-1}$ dry matter, except pH, moisture (fresh matter) and C/N ratio.

## 3 RESULTS AND DISCUSSION

### 3.1 Analytical characterization of the base materials used and the substrates made

The chemical characteristic results of the different source materials, substrates made, and commercially controlled based substrates are shown in Table 2. It can be concluded that the final values obtained after the SAS recomposing process were adjusted to the optimum range considered for this process (Lohr et al., 1984; Szmidt, 1994; Raymond et al., 1997). Due to high concentrations of P, K, Ca, and Mg in the cover layer (Raymond et al., 1997), a high ash (which contributes greatly to the cover layer of mineral soil), and high values of electrical conductivity (due to release of salts from the decomposed organic matter) (Stewart and Meek, 1977; Lohr et al., 1984) are observed.

The chemical characterization (moisture content, total nitrogen, protein, ash, organic matter, C/N ratio, crude fibre, NFE, NDS, and cellulose) was studied using two different base materials in the made substrates. With increasing SAS doses, the pH increases to 8.07 and the ash content to 50.88 %. In contrast, the moisture content decreases to 72.50 %, organic matter to 49.12 %, C/N to 33.10, crude fiber to 23.10 %, NFE to 20.40 %, cellulose to 15.33 % and NDS to 11.18 %.

### 3.2 Principal component analysis

The results of the experiment are presented using the Multivariate Statistical Technique of Principal Component Analysis (PCA), according to the physicochemical characterization of the substrates made (Table 3).

Total nitrogen of the tested substrates shows a negative correlation with moisture (r = - 0.895) and NDS (r = - 0.747), and a positive correlation with pH (r = 0.786) and hemicellulose content (r = 0.730); the correlation values are lower with the C/N (r = - 0.544) and crude fibre content (r = 0.548) and lignin (r = 0.527).

C/N ratio is highly correlated with the values of NFE (r = 0.921) and NDS (r = 0.848), but not, with pH (r = - 0.816), ash content (r = - 0.682), cellulose (r = 0.649) and, as stated above, total nitrogen (r = - 0.544).

The crude fat content of the substrates has an average negative correlation with moisture (r = - 0.783) and low correlation with lignin content (r = 0.590), ash (r = - 0.604), hemicellulose (r = 0.648) and cellulose (r = 0.652).

As expected, the correlation between the crude fibre and ash content is high, with a negative correlation value of r = - 0.931, and a positive value of r = 0.948 with cellulose. When the crude fibre is correlated with hemicellulose (r = 0.831), moisture (r = - 0.747), lignin (r = 0.776), and crude fat (r = 0.705), the values are lower. This correlation decreases to r = 0.660 when considering NFE, and to r = 0.548, when considering the total nitrogen content.

Finally, the ash content correlates with a large number of analytical parameters of the processed substrates; in all cases, these correlations are negative: cellulose (r = - 0.987), crude fibre (r = - 0.931), NFE (r = - 0.889), lignin (- 0.767), hemicellulose (- 0.714), C/N (r = - 0.682) and crude fat (r = - 0.604).

**Table 3:** Substrate analytical parameters correlation matrix

| | pH | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| pH | 1.000 | Moisture | | | | | | | | | | |
| Moisture | - 0.601* | 1.000 | $Nitrogen_T$ | | | | | | | | | |
| $Nitrogen_T$ | 0.786** | - 0.895*** | 1.000 | Ash | | | | | | | | |
| Ash | 0.228 | 0.484 | - 0.229 | 1.000 | C/N ratio | | | | | | | |
| C/N ratio | - 0.816** | 0.252 | - 0.544* | - 0.682* | 1.000 | Crude fibre | | | | | | |
| Crude fibre | 0.058 | - 0.747** | 0.548* | - 0.931*** | 0.390 | 1.000 | Crude fat | | | | | |
| Crude fat | 0.305 | - 0.783** | 0.490 | - 0.604* | 0.097 | 0.705** | 1.000 | NFR | | | | |
| NFE | - 0.558* | - 0.052 | - 0.225 | - 0.889*** | 0.921*** | 0.660* | 0.352 | 1.000 | Hemi | | | |
| Hemi | 0,495 | -0,766** | 0,730** | -0,714** | 0,035 | 0,831** | 0,648 | 0,411 | 1.000 | Cellu | | |
| Cellu | - 0.206 | - 0.558* | 0.271 | - 0.987*** | 0.649* | 0.948*** | 0.652* | 0.843** | 0.689* | 1.000 | Lignin | |
| Lignin | 0,319 | -0,601* | 0,527* | -0,767** | 0,233 | 0,776** | 0,590* | 0,583* | 0,942*** | 0,717** | 1 | NDS |
| NDS | - 0.988*** | 0.545* | - 0.747** | - 0.316 | 0.848*** | 0.034 | - 0.206 | 0.625* | -0.406 | 0.287 | -0,243 | 1.000 |

NFE, nitrogen free extractives; Hemi, hemicellulose; Cellu, cellulose; NDS, neutral detergent-soluble; $Nitrogen_T$, total nitrogen. g $kg^{-1}$ dry matter, except pH, moisture (over fresh matter) and C/N ratio.
Absolute value of the correlation coefficient between 0.50 and 0.69 (*), from 0.70 to 0.84 (**) or equal to or greater than 0.85 (***).

**Table 4:** Total variance explained by each factor.

| Factor | Initial eigenvalues | | |
|---|---|---|---|
| | Total | % variance | % cumulated variance |
| 1 | 6.33 | 52.77 | 52.77 |
| 2 | 4.58 | 38.20 | 90.97 |
| 3 | 0.62 | 5.15 | 96.12 |
| 4 | 0.37 | 3.09 | 99.21 |
| 5 | 0.06 | 0.48 | 99.69 |
| 6 | 0.04 | 0.31 | 100.00 |
| 7 | 0.00 | 0.00 | 100.00 |
| - | - | - | - |
| - | - | - | - |
| 12 | 0.00 | 0.00 | 100.00 |

According to the results, there are two main factors that explain the total variance (90.97 %) of the experiment (Table 4).

**Table 5:** Rotated Component Matrix for analytical parameters and factors

| Analytical parameter | Factor 1 | Factor 2 |
|---|---|---|
| pH | 0.174 | - 0.962*** |
| Moisture | - 0.768** | 0.544 |
| Total nitrogen | 0.566 | - 0.768** |
| Ash | - 0.915*** | - 0.397 |
| C/N ratio | 0.346 | 0.929*** |
| Crude fibre | 0.971*** | 0.078 |
| Crude fat | 0.779** | - 0.181 |
| NFE | 0.657 | 0.732** |
| Hemicellulose | 0,905*** | -0,303 |
| Cellulose | 0.920*** | 0.358 |
| Lignin | 0,885*** | -0,104 |
| NDS | - 0.080 | 0.971*** |

NFE, nitrogen free extractives; NDS, neutral detergent-soluble.
Extraction method: principal component analysis; rotation method: Varimax Normalization with Kaiser; the rotation converged in 5 iterations.
Extent of participation, in absolute value, between 0.70 and 0.84 (**) or equal to or greater than 0.85 (***).

In Table 5 the "Rotated Component Matrix" for analytical parameters and experiment factors are given. The variables: ash, crude fibre, hemicellulose, cellulose, and lignin content of the substrates are those with a higher degree of participation in shaping this first factor, with factor values of - 0.915, 0.971, 0.905, 0.920 and 0.885, respectively. The mean values of participation in the Factor 1 are expressed with moisture and crude fat, with load factor values of - 0.768 and 0.779, respectively. Factor 2 is defined by pH, C/N and NDS with a load factor of - 0.962, 0.929 and 0.971, respectively; the average values of participation in the Factor 2 are manifested in total nitrogen and NFE, with values of load factor - 0.768 and 0.732, respectively.

**Table 6:** ANOVA of substrate germination index

| Substrate | Germination index |
|---|---|
| T2 | 5.00 |
| T3 | 5.00 |
| T4 | 5.00 |
| T5 | 4.75 |
| T6 | 4.25 |
| T7 | 4.75 |
| Average | 4.79 |
| Fisher F | 2.73 |
| Significance level F Fisher | 0.052 $^{ns}$ |

T, treatment; SPS, spent *Pleurotus ostreatus* (Jacq.) P. Kumm. substrate; SAS, spent *Agaricus bisporus* (Lange) Imbach. substrate; T2, SPS 5,400 g + SAS 600 g; T3, SPS 4,800 g + SAS 1,200 g; T4, SPS 4,200 g + SAS 1,800 g; T5, SPS 3,600 g + SAS 2,400 g; T6, SPS 3,000 g + SAS 3,000 g; T7, Commercially controlled based substrates.
ns, no significant difference, $p > 0.05$.

### 3.3 Germination rate. Descriptive statistics and analysis of variance

In Table 6 the results obtained for the GI of evaluated substrates are given. In this experiment, we obtained up to three flushes, the same as those achieved by Pardo and Lopez Mondéjar (2004). Mata and Gaitán-Hernádez (1995) and Salmones et al. (1997), also, obtained four harvests crops in their experiments. As in the experiments conducted by Bonilla-Lavado et al. (2006), where the production of *P. ostreatus* had four flushes (in the third flush, all treatments were productive although at a lower rate than the first two flushes, which only enriched *Tithonia diversifolia* (Hemsl.)), coinciding with the studies by Lozano (1990), who obtained harvests for two months. Gea et al. (2009) reported, on experiments based on *P. ostreatus*, a first flush, lasting approximately 10 days, from which primordia was formed constantly and uninterrupted, for more than a month, until the end of the trial; therefore, a clearly defined second flush was not necessary. Bernabé-González et al. (2004), evaluated three flushes of *P. pulmonarius* (Fr.) Quél., which were unstable, except in dry jicama stover where only two flushes occured. Philippoussis et al. (2001) achieved the same flushes with *P. ostreatus*, *P. pulmonarius* and *Pleurotus eryngii* (DC.) Quél.. In general, and in the present study, the higher yields were obtained between the first and second flush (80 to 90 % of total production), except Zeng-Chin et al. (2009) who through growing of *P. citrinopileatus* Singer, achieved five flushes and obtained higher BE in the second flush.

Of the six different treatments that have been generated with different mixtures, treatment 1, consisting of 6,000 g of SPS, showed difficulties in germination and a high degree of contamination by *Gliocladium* spp. (which can be associated to the low pH of the substrate (Table 2)), which produced difficulties in mycelial development, growth arrest, and the absence of production of *Pleurotus ostreatus*. In the rest of the processed substrates, GI was adequate, and was found that the two treatments with lower SAS were the earliest in the first flush (difference that was cancelled when the earliness referred to days from inoculation to full induction).

The substrates of T2, T3 and T4 have the highest GI (5.00), higher than the average of the 24 substrates (4.79). The substrates of T5 and T7 (commercial) have a GI of 4.75, and the substrate with the GI lowest is T6 (4.25).

### 3.4 Quantitative production parameters. Descriptive statistics and analysis of variance

The most noteworthy aspects according to quantitative production parameters are presented in Table 7. The duration of the commercial crop cycle was 70 days, 16 of which were for the incubation period.

**Table 7:** ANOVA of the quantitative parameters of the Experiment

| Substrate | Earliness (days) | | Gross yield (g bag$^{-1}$) | Index fructification Number fruit.bodies hole$^{-1}$ | Number mushrooms bag$^{-1}$ | UM | BE |
|---|---|---|---|---|---|---|---|
| | 1st Flush "spawning" | Total "spawning" | | | | | |
| T2 | 23.40cd | 35.93b | 573.75bc | 1.19 | 49.00ab | 8.95b | 35.98ab |
| T3 | 21.35d | 33.13b | 637.50ab | 1.19 | 63.00a | 7.50b | 39.68ab |
| T4 | 28.15bc | 33.85b | 472.50bc | 1.00 | 34.75bc | 10.79b | 27.23bc |
| T5 | 30.23b | 35.18b | 341.75bc | 0.69 | 22.75bc | 11.89ab | 22.58bc |
| T6 | 32.55ab | 39.15ab | 260.75c | 0.69 | 19.25c | 10.92b | 15.88c |
| T7 | 36.73a | 43.80a | 938.75a | 1.00 | 41.50abc | 19.54a | 46.18a |
| Average | 28.73 | 36.84 | 537.50 | 0.96 | 38.37 | 11.60 | 31.25 |
| Fisher F | 16.24 | 6.01 | 9.61 | 2.56 | 7.00 | 4.91 | 8.19 |
| $S_L$ | 0.00*** | 0.002** | 0.00*** | 0.06ns | 0.001*** | 0.005** | 0.00*** |

T, treatment; SPS, spent *Pleurotus ostreatus* (Jacq.) P. Kumm. substrate; SAS, spent *Agaricus bisporus* (Lange) Imbach. substrate; T2, SPS 5,400 g + SAS 600 g; T3, SPS 4,800 g + SAS 1,200 g; T4, SPS 4,200 g + SAS 1,800 g; T5, SPS 3,600 g + SAS 2,400 g; T6, SPS 3,000 g + SAS 3,000 g; T7, commercially controlled based substrates; UM, unit mass of uncut mushrooms (g); BE, biological efficiency (kg/100 kg of dry substrate); $S_L$, F significance level Fisher.
ns, no significant difference, $P > 0.05$; ** P-value < 0,01; *** P-value < 0,001. For each column, values followed by different letters are significantly different from each other (P = 0.05, Tukey-HSD).

According to Patra and Pani (1995) and Sánchez et al. (2006), the quality production of a substrate is acceptable from BE of 50 %. In this experiment reduction of BE was observed as the percentage share of SAS in prepared substrate increased (up to 15.88 %). In the observed differences among treatments comprised of different mixtures, the SAS could have worsened some physical characteristics of the substrate such as aeration and drainage, as its particles are much smaller with respect to the SPS. This may have reduced porosity in the substrate and increased compaction in treatments with higher rate of SAS; reducing mycelium growth. Air exchange was especially difficult due to closed bags, favouring increases in $CO_2$ concentrations and decrease of $O_2$ within the substrates. It can be seen that in the substrates with lower SAS content, mycelial colonization occurred in the centre of the bag, whereas in the substrates which contained higher SAS content, the fungus colonized a few centimetres of depth only.

T2 (SPS 5,400 g + SAS 600 g) and T3 substrates (SPS 4,800 g + SAS 1,200 g), provided acceptable yields, with BE values of 35.98 % and 39.68 %, respectively, lower than the control (46.18 %) (Table 7). Several authors have succeeded in studying the growth of *P. ostreatus* with different types of substrates resulting in higher BE values than those obtained in this experiment with BE values up to 125 % (Klibanski et al., 1993; Gaitán-Hernádez and Salmones, 1996), up to 138 % with *P. pulmonarius* on coffee pulp (Velázquez-Cedeño et al., 2002), and up to 164 % on mixtures of jicama stover and corn stover (Bernabé-González et al., 2004). BE is mainly determined by *Pleurotus* spp. species and the type of substrate used. BE increases when the substrates are enriched with nutrients of natural or synthetic origin (Bonilla-Lavado et al., 2006); these researchers, in their experiments, obtained between 0.60 % (on coir) and 36.40 % (on sawdust mixture and *Tithonia diversifolia* (Hemsl.) A.Gray). Pardo et al. (2003), working with straw, vine shoot, kenaf, and various combinations of these raw

materials, obtained BE values ranging between 6.75 and 14.02 %. In a later work, Pardo et al. (2005b) only achieved values of 23.00 and 26.90 % when they combined winter cereal straw and kenaf and then subjected the substrate to pasteurization and thermophilic conditioning. Perez and Mata (2005) with *P. ostreatus* obtained BE values of 28.10 % based on pine shavings.

Salmones et al. (1997) obtained BE ranging between 16.80 % and 71.90 %, using barley straw substrate. Guzman et al. (2003) obtained BE in *P. ostreatus* up to 59.00 % with waste sugarcane. Garzón and Cuervo (2008) in their research show how wood sawdust and corn stalk BE are improved up to values of 29.10 % to 48.40 % when they are combined with coffee pulp and bagasse sugarcane in different proportions. Varnero et al. (2010) obtained BE values of 2.97 % on poplar chips and 32.94 % on wheat straw. According to the results obtained in this work and those offered by the literature, low BE values are observed when the degree of participation in the prepared substrate SAS is greater; due to the prepared substrates not receiving nutritional supplementation.

Pardo et al. (2005b), working with various substrates made different combinations of WS, barley straw, kenaf, vine-and grape seed flour. The total quantity of mushrooms bag$^{-1}$ obtained varied between 42 and 82, with the lowest yields of 20.50 g and 32.70 g on combination of straw and kenaf. While the first yield component value is less than the values obtained in this experiment (19.25 to 63 mushrooms), the average unit mass is much higher (between 7.50 and 11.89 g, excluding the one offered by the commercial substrate which was 19.54 g) (Table 7). In another work of Pardo et al. (2005a) where scrape, straw, kenaf, vine shoot and “alperujo" were combined the average mass of an upper part of the mushroom ranged between 12 g and 93 g. However, the number of fruiting bodies hole$^{-1}$ ranged between 0.02 and 1, in general and was lower compared to results of this experiment 0.69 and 1.19 (Table 7). Using pasteurization and thermophilic conditioning treatments, benomyl moisturization and pasteurization, and semianaerobia fermentation with the same substrates in the same bags, Pardo et al. (2007) obtained average mushroom upper part mass ranging between 14.60 and 25.90 g (higher than those presented in Table 7).

Gea et al. (2009), using parallelepipedic packages with 7 holes of 25 mm in diameter and a specific substrate for *P. ostreatus* cultivation which separates supplements made from aerobic fermentation, achieved a range of 88 to 136 mushrooms bag$^{-1}$ and an average fruiting body mass of 12.40 g to 14.50 g (each values higher than those presented in Table 7). This research offered fruiting index values between 1.21 and 1.57 fruiting bodies per hole. As is the case in this experiment, the literature confirms that increasing the quantity of fruiting bodies per hole and the quantity of mushrooms per bag, decreases the value of a mushroom mass.

T1, consisting of 6,000 g of SPS, showed difficulties in germination and a high degree of contamination by *Gliocladium* spp. Due to these reasons, T1 was not included in the statistical analysis of this experiment.

### 3.5 Correlation matrix and "step by step" regression models

Table 8 presents the correlation matrix between GI, earliness, quantitative production parameters, and physicochemical characteristics of the substrates made. With statistical significance, the cellulose content has positively favoured both the GI and the BE (r = 0.913 and r = 0.948, respectively) and negatively days from inoculation until the formation of the first primordia (r = - 0.922) (Table 8). Although not statistically significant (P = 8.2 %) a high positive correlation was found between C/N and BE (r = 0.830) and negative with ash content (P = 7.2 %; r = - 0.844). Also, the number of yield component of mushrooms (r = 0.854) with the cellulose content was positively correlated, but without statistical significance (P = 6.6 %).

**Table 8:** Correlation matrix between germination index, earliness, and production of quantitative parameters, and physicochemical characteristics.

| | Germination Index | 1st Flush "spawning" | Total "spawning" | Total quantity of mushrooms | UM | BE |
|---|---|---|---|---|---|---|
| pH | - 0.600 (0.284) | 0.789 (0.113) | 0.247 (0.689) | - 0.703 (0.186) | 0.659 (0.227) | - 0.784 (0.117) |
| $Nitrogen_T$[1] | - 0.854 (0.065) | 0.695 (0.193) | 0.864 (0.059) | - 0.621 (0.263) | 0.384 (0.524) | - 0.743 (0.150) |
| Ash | - 0.722 (0.168) | 0.832 (0.080) | 0.582 (0.304) | - 0.749 (0.145) | 0.622 (0.263) | - 0.844 (0.072) |
| C/N ratio | 0.784 (0.116) | - 0.805 (0.100) | - 0.712 (0.177) | 0.726 (0.165) | - 0.556 (0.331) | 0.830 (0.082) |
| Crude fibre[1] | 0.582 (0.304) | - 0.648 (0.237) | - 0.163 (0.793) | 0.545 (0.343) | - 0.478 (0.415) | 0.657 (0.228) |
| Crude fat[1] | 0.822 (0.088) | - 0.662 (0.223) | - 0.809 (0.098) | 0.693 (0.195) | - 0.533 (0.355) | 0.700 (0.189) |
| NFE[1] | 0.683 (0.204) | - 0.776 (0.123) | - 0.661 (0.225) | 0.709 (0.180) | - 0.570 (0.315) | 0.789 (0.112) |
| Cellulose[1] | 0.913* (0.031) | - 0.922* (0.026) | - 0.721 (0.170) | 0.854 (0.066) | - 0.690 (0.197) | 0.948** (0.014) |
| NDS[1] | 0.666 (0.220) | - 0.782 (0.118) | - 0.317 (0.603) | 0.684 (0.203) | - 0.603 (0.281) | 0.788 (0.113) |

UM, unit mass of uncut mushrooms (g); BE, biological efficiency; $Nitrogen_T$, total nitrogen; NFE, nitrogen free extractives; NDS, neutral detergent-soluble; [1], g $kg^{-1}$ dry matter.
Results in parentheses indicate statistical significance. No significant ($P > 0.05$) (non *); significant at 95 % ($0.01 < P \leq 0.05$) (*); significant at 99 % ($0.001 < P \leq 0.01$) (**).

The highest cellulose content in the previously mentioned mixtures favoured the fruition of oyster mushroom (number of flushes, total yield and BE) due to a higher SPS content. The opposite occurred in the time period between inoculation and the appearance of the first primordia. Although not significant from the statistical point of view, higher C/N ratios have also favoured BE, but not the higher total nitrogen contents. Several researches have shown that low nitrogen contents can be a depressive factor for growing of many edible mushrooms (Boyle, 1998; Philippoussis et al., 2002, 2003). The quantity of mushrooms is positively correlated with the BE in this experiment (r = 0.975), while the correlation is negative with the second yield component, unit mass (r = - 0.952). This last yield component, although not significant, is negatively correlated with the BE (P = 5.40 %; r = - 0.872) (Table 9).

**Table 9:** Correlation matrix between the rate of germination, earliness, yield components, and biological efficiency.

| | Germination Index | 1rs Flush "spawning" | Total "spawning" | Total quantity of mushrooms | UM | BE |
|---|---|---|---|---|---|---|
| Germination Index | 1.000 | | | | | |
| 1rs Flush "spawning" | - 0.793 (0.109) | 1.000 | | | | |
| Total "spawning" | - 0.887* (0.045) | 0.667 (0.219) | 1.000 | | | |
| Total quantity of mushrooms | 0.738 (0.154) | - 0.987** (0.002) | - 0.658 (0.228) | 1.000 | | |
| UM | - 0.502 (0.389) | 0.912* (0.031) | 0.420 (0.481) | - 0.952** (0.013) | 1.000 | |
| BE | 0.847 (0.070) | - 0.996*** (0.000) | - 0.720 (0.170) | 0.975** (0.005) | - 0.872 (0.054) | 1.000 |

UM, unit mass of uncut mushrooms (g); BE, biological efficiency (kg/100 kg of dry substrate).
Results in parentheses indicate statistical significance. Not significant ($P > 0.05$) (non *); significant at 95 % ($0.01 < P \leq 0.05$) (*); significant at 99 % ($0.001 < P \leq 0.01$) (**); 99.9 % significant ($P \leq 0.001$) (***).

Table 10 shows the "step by step" regression analysis to the physical - chemical properties of substrates, GI, earliness, and quantitative production parameters of the current experiment. Cellulose explains a high percentage of variability in both GI and number of days from inoculation until the formation of the first primordia, although in the first case, there is a positive coefficient and in the second, the coefficient accompanying the model is negative. GI, in turn, is involved in the mathematical model explaining the variability of the days from inoculation until full induction, but the coefficient accompanying the model is negative. With a high fit value, the model which explains the variability of yield component, quantity of mushrooms, includes a negative coefficient for the days from inoculation until the appearance of the first primordia. The other yield component, average unit mass of mushrooms, is well explained with a negative coefficient for the quantity of mushrooms. Finally, the BE is explained by a model including, with positive coefficients, the quantity of mushrooms and the cellulose content of made substrates. The days from inoculation until the appearance of the first primordia have a negative correlation with a high determination coefficient value for BE.

**Table 10:** Models obtained by regressing "step by step".

| Explained variable | Independent variable | Equation | $R^2$ corrected | SE |
|---|---|---|---|---|
| GI | PCC | GI = 2.474* + 0.012* · C | 77.70* | 0.15390 |
| P2 | PCC + GI | P2 = 60.844** – 0.177* · C | 80.10* | 2.08320 |
| P4 | PCC + GI + P2 | P4 = 66.049** – 6.375* · GI | 71.70* | 1.24657 |
| TQM | PCC + QPP (– BE) | Nº mushrooms = 142.673*** – 3.867** · P2 | 96.60** | 3.39052 |
| UM | PCC + QPP (– BE) | UM = 13.465*** – 0.092** · TQM | 87.50** | 0.62358 |
| BE | PCC + QPP | BE = 84.364*** – 2.067*** · P2<br>BE = 55.457** – 1.813*** · P2 + 4.587** · GI | 98.80***<br>100.00*** | 1.05719<br>0.14023 |
| | PCC + P4 + UM + TQM | BE = - 16.380** + 0.323*** · TQM + 0.171** · C | 99.90*** | 0.22397 |

C, cellulose; TQM, Total quantity of mushrooms; $R^2$, determination coefficient (%); SE, standard error of the estimate.

**Physical-chemical characteristics of substrate (PCC):** pH (aq. 1:5, w/w), total nitrogen (g $kg^{-1}$, odm), ash (g $kg^{-1}$, odm), C/N ratio, crude fibre (CFi; g $kg^{-1}$, odm), crude fat (CFa; g $kg^{-1}$, odm), nitrogen free extractives (NFE; g $kg^{-1}$, odm), hemicellulose (g $kg^{-1}$, odm), cellulose (g $kg^{-1}$, odm), lignin (g $kg^{-1}$, odm), neutral-detergent soluble (NDS; g $kg^{-1}$, odm); odm = on dry matter.

**Index germination, earliness and quantitative production parameters (QPP):** germination index (GI), days from inoculation to the formation of the first primordia (P2), days from inoculation to the onset of harvest (P4), total quantity of mushrooms, average unit mass of uncut mushrooms (UM, g), biological efficiency (BE, kg/100 kg of dry substrate).

Significant at 95 % ($0,01 < P \leq 0,05$) (*); 99 % ($0.001 < P \leq 0.01$) (**); 99.9 % significant ($P \leq 0.001$) (***). Regressions include only those whose coefficients accompanying the independent variables are significant, provided that the significance of the model is significant.

## 4 CONCLUSIONS

According to the results obtained for the combinations tested, the biological efficiencies achieved on tested substrates are lower than those obtained with the commercial reference substrate, so initially, there would be a lack of interest in the spent *Pleurotus ostreatus* and *Agaricus bisporus* substrates. However, since they are waste materials, they can be obtained with no or little money. Following treatments showed the highest BE (35.98 % and 39.68 %, respectivelly) - 5,400 g SPS + 600 g SAS and 4,800 g SPS + 1,200 g SAS, compared to 46.18 % of the commercial substrate.

Consequently substrate formulations based on spent *Pleurotus ostreatus* and *Agaricus bisporus* composts could be a low-cost substrate with selective and balanced nutrients for growth and development of oyster mushrooms.

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**Agrovoc descriptors:** Oganic fertilizers, inorganic fertilizers, turf, lawn grasses, fertilizer application, growth, production

**Agris category code**: F04, f01

# The comparison of organic and inorganic fertilizers influence on selected indicators of turf growth-production process

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## ABSTRACT

The aim of this experiment was to compare the influence of organic and inorganic fertilizers on selected indicators of turf growth-production process under non–irrigated conditions. The experiment was carried out in warm and dry conditions in the area of Nitra (Slovak Republic). In the experiment were included 5 treatments: 1.Without fertilization, 2. Turf NPK fertilizer 15–3–8 (+3 MgO +0.8 Fe +18 S), 3. Slow release NPK fertilizer 14–5–14 (+4 CaO +4 MgO +7 S), 4. Organic NPK fertilizer 5-1-1 and 5. Organic NPK fertilizer 3-2-1. Determination of the average height of turf, total height of turf and the annual average daily gain of height showed that best treatment was application of slow release fertilizer. Turf fertilized by Organic NPK fertilizer 5-1-1 reached the highest values of the average height of turf, total height of turf and the annual average daily gain of height, the same as treatment without fertilization. These finding were statistically significant. Treatment without fertilization reached the lowest values in evaluated growth-production parameters.

**Key words:** turf, fertilizing, organic fertilizers, inorganic fertilizers, growth-production process

## IZVLEČEK

### PRIMERJAVA VPLIVA ORGANSKIH IN MINERALNIH GNOJIL NA IZBRANE KAZALNIKE RASTI IN PRIDELKA TRAVNE RUŠE

Namen tega poskusa je bil primerjati vpliv gnojenja z organskimi in mineralnimi gnojili na izbrane kazalnike rasti in pridelka travne ruše v razmerah brez namakanja. Poskus je potekal v toplih in sušnih razmerah na območju Nitre (Republika Slovaška). V poskus je bilo vključenih 5 obravnavanj: 1. Brez gnojenja, 2. Gnojenje ruše z NPK gnojili v razmerju 15–3–8 (+3 MgO +0.8 Fe +18 S), 3. Gnojenje z gnojili s počasnim sproščanjem NPK hranil 14–5–14 (+4 CaO +4 MgO +7 S), 4. Gnojenje z organskimi NPK gnojili v razmerju 5-1-1 in 5. Gnojenje z organskimi NPK gnojili v razmerju 3-2-1. Meritve povprečne višine travne ruše, celokupne višine travne ruše in povprečnega letnega dnevnega prirastka v višino so pokazale, da je bilo najboljše obravnavanje gnojenje z gnojili, ki počasi sproščajo hranila. Ruša, ki je bila gnojena z organskimi gnojili v razmerju 5-1-1, je dosegla enake največje vrednosti povprečne višine, celokupne višine in povprečnega letnega dnevnega prirastka v višino kot obravnavanje brez gnojenja. Izsledki so bili statistično značilni. Obravnavanje brez gnojenja je imelo najmanjše vrednosti ovrednotenih rastno-produkcijskih parametrov.

**Ključne besede:** travna ruša, gnojenje, organska gnojila, mineralna gnojila, rastno-produkcijski procesi

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## 1 INTRODUCTION

Plant nutrition and turfs fertilizing have important position in system of caespestechnical measures (ways of exploiting and turf care) (Ondřej and Opatrná, 1997). Balanced and sufficient nutrition is a precondition for the turfs quality, their durability and resistance to disease and action of other stressors. Frequent mowing of ornamental and sport turfs demands fertilization (Svobodová, 1998).

For fertilizing of turfs various forms and types of fertilizers are used (Gregorová, 2001). Nitrogen fertilizers with nitrate ($NO_3^-$), eg. ammonium nitrate are acting quickly, but they are quickly leached out from the soil. Nitrogen is flushed into groundwater or escapes as a gas compound into the atmosphere (Míka, 1991).

Slow release fertilizers (SRF) release nutrients slowly and uncontrolled (Wu et al., 2008). The greatest advantage is that the required dose of fertilizer is applied to a reduced numbers of applications compared to other forms of fertilizers (Magni et al., 2008).

In turf management use of classical organic fertilizers is not frequent (slurry, dung-water, etc.). The classical organic fertilizers are replaced by organic fertilizers which are in the dried form and do not contain live weed seeds and harmful microorganisms. Due to the gradual release of nutrients, fertilizer supply plant nutrients during the whole vegetation (Nardi et al., 2004; Rasmussen and Harold, 2008).

The aim of the experiment was to compare the influence of organic and inorganic fertilizers on selected traits of turf growth and production process under non-irrigated conditions.

## 2 MATERIAL AND METHODS

### 2.1 Characteristics of experimental site

In period 2012 – 2014 a turf experiment located in moderate climatic zone of warm and dry area in Nitra (Slovak Republic) was conducted. Average annual temperature is 9.7 °C and annual rainfall is 560 mm (Babošová and Noskovič, 2014). Weather conditions during vegetation periods are shown in Table 1.

**Table 1:** Weather condition on vegetation periods in 2012 – 2014

| Year | Indicator | Month | | | | | | | | Vegetation period | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | III. | IV. | V. | VI. | VII. | VIII. | IX. | X. | Σ | Ø |
| 2012 | Ø temperature (°C) | 7.41 | 11.23 | 17.29 | 20.86 | 22.77 | 21.47 | 18.02 | 10.77 | - | 16.23 |
| | Σ rainfall (mm) | 2.80 | 36.10 | 19.60 | 70.10 | 61.40 | 7.30 | 31.40 | 80.60 | 309.30 | - |
| 2013 | Ø temperature (°C) | 3.20 | 12.10 | 15.50 | 19.30 | 22.70 | 21.80 | 14.70 | 12.10 | - | 15.18 |
| | Σ rainfall (mm) | 106.20 | 20.40 | 77.80 | 46.70 | 2.10 | 73.90 | 60.00 | 30.50 | 417.60 | - |
| 2014 | Ø temperature (°C) | 9.33 | 12.37 | 15.24 | 19.35 | 21.81 | 18.86 | 16.78 | 12.10 | - | 15.73 |
| | Σ rainfall (mm) | 15.40 | 48.90 | 57.60 | 52.50 | 64.10 | 55.90 | 122.00 | 34.60 | 451.00 | - |

Source: Department of Biometeorology and Hydrology, HLEF SUA in Nitra. Ø – average, Σ – sum.

The experiment was conducted on clay-loam fluvisol. In the autumn before the foundation of experiment we collected soil samples (app. 250 g) from depth 0 – 200 mm. The samples were analysed for:

- $N_t$ – Kjeldahl method,
- P – spectrophotometrically by phosphomolybdic method in the leachate by Mehlich III,
- K and Ca – flame-photometrically in the leachate by Mehlich III,
- Mg – spectrophotometrically in the leachate by Mehlich III,
- oxidizable carbon ($C_{ox}$) – by Tjurin method in modification by Nikitin,
- pH – exchangeable in KCl.

Soil chemical characteristics of the experimental site are documented in Table 2.

**Table 2:** Weather condition on vegetation periods in 2012 – 2014

| $N_t$ | P | K | Mg | Ca | $C_{ox}$ | pH/KCl |
|---|---|---|---|---|---|---|
| mg $kg^{-1}$ | | | | | g $kg^{-1}$ | |
| 1 823.2 | 58.3 | 336 | 541 | 6 067 | 7.7 | 6.78 |

## 2.2 Characteristics of experiment

The experiment was established in early October 2011. We used turf mixture designed for low slowly growing turfs with following composition: *Lolium perenne* L. (30 %), *Festuca rubra* L. (50 %) and *Festuca ovina* L. (20 %).

Experimental plots area was 2.4 $m^2$ and each treatment was in 3 random replications.

In the experiment 5 treatments were used:

1. Without fertilizing („Control“),
2. Turf fertilizer NPK 15-3-8 (+3 MgO + 0.8 Fe + 18 S) („Turf fertilizer“),
3. Slow release fertilizer NPK 14-5-14 (+4 CaO + 4 MgO + 7 S) („SRF“),
4. Organic fertilizer NPK 5-1-1 („OF 1“),
5. Organic fertilizer NPK 3-2-1 („OF 2“),

For the recommended N dose of fertilizer the value 18 g $m^{-2}$ was taken, which meets the requirements for intensively used turfs (Svobodová, 2004).

System of fertilizing is presented in Table 3.

**Table 3:** System of fertilizing

| Type of fertilizer (number of applications per year) | Yearly dose (g $m^{-2}$) | Date of application | | | |
|---|---|---|---|---|---|
| | | Beginning of vegetation | Around 20.6. | Half of July | Half of August |
| | | Dose of fertilizer to variant (g $m^{-2}$) | | | |
| Turf fertilizer (3x) | 120 | 40 | 40 | | 40 |
| SRF (2x) | 128,6 | 64,3 | | 64,3 | |
| OF 1 (1x) | 360 | 360 | | | |
| OF 2 (1x) | 600 | 600 | | | |

### 2.3 Characteristics of fertilizers used in the experiment

Turf fertilizer 15-3-8 (+3 MgO +0.8 Fe +18 S) is the granulated fertilizer intended for use for turfs throughout the year in the form for multiple fertilization (three-fivefold) during the growing season. Nitrogen is in the ammonium form.

SRF NPK 14-5-14 (+4 CaO +4 MgO +7 S) is a complex NPK fertilizer containing urea formaldehyde component as a source of nitrogen enriched with micronutrients. Part of major NPK nutrients is founded in fast-dissolving form.

OF 5-1-1 content is comprising C, H, O, N, P, K, Ca, Mg, S, Fe etc., in the form of organic components of the starch material from the milled cereals (30 %), enriched hydrolyzate of whey (30 %), lignocelluloses raw material from wood processing (30 %), by hydrolysis of whey enriched (30 %) and in 10 % mineral constituent zeolite of sodium aluminium silicate. Philosophy of this fertilizer is unlike mineral fertilizers aimed at improving the carbon balance.

OF 3-2-1 is produced by modern technology from natural materials without the use of chemicals and preservatives. Production procedure at high temperature leads to inactivation of pathogens and weed seeds. This fertilizer is characterized as high-quality organic fertilizer with gradual release of the main nutrients and essential trace elements. Its high biological value is increased due to harmless processing, content balance, easy handling and hygiene applications in practice. Compared with manure it constitutes a modern compensation for of manure.

### 2.4 Monitored parameters and analysis

Experiment was realized under non–irrigated conditions. When turf reached height of approximately 80 – 100 mm, it was mowed to height 50 mm. The turf height (mm) was determined as an average of 10 measurements in plots before each mowing. We used for the measurement ruler. Production of above-ground phytomass (g $m^{-2}$) was determined by sampling the above-ground phytomass by means of accumulation scissors from the surface of 0.1 × 1 m and subsequently dried at 105 °C.

The annual average daily gain of height (mm $day^{-1}$) was calculated according to the formula:

$$\text{The average daily gain of height} = \frac{\text{Ø height of mowing (mm)} - 50 \text{ mm}}{\text{number of accretion days}}$$

$$\text{The annual average daily gain of height} = \frac{\text{the sum of average daily gains of height}}{\text{number of the average daily gains of height}}$$

The annual average daily gain of mass (g $m^{-2}$ $day^{-1}$) was calculated according to the formula:

$$\text{The average daily gain of phytomass} = \frac{\text{Ø production on mowing } (g m^{-2})}{\text{number of accretion days}}$$

$$\text{The annual average daily gain of phytomass} = \frac{\text{the sum of average daily gains of mass}}{\text{number of the average daily gains of mass}}$$

### 2.5 Statistical analysis

Results were statistically evaluated by the Analysis of Variance (ANOVA – Multiple Range Tests, Method: 95.0 percent LSD) using statistical software STATISTICA 7.1 (Stat Soft. Inc. 2007).

## 3 RESULTS AND DISCUSSION

### 3.1 The average height of turf

The average height of turf is indicated in Table 4. The highest turfs were fertilized by Turf fertilizer (103.59 mm) and SRF (105.41 mm) in 2012. Treatment "OF 2" (94.66 mm) was lower in comparison to the Control (95.99 mm). Turf fertilized OF 1 reached the average height of turf 99.25 mm. Comparing the values of standard deviation (δ) showed that the effect of the fertilizer SRF (δ = 1.59) on turf is more evenly developed as treatments fertilized by Turf fertilizer (δ = 1.68), OF 1 (δ = 1.71), and OF 2 (1.83). The least balanced growth was at Control (δ = 2.16).

We registered increase of the average height on turfs fertilized by Turf fertilizer, SRF and OF 1 (115.59 – 120.05 mm) in the second year (2013). There was also higher variability of measured values (1.97 – 3.49) in compare to the other variants. The sharp decline reached Control (72.03 mm).

In the last evaluated year (2014) again higher average height of turf was reached by application Turf fertilizer, SRF and OF 1 (112.10 – 118.79 mm). Treatment "OF 2" didn't reach the average height 100 mm not even in 2014.

The values of the average turf height for the whole period (2012 – 2014) fertilized by organic and inorganic fertilizers were statistically higher than Control. The smallest growth had turf fertilized by OF 1 (δ = 2.93). Between 2012 and 2014 we found statistically significant effect on the average height of turf.

**Table 4**: The average height of turf

| Year/variant | 2012 | | 2013 | | 2014 | | 2012 – 2014 | |
|---|---|---|---|---|---|---|---|---|
| | $\bar{x}$ height (mm) | δ | $\bar{x}$ height (mm) | δ | $\bar{x}$ height (mm) | δ | $\bar{x}$ height (mm) | δ |
| Control | 95.99 | 2.16 | 72.03 | 1.30 | 83.24 | 1.52 | $83.75^{b}$ | 2.02 |
| Turf fertilizer | 103.59 | 1.68 | 119.78 | 2.03 | 112.10 | 2.30 | $111.82^{a}$ | 2.13 |
| SRF | 105.41 | 1.59 | 115.59 | 1.97 | 118.79 | 2.05 | $113.26^{a}$ | 1.99 |
| OF 1 | 99.25 | 1.71 | 120.05 | 3.49 | 117.45 | 2,87 | $112.25^{a}$ | 2.93 |
| OF 2 | 94.66 | 1.83 | 94.97 | 1.76 | 97.94 | 1.96 | $95.86^{c}$ | 1.86 |
| Ø | $80.58^{A}$ | - | $104.48^{AB}$ | - | $105.90^{B}$ | - | - | - |

Different index (a, A, b, B, c) means statistically significant differences within column (Fisher LSD test, α= 0.05), δ – standard deviation, $\bar{x}$, – arithmetic mean, Ø – mean.

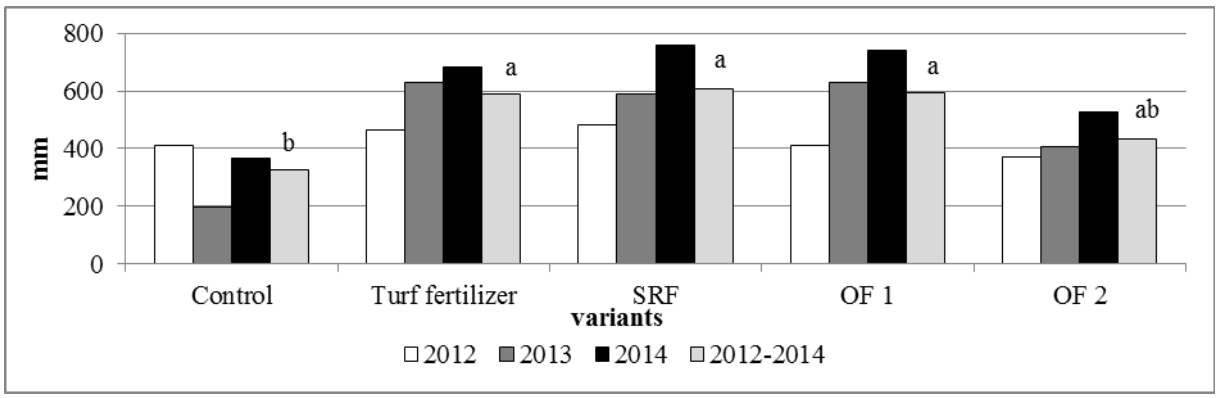
### 3.2 Total height of turf

The results of total turf height are presented on Figure 1. Turfs fertilized by inorganic fertilizers Turf fertilizer (463.70 mm) and SRF (481.00 mm) had a high growth in 2012. Treatments with application of organic fertilizers grew more slowly (370.60 and 409.70 mm) than Control (412.20 mm). In this year we observed a negative effect of rainfall deficiency on the turf growth (Table 1). Lack of water affected the functional manifestations of the plants and realization of their growth-production process (Kostrej et al., 2000; Brestič and Olšovská, 2001; Brestič et al., 2008).

We observed marked increase of the total height of turf at all variants in the following year (2013). The exception was control where total turf height decreased (198.30 mm). Maximum turf height was reached when fertilized by OF 1 (630.47 mm).

The values of total turf height again increased at all treatments in 2014. The biggest increase was observed on turf fertilized by SRF (about 166.34 mm). Conversely, markedly less than it was on the variant with application Turf fertilizer (about 55 mm).

In years 2012 – 2014 the most intensively grew turfs fertilized by Turf fertilizer (591.57 mm), OF 1 (594.06 mm) and SRF (609.33 mm). Statistical evaluation showed that fertilization with these fertilizers had significant effect on the total height of fertilized turfs compared with control.




Different index (a, b) means statistically significant differences within column (Fisher LSD test, α= 0.05).

**Figure 1:** Total height of turf

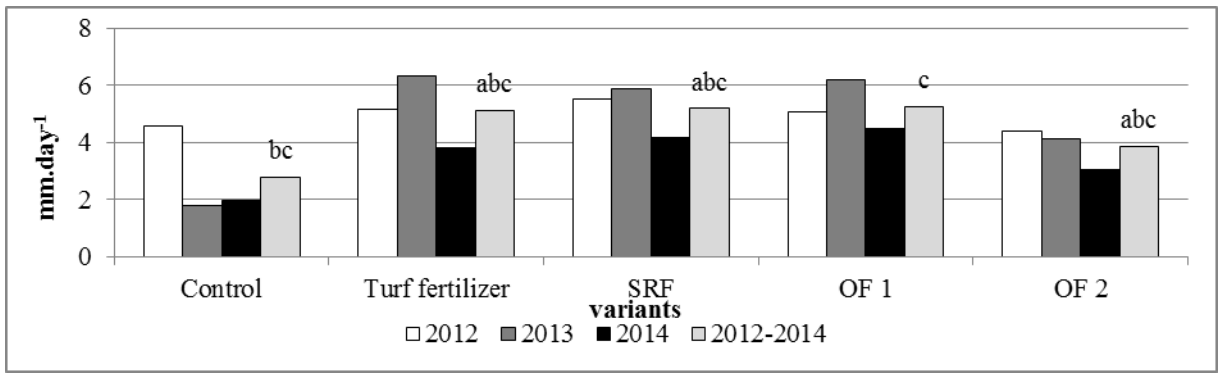
### 3.3 The annual average daily gain of height

The development of the average annual daily gain of height in 2012 is presented on Figure 2. The highest intensity of growth (more than 5 mm day$^{-1}$) was observed on turfs with application Turf fertilizers (more than 5.18 mm day$^{-1}$), SRF (5.55 mm day$^{-1}$) and OF 1 (5.09 mm day$^{-1}$). Comparison of the average daily gain of height values with the Descriptor for Poaceae (Ševčíková et al., 2002) showed that turfs had "very fast" growth, i.e. achieved 1 point on the scale, where 1 is the worst and 9 is the best rating level of evaluated characteristics. "Moderate" growth (i.e. 3 points according to this scale) had control treatment, turfs fertilized by Turf fertilizer and OF 2. It is considered positive from the point of view of grass turf management (Turgeon, 2002; Cagaš and Macháč, 2005).

In the second year of monitoring (2013) the growth in treatments "Turf fertilizer", "SRF" and "OF 1" was even faster (until 6.35 mm day$^{-1}$) than in 2012. We consider a possible gradual release of nutrients in the environment during the whole season (Gregorová 2001; Svobodová, 2003), when the weather conditions were better than in the previous year (Table 1). Similar conclusion in turf experiments reached Zhang and Nyborg (1998) too. They found that better weather conditions improved the release of nutrients from these fertilizers. This results in improving growth-production process of turf. Slowly developed turf with application OF 2 and control. According to the Descriptor for Poaceae (Ševčíková et al., 2002), can be argued that turfs fertilized by Turf fertilizer, SRF and OF 1 reached "very fast" growth.

In 2014 we found a decrease in the rate of turfs growth. The most remarkable change occurred in the treatment "Turf fertilizer" (about 2.55 mm day$^{-1}$). The most intensive growth had turfs fertilized by SRF (4.17 mm day$^{-1}$) and OF 1 (4.47 mm day$^{-1}$). According to the Descriptor for Poaceae (Ševčíková et al., 2002), we may evaluate these turfs as "fast" growing. Other fertilization treatments could be evaluated as "moderate" growing.

Comparing the monitored years 2012 – 2014, we found the highest annual average daily height gain on turfs fertilized with Turf fertilizer (5.11 mm day$^{-1}$), SRF (5.20 mm day$^{-1}$) and OF 1 (5.26 mm day$^{-1}$). Treatment "OF 2" had lower average height daily gain (3.86 mm day$^{-1}$). The lowest growth rate was observed in Control (2.78 mm day$^{-1}$). These findings were not statistically significant.




Different index (a, b, c) means statistically significant differences within column (Fisher LSD test, α= 0.05).

**Figure 2:** The annual average daily height gain

### 3.4 The average of above-ground dry phytomass

The values of the average of dry above-ground phytomass (Table 5) seems that turfs fertilized by inorganic fertilizers Turf fertilizer and SRF produced about 2.11 – 3.50 g $m^{-2}$ more phytomass as control. Treatments with application of organic fertilizers had the average of dry above-ground phytomass smaller than 30 g $m^{-2}$. The smallest above-ground phytomass created control (30.81 g $m^{-2}$), which was also characterized by lowest variability of phytomass production (δ = 1.4). Conversely the variant of application, in which the highest production of above-ground phytomass was observed, was the least suitable from this perspective (Bigelow and Walker, 2005).

Relatively high production of phytomass was characterized for variant fertilized by OF 1 (44.44 g $m^{-2}$) in 2013. Turfs with application of inorganic fertilizers had almost identical average of dry above-ground phytomass (38.68 – 38.96 g $m^{-2}$). The least productive treatment was OF 2 (27.71 g $m^{-2}$) and control (15.57 g $m^{-2}$). Control had also the lowest variability of phytomass production (δ = 1.28 and 1.72).

Variants fertilized by inorganic fertilizers and OF 1 reached almost identical production of phytomass (30.47 – 32.40 g $m^{-2}$) in the last evaluated year (2014). The least productive were treatment with application OF 2 (23.97 g $m^{-2}$) and control (12.22 g $m^{-2}$), which also had the lowest variability of production of aboveground phytomass (δ = 0.79 and 1.64).

The average of years 2012 – 2014 seems that the average of dry above-ground phytomass on fertilized turfs as control was statistically significant. Approximately the same production of phytomass produced treatments fertilized by inorganic fertilizers and OF 1 (34.55 and 34.92 g $m^{-2}$). Turf with application of OF 2, compared to other fertilized turfs, produced less above-ground phytomass (about 7.65 – 8.02 g $m^{-2}$). Treatments fertilized by organic fertilizers were characterized by the lowest and highest variability of phytomass (δ = 1.63 and 2.57). Given the objective of turf growing, which is to achieve and maintain suitable turf, e. g. adequate density, balanced colour, uniformity, recuperative capacity without achieving high production of aboveground phytomass (Gregorová, 2001; Turgeon 2002) the variants Control, SRF and OF 2 were positively assessed. For the year 2014 was proved statistically significant effect of treatments on the average of dry above-ground phytomass.

**Table 5**: The average of dry above-ground phytomass

| Year/variant | 2012 | | 2013 | | 2014 | | 2012 – 2014 | |
|---|---|---|---|---|---|---|---|---|
| | $\bar{x}$ phytomass (g m$^{-2}$) | δ | $\bar{x}$ phytomass (g m$^{-2}$) | δ | $\bar{x}$ phytomass (g m$^{-2}$) | δ | $\bar{x}$ phytomass (g m$^{-2}$) | δ |
| Control | 30.81 | 1.24 | 15.57 | 1.28 | 12.22 | 0.79 | 19.53$^{b}$ | 1.36 |
| Turf fertilizer | 34.31 | 1.93 | 38.96 | 1.95 | 30.47 | 2.43 | 34,58$^{a}$ | 2.14 |
| SRF | 32.92 | 1.26 | 38.68 | 2.34 | 32.05 | 1.75 | 34,55$^{a}$ | 1.83 |
| OF 1 | 27.93 | 1.46 | 44.44 | 3.51 | 32.40 | 2.21 | 34.92$^{a}$ | 2.57 |
| OF 2 | 29.02 | 1.52 | 27.71 | 1.72 | 23.97 | 1.64 | 26.90$^{c}$ | 1.63 |
| Ø | 31.0$^{A}$ | - | 38.21$^{A}$ | - | 26.22$^{B}$ | - | - | - |

Different index (a, A, b, B, c) means statistically significant differences within column (Fisher LSD test, α= 0.05), δ – standard deviation, $\bar{x}$ – arithmetic mean, Ø – mean.

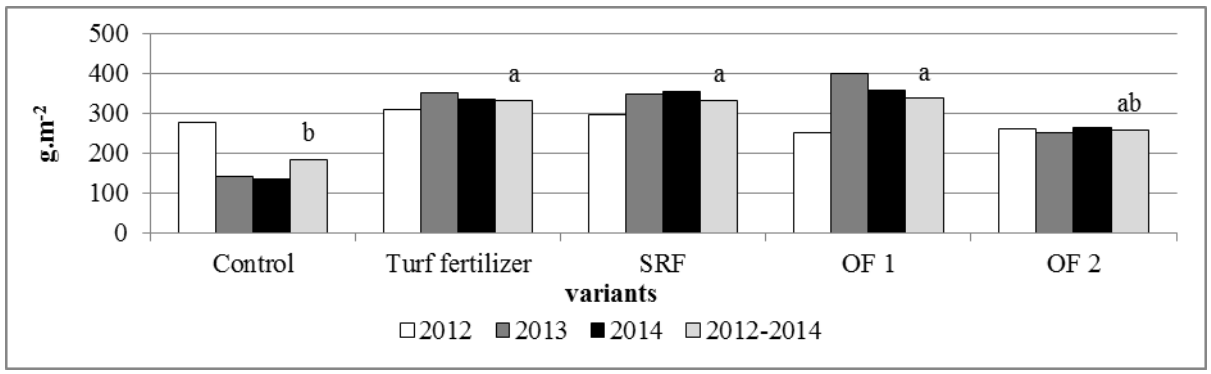
### 3.5 Total production of dry above-ground phytomass

The total production of dry above-ground phytomass (2012) was the highest on treatments fertilized by inorganic fertilizers Turf fertilizer (308.80 g m$^{-2}$) and SRF (296.30 g m$^{-2}$). Also Cagaš et al. (2011) found out in turf experiments that at different dose of nitrogen higher production of phytomass was achieved on turf fertilized by slow release fertilizer than on turf fertilized by long-acting fertilizer. The smallest production had treatments with application of organic fertilizers (251.40 – 261.20 g m$^{-2}$).

The lowest production had control (140.20 g m$^{-2}$) in the second year (2013). Relatively higher production was observed also on treatment "OF 2" (249.43 g m$^{-2}$). Approximately 350 g m$^{-2}$ of above-ground phytomass produced turfs with application of inorganic fertilizers. Treatment "SRF" reached lower production as treatment "Turf fertilizer". This finding was not confirmed in an experiment carried out by Bilgili and Açikgöz (2011). The highest total production of dry above-ground phytomass had turf with application of OF 1 (399.97 g m$^{-2}$).

Fertilized treatments, without turf fertilized OF 2 (263.67 g m$^{-2}$), produced phytomass bigger than 300 g m$^{-2}$ in 2014. Control treatment had production of above-ground phytomass 134.37 g m$^{-2}$.

Comparison of total production values of dry above-ground phytomass (2012 – 2014) with Descriptor for Poaceae (Ševčíková et al., 2002), has shown that all evaluated treatments were characterized from "very low" until "low" production of phytomass (250 – 400 g m$^{-2}$). The exception was control which is characterized by "very low" production of phytomass (< 250 g m$^{-2}$). Improvement in production of turfs fertilized by inorganic fertilizers and OF 1 was compared with control, and the difference was statistically significant. Treatment "SRF" was one of the most productive in our experiment. However, this is not matched with the statement of Lošák and Ševčíková (2012), who in their experiment after using slow release fertilizer recorded the lowest production of above-ground phytomass.




Different index (a, b) means statistically significant differences within column (Fisher LSD test, α= 0.05).

**Figure 3:** Total production of dry above-ground phytomass

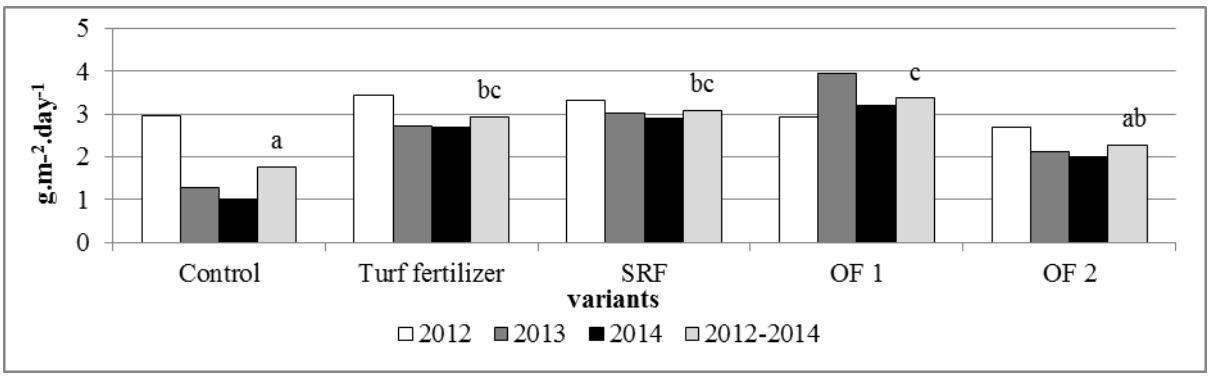
### 3.6 The annual average daily phytomass gain

Analysis of the annual average daily gain of above-ground phytomass (2012) (Figure 4) was observed the highest on treatments fertilized by inorganic fertilizers Turf fertilizer (3.43 g $m^{-2}$ $day^{-1}$) and SRF (3.31 g $m^{-2}$ $day^{-1}$). Martincová (2007) found out increasing of phytomass production on treatment fertilized by slow release fertilizer in drier seasons as on treatments with long-acting forms of fertilizers. This claim was not confirmed in our experiment. Conversely the lowest annual average daily phytomass gain was achieved on turfs fertilized by organic fertilizers OF 1 (2.94 g $m^{-2}$ $day^{-1}$), OF 2 (2.68 g $m^{-2}$ $day^{-1}$) and control (2.95 g $m^{-2}$ $day^{-1}$).

In the following year 2013 the most productive were treatments fertilized by inorganic fertilizers Turf fertilizer (2.73 g $m^{-2}$ $day^{-1}$), SRF (3.02 g $m^{-2}$ $day^{-1}$) and OF 1 (3.96 g $m^{-2}$ $day^{-1}$). The lowest production we observed on control (1.29 g $m^{-2}$ $day^{-1}$).

In 2014 the most productive treatments were again those fertilized by inorganic fertilizers (2.68 g $m^{-2}$ $day^{-1}$ and 2.89 g $m^{-2}$ $day^{-1}$) and OF 1 (3.21 g $m^{-2}$ $day^{-1}$). Turf with application of OF 2 produced about 50 % more above-ground phytomass as control. This increase wasn´t considerable as in experiment of Sloboda (2000), who observed increase in phytomass for about 294.6 %, also Bošanská (1999) found out using OF 2 fertilizer increased production for about 33 % versus control.

In comparing the monitored years 2012 – 2014, we found yearly reduction of annual average daily phytomass gain. The treatment OF 1, where the production of phytomass in 2013 was about 1.02 g $m^{-2}$ $day^{-1}$ was higher than in 2012. The most remarkable decline in production was seen on control (about 1.66 g $m^{-2}$ $day^{-1}$). Relatively balanced average of above-ground phytomass gain was on turf with application of SRF. The annual average daily phytomass gain increased on all fertilized treatments compared with control. Statistically significant effect was recorded on turf fertilized by OF 1.




Different index (a, b, c) means statistically significant differences within column (Fisher LSD test, α= 0.05).

**Figure 4:** The annual average daily phytomass gain

## 4 CONCLUSION

There was observed the influence of organic and inorganic fertilizers on selected characteristics of turf growth-production process. The highest turf height and production of above-ground phytomass were gained by effect of Turf fertilizer 15-3-8 (+3MgO +0.8Fe +18S), slow release fertilizer 14-5-14 (+4CaO +4MgO +7S) and organic fertilizer 5-1-1. Organic fertilizer 3-2-1 and control treatment reached lower parameters of turf growth-production process. Given the objective of turf growing, which is to achieve and maintain suitable turf, i.e. adequate density, balanced colour, uniformity, recuperative capacity without achieving high production of aboveground phytomass, the treatments implemented were appropriate. Treatments fertilized by slow release fertilizer 14-5-14 (+4CaO +4MgO +7S) and organic fertilizer 5-1-1 had the most balanced growth and the highest production of turf above-ground phytomass in comparison with others treatments.

## 5 ACKNOWLEDGEMENTS

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**Agris category code**: F60, f62, f40

# Effects of $TiO_2$ nanoparticles and water-deficit stress on morpho-physiological characteristics of dragonhead (*Dracocephalum moldavica* L.) plants

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**ABSTRACT**

Water-deficit stress is the most important environmental factors limiting plant growth, and production. Nano-titanium dioxide (nano anatase$TiO_2$) can have various profound effects on the crop physiological, biochemical and morphological characteristics. In the present research, the influences of different concentrations of$TiO_2$ nanoparticles (NPs) (0, 10 and 40 ppm) and water-deficit stress on Dragonhead (*Dracocephalum moldavica* L.) were investigated in a factorial experiment based on randomized complete block design with three replications. Results showed that under normal irrigation, foliar application of 10 ppm $TiO_2$ NPs increased plant shoot dry mass and essential oils content. Under water-deficit stress condition, plants treated with 10 ppm $TiO_2$ NPs had more proline and much less $H_2O_2$ and malondialdehyde content as compared to untreated plants. Therefore, it can be concluded that proper concentration of $TiO_2$ NPs probably can be used as an exogenous stimuli for improvement of shoot growth and essential oil content in plants. Furthermore, water-deficit stress-induced damages such as oxidative stress and membrane damage can be ameliorated by foliar application of $TiO_2$ NPs at appropriate concentrations.

**Key words:** aromatic plants, drought stress, malondialdehyde, reactive oxygen species,$TiO_2$NPs

**IZVLEČEK**

**UČINKI NANO DELCEV $TiO_2$ IN SUŠNEGA STRESA NA IZBRANE MORFOLOŠKE IN FIZIOLOŠKE LASTNOSTI MOLDAVSKE KAČJEGLAVKE (*Dracocephalum moldavica* L.)**

Sušni stres je eden izmed najpomembnejših okoljskih dejavnikov, ki omejujejo rast in produktivnost rastlin. Uporaba nano delcev titanovega dioksida ($TiO_2$ nano anataza) ima lahko velike učinke na fiziološke, biokemične in morfološke lastnosti gojenih rastlin. V tej raziskavi je bil v naključnem faktorskem bločnem poskusu preučevan vpliv različnih koncentracij nano delcev $TiO_2$ (0, 10 in 40 ppm) in sušnega stresa na moldavsko kačjeglavko (*Dracocephalum moldavica* L.) v treh ponovitvah. Rezultati so pokazali, da sta se suha masa in vsebnost eteričnih olj povečali pri normalnem namakanju in listnem dodajanju 10 ppm $TiO_2$ nano delcev. V razmerah sušnega stresa so vsebovale rastline, ki so bile tretirane z 10 ppm $TiO_2$ več prolina in mnogo manj $H_2O_2$ in malonildialdehida v primerjavi z netretiranimi rastlinami. Na osnovi tega bi lahko zaključili, da bi z uporabo primerne koncentracije nano delcev $TiO_2$ kot zunanjega stimulanta, lahko izboljšali rast poganjkov in vsebnost eteričnih olj pri tej rastlini. Poškodbe kot sta oksidativni stres in poškodovanost membran, ki nastanejo zaradi sušnega stresa, bi lahko ublažili s foliarno aplikacijo primerne koncentracije nanodelcev $TiO_2$.

**Ključne besede:** aromatične rastline, sušni stres, malondialdehid, reaktivne oblike kisika, $TiO_2$ nano delci

## 1 INTRODUCTION

Dragonhead (*Dracocephalum moldavica* L.), a perennial aromatic herb belonging to Lamiaceae family, has antioxidative properties and can be used as food and cosmetic related preservatives

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(Dastmalchi et al., 2007). It is also known as medicinal herb which is used for treatment of stomach and liver disorders. Dragonhead shoot contains various secondary metabolites such as phenolic compounds (flavonoids), essential oils and etc. which are responsible for medicinal characteristics of this plant (Dastmalchi et al., 2007; Yang et al., 2014). Production and accumulation of these metabolites depends highly on the plant growing conditions such as available soil water (Selmar and Kleinwachter, 2013; Yousefzadeh et al., 2013).

Drought stress is widespread and upcoming limiting environmental factor which adversely affects crop production especially in drought prone areas. The most common plant responses to drought stress are stomata closure and overproduction of different types of secondary metabolites in order to prevent water losses and oxidative damage, respectively (Serraj and Sinclair, 2002). Upon stomata closure, $CO_2$ supply to Calvin cycle is severely declined which in turn can finally result in reduced biomass production. It has been reported that severe drought stress significantly reduced root and shoot dry mass, leaf chlorophyll pigments and leaf relative water content (RWC) of Dragonhead plants (Alaei et al., 2013). Under severe drought stress condition, plant cells undergo oxidative damage due to production of Reactive Oxygen Species (ROS). Parts of these free radicals may be detoxified by antioxidant enzyme such as super oxide dismutase producing $H_2O_2$. However, ROSs may attack the phospholipids of the cell membrane causing lipid peroxidation and electrolyte leakage. In this case, malondialdehyde (MDA) which is one of the final products of lipid peroxidation can be considered as an evaluation factor for membrane damage. Concentration of compatible organic solutes such as proline which contributes to stabilizing sub-cellular structures and osmotic adjustment in cell cytosol are also generally enhanced in plants suffering drought stress (Ashraf and Foolad, 2007). Besides, an increased synthesis of essential oils in response to drought stress is reported in aromatic plants (Kleinwachter et al., 2015). These metabolites may contribute to prevent damage caused by free radicals. However, drought stress-related increase in the concentration of essential oils may be compensated by the related loss in biomass, resulting in almost the same overall essential oil content in both drought-stressed and well-watered plants (Selmar and Kleinwachter, 2013).

$TiO_2$ nanoparticles (NPs) have various profound effects on the crop physiological, biochemical and morphological characteristics (Mishra et al.2014). Exogenous application of $TiO_2$ NPs during spinach growth stage promoted chlorophyll formation, Rubisco activase activity and photosynthetic rate which finally lead to increase in plant dry mass (Gao et al., 2008). It is also reported that foliar application of $TiO_2$ NPs increased seed yield of cowpea (*Vigna unguiculata* (L.) Walp.), possibly due to increased photosynthetic rate (Owolade et al., 2008). Also, activity of antioxidant enzymes such as catalase and peroxidase has been boosted in response to $TiO_2$NPs application. As a result, accumulation of MDA lessened due to induction of plant antioxidant systems (Lei et al., 2008). In addition, low concentration of $TiO_2$ NPs reliably improved resistance of chickpea genotypes to cold stress and also alleviated cold-induced damages via activating defense mechanisms (Mohammadi et al., 2013). Therefore, $TiO_2$ has opened new and interesting horizon for plant physiologists in order to improve plant performance even under stress conditions. Since, the effects of $TiO_2$ NPs may not be the same in all environmental conditions and would be vary between different plant species and applied concentrations (Feizi et al., 2012). Thus, in the present research the influences of $TiO_2$ NPs concentrations on morpho-physiological and biochemical characteristics of dragonhead, as an aromatic and medicinal plant, were investigated under both water stress and non-stress conditions.

## 2 MATERIALS AND METHODS

### 2.1 Characterization and scanning electron microscopy (SEM) image of nano $TiO_2$

Nano $TiO_2$ (namely nano-anatase) was provided from the Nanomaterials Pioneers Company, NANOSANY (Mashhad, IRAN). The provided pack was characterized by laboratory analytical methods. The specific surface area (SSA) of nano $TiO_2$ was approximately 200-240 $m^2g^{-1}$, with a pore size of 0.1 ml g and purity of >99 %. The size of $TiO_2$ NPs were specified by Scanning Electron Microscope (SEM), and estimated to be 10-25 nm (Figure 1). The crystal characteristics of nano $TiO_2$ particles were determined by X-Ray Diffraction (XRD) method (XPert PRO MPD, PANalytical) in the 2θ range of 30°-120° operated at a voltage of 40 kV and a current of 40 mA with Cu Kα radiation. The XRD analytical procedure revealed that employed nano$TiO_2$ particles were all exhibited in the anatase form (Figure 2).

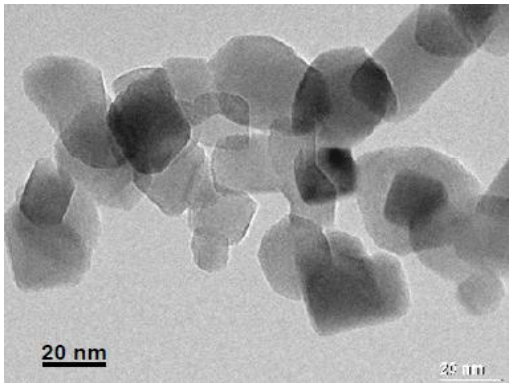


**Figure 1:** SEM micrograph of $TiO_2$ NPs. Average size of the nanoparticles was 10-25 nm

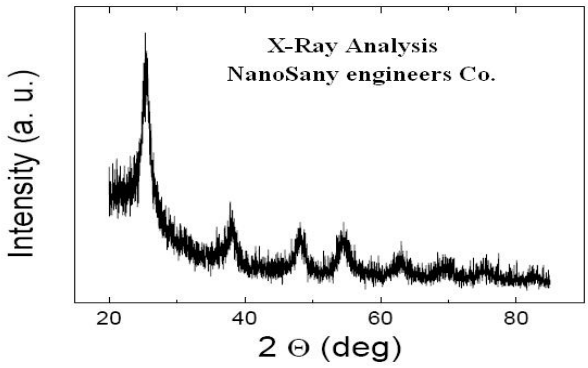


**Figure 2:** XRD pattern of $TiO_2$ NPs. XRD measurement showed that used $TiO_2$ NPs were all in the anatase phase

### 2.2 Plant materials, treatments and experimental setup

Dragonhead (*Dracocephalum moldavica* L.) is distributed in the north and northwestern parts of Iran, especially in the western parts of Azarbaijan province, and Albourz Mountains. Landrace Dragonhead seeds were prepared from Urmia University, Iran. Seeds were surface sterilized with 1 % sodium hypochlorite (NaOCl) for 5 min and then were washed three times, soaked in distilled water for 10 min. Ten seeds were directly grown in pots, containing approximately 4 kg of soil

comprising a mixture of clay, silt and sand in the ratios of 4.5, 71.5 and 24 percent, respectively, with an electric conductivity (EC) of 1.52 $dSm^{-1}$ and pH of 7.2. The concentrations of total N, P, and K were 0.07 %, 12.8 mg $kg^{-1}$, and 7.4 mg $kg^{-1}$, respectively. At the 3-4 leaf stage, plants were thinned to five per pot. Natural light supplemented with fluorescent lamps was provided in the greenhouse for 16 h per day with an irradiance of 250 $\mu molm^{-2}s^{-1}$, and temperature of 28/18 ˚C (day/night).

A factorial experiment based on randomized complete block design was carried out with three replications. Treatments were NPs of $TiO_2$ solutions (three levels), and water stress (two levels). NPs of $TiO_2$ solutions were prepared at concentrations of 0, 10 and 40 $mgl^{-1}$ with filtered, double-distilled water. Working solutions were made by vigorous vortexing (using ultrasonic) when required. Plants were exposed to water stress at the initiation of flowering stage with daily weighting pots. Plants were irrigated every two days to achieve field capacity (FC) and 50 % of FC by pressure plate set, for control and water-deficit conditions, respectively. At the beginning of flowering stage, 50 ml of $TiO_2$ NPs were sprayed on the plant shoots in each pot for three successive days using hand atomizer. The plants sprayed with the same volume of distillated water were considered as control. Leaf samples of each treatment were taken at complete flowering stage and were separated into two parts. Part of each sample was immediately frozen in liquid nitrogen for two minutes and then stored at -70 ˚C for all measurements such as plastid pigments, MDA, proline and $H_2O_2$ contents. The other part was shade dried for a week and then used for extraction of essential oils. Plant morphological traits including shoot dry mass (SDM), root dry mass (RDM), stem branch number (SBN) and plant leaf number (PLN) were recorded at full flowering stage.

## 2.3 Plant physiological parameters assays

2.3.2 Leaf relative water content determination

RWC was determined according to Barr and Weatherley (1962):

$$RWC\ (\%) = (FM-DM)/(TM-DM)\ 100$$

Where: FW: fresh mass; DW: dry mass; TW: turgor mass.

2.3.2 Plastid pigment measurements

Chlorophyll (Chl) and carotenoids were extracted from 0.5 g of the youngest fully expended fresh leaves by grounding them in 0.5 ml of acetone (80 % V/V). The absorption was recorded at 645 nm (Chl*a*), 663 nm (Chl*b*) and 470 nm (carotenoids) in a spectrophotometer (PG Instrument LTD T80+UV/VIS. Photosynthetic pigment contents were calculated from the following equations as described by Lichtenthaler and Wellburn (1983).

$$Chla\ (mg.g^{-1}FM) = 11.75 \times A_{663} - 2.35 \times A_{645}$$

$$Chlb\ (mg.g^{-1}FM) = 18.61 \times A_{645} - 3.96 \times A_{663}$$

$$Carotenoids\ (mg.g^{-1}FM) = 4.69 \times A_{470} - 0.268 \times (20.2 \times A_{645} + 8.02 \times A_{663})$$

2.3.3 Determination of $H_2O_2$ content

Hydrogen peroxide ($H_2O_2$) content in the leaves of Dragonhead plant was specified according to Velikova et al. (2000). Briefly, fresh tissues of leaves (0.5 g) were homogenized in an ice bath with 5 ml of TCA (0.1 % w/v). The homogenate was centrifuged at 12,000 × g for 15 min. Then 0.5 ml of the supernatant was supplemented to 0.5 ml of 10 mm potassium phosphate buffer (pH 7.0) and 1 ml of 1 m KI. Finally, the absorbance of the supernatant was recorded at 390 nm in a spectrophotometer (PG Instrument LTD $T80^{+}$ UV/VIS). The content of $H_2O_2$ was estimated by comparison with a standard calibration curve previously made by various $H_2O_2$ concentrations.

2.3.4 Determination of the MDA content

The level of MDA content (as an end product of lipid peroxidation) was assessed according to Heath and Packer (1968).Briefly, 0.5 g of fresh tissues of leaves were homogenized in 5 ml of 0.1 % (w/v) TCA solution and centrifuged at 12,000 × g for 15 min at 25 °C. Then, 2 ml of supernatant was added to 2 ml of 0.6 % (w/v) TBA. The mixture was incubated at 95 °C for 30 min, then cooled on ice and then samples were centrifuged at 4,000 × g for 20 min. Thereafter, the absorbance of supernatant was recorded at 532 nm.

The MDA content was calculated based on its extinction coefficient of 155 $mM^{-1}$ $cm^{-1}$.

2.3.5 Determination of the proline content

Proline contents in leaf tissue were measured by the Bates et al. (1973) method. Fresh leaf material (0.5 g) was homogenized in 10 ml of 3 % aqueous sulfosalicylic acid and the homogenate was centrifuged at 10,000 rpm. 2 milliliter of the supernatant was mixed with 2ml of acid ninhydrin and 2ml of glacial acetic acid in a test tube. The mixture was placed in a water bath for 1 h at 100 $^{o}$C. The reaction mixture was extracted with 4 ml toluene and the chromophore containing toluene was aspirated, cooled to room temperature, and the absorbance was measured at 520 nm with a spectrophotometer (PG Instrument LTD T80+UV/VIS). Appropriate proline standards were included for the calculation of proline in the samples.

2.3.6 Determination essential oil yield

Shade-dried aerial parts of the plants (50 g) were subjected to hydro-distillation for 4 h using a Clevenger type apparatus to extract essential oils (Sefidkon et al., 2004).

### 2.4 Statistical analysis

The data were analyzed using the SAS statistical software. A factorial experiment based on randomized complete block design was carried out with three replicates (n = 3). Duncan's Multiple Range Test ($P < 0.01$) was used to compare the means.

## 3 RESULTS AND DISCUSSION

### 3.1 Foliar application of $TiO_2$NPs under normal irrigation

Analysis of variance (ANOVA) showed that 10 ppm foliar application of $TiO_2$ NPs had various effects on morphology and physiology of dragonhead plants. Under normal irrigation, RDM, PLN, SBN and RWC of the plants sprayed with 10 ppm $TiO_2$ didn't changed, while their SDM increased significantly in comparison with untreated plants (Table 1, 2). However, application of 40 ppm $TiO_2$ decreased RDM, PLN, SBN, SDM and RWC (Table 2). Increase in spinach dry mass and mung bean (*Vigna radiata* L.) shoot and root length in response to $TiO_2$ NPs has been reported (Raliya et al., 2015). It has been shown that foliar applied NPs can enter the leaves through stomatal openings and then are translocated to various tissues via the symplast and/or apoplast (cell wall and intercellular space) pathways (Larue et al., 2012).When located inside the photosynthetic cells, $TiO_2$ could increase Rubisco activase activity and even its mRNA expression (Gao et al., 2008; Ma et al., 2008) and promote photosynthesis rate (Zhang et al., 2008), the possible reasons behind increase in shoot dry mass of treated dragonhead plants. In addition, NPs can sequester nutrient elements on their surface and serve as a nutrient stock to the plants (Navarro et al., 2008). Also, $TiO_2$ NPs *per se* may act as nano-nutrient fertilizer to improve biomass production by stimulating plant metabolic activities (Raliya et al., 2015). In the other hand, $TiO_2$ NPs induce production of hydroxyl radicals. These radicals have been described as potential cell wall-loosening agent by unspecific cleavage of polysaccharides (Larue et al., 2012). The $TiO_2$ NPs which are passed through apoplast in an appropriate concentration would thus possibly loosen cell wall structure indirectly which may leads to stimulate cell enlargement and growth of the treated plant. Also, it has been demonstrated that high concentrations of the NPs which are toxic for the plants may have positive effects on physiological performance of plants in low concentrations (Khodakovskaya and Lahiani, 2014).

**Table 1:** Analysis of variance (ANOVA) for studied traits in treated dragonhead plants with $TiO_2$ nanoparticles under water-deficit stress

| Traits | Mean squares for source of variation | | | | |
|---|---|---|---|---|---|
| | block | Factor a ($TiO_2$) | Factor b (Water deficit) | Interaction a×b | Error |
| Stem branch Number | 1.16 $^{ns}$ | 38.00 $^{**}$ | 138.88 $^{**}$ | 14.88 $^{**}$ | 0.3 |
| Leaf number | 56.00 $^{ns}$ | 68085.50 $^{**}$ | 99755.55 $^{**}$ | 19590.05 $^{**}$ | 105.73 |
| Root dry mass | 0.00001 $^{ns}$ | 0.1375 $^{**}$ | 0.5408 $^{**}$ | 0.0337 $^{**}$ | 0.0002 |
| Shoot dry mass | 0.04 $^{ns}$ | 5.53 $^{**}$ | 15.01 $^{**}$ | 0.89 $^{**}$ | 0.02 |
| RWC | 1.05 $^{ns}$ | 226.05 $^{**}$ | 329.38 $^{**}$ | 77.72 $^{**}$ | 1.32 |
| Chlorophyll *a* | 0.0003 $^{*}$ | 0.208 $^{**}$ | 0.011 $^{**}$ | 0.024 $^{**}$ | 0.000006 |
| Chlorophyll *b* | 0.00004 $^{**}$ | 0.0285 $^{**}$ | 0.0008 $^{**}$ | 0.0046 $^{**}$ | 0.000001 |
| Total chlorophyll | 0.0006 $^{**}$ | 0.3912 $^{**}$ | 0.0186 $^{**}$ | 0.0493 $^{**}$ | 0.00001 |
| Carotenoid | 0.0002 $^{**}$ | 0.1299 $^{**}$ | 0.0126 $^{**}$ | 0.0155 $^{**}$ | 0.000004 |
| Shoot MDA contents | 0.0005 $^{**}$ | 0.2467 $^{**}$ | 3.0258 $^{**}$ | 0.1686 $^{**}$ | 0.00005 |
| Shoot $H_2O_2$ contents | 0.0005 $^{ns}$ | 3.5325 $^{**}$ | 14.8512 $^{**}$ | 1.4541 $^{**}$ | 0.0102 |
| Shoot proline contents | 7.79 $^{*}$ | 49.97 $^{**}$ | 424.27 $^{**}$ | 220.11 $^{**}$ | 1.53 |
| Essential oil yield | 0.0007 $^{ns}$ | 1.5840 $^{**}$ | 0.0005 $^{**}$ | 0.0369 $^{**}$ | 0.0073 |

*,** Significant at the 0.05 and 0.01 probability level, respectively. $^{ns}$ Not significant.

**Table 2:** Mean comparison of physio-morphological and biochemical traits in treated dragonhead plants with $TiO_2$ nanoparticles under water-deficit stress

| Traits | $TiO_2$ (ppm) | | | | | |
|---|---|---|---|---|---|---|
| | Untreated (0) | | 10 | | 40 | |
| | Normal irrigation | Water deficit | Normal irrigation | Water deficit | Normal irrigation | Water deficit |
| Stem branch Number | 15.33$^{a}$ | 7.33$^{c}$ | 15.33$^{a}$ | 13.33$^{b}$ | 12.67$^{b}$ | 6.00$^{d}$ |
| Leaf number | 418.33$^{a}$ | 137.67$^{d}$ | 427.00$^{a}$ | 350.00$^{b}$ | 220.00$^{c}$ | 131.00$^{d}$ |
| Root dry mass (g) | 0.65$^{a}$ | 0.15$^{c}$ | 0.67$^{a}$ | 0.47$^{b}$ | 0.44$^{b}$ | 0.10$^{d}$ |
| Shoot dry mass (g) | 3.47$^{b}$ | 0.77$^{f}$ | 4.53$^{a}$ | 3.00$^{c}$ | 2.70$^{d}$ | 1.46$^{e}$ |
| RWC (%) | 76.00$^{a}$ | 60.67$^{c}$ | 77.67$^{a}$ | 68.33$^{b}$ | 61.33$^{c}$ | 60.33$^{c}$ |
| Leaf chlorophyll *a* (mg $g^{-1}$ FM) | 0.77$^{a}$ | 0.57$^{d}$ | 0.40$^{f}$ | 0.44$^{b}$ | 0.30$^{c}$ | 0.31$^{e}$ |
| Leaf chlorophyll *b* (mg $g^{-1}$ FM) | 0.30$^{a}$ | 0.23$^{b}$ | 0.15$^{d}$ | 0.19$^{c}$ | 0.13$^{e}$ | 0.12$^{f}$ |
| Total chlorophyll (mg $g^{-1}$ FM) | 1.078$^{a}$ | 0.809$^{b}$ | 0.561$^{d}$ | 0.641$^{c}$ | 0.446$^{e}$ | 0.442$^{e}$ |
| Leaf carotenoids (mg $g^{-1}$ FM) | 0.66$^{a}$ | 0.49$^{b}$ | 0.36$^{d}$ | 0.39$^{c}$ | 0.30$^{e}$ | 0.28$^{f}$ |
| Shoot MDA contents (nmol$g^{-1}$FM) | 0.13$^{f}$ | 1.16$^{b}$ | 0.19$^{e}$ | 0.63$^{c}$ | 0.31$^{d}$ | 1.31$^{a}$ |
| Shoot $H_2O_2$ contents (µmol$g^{-1}$FM) | 0.73$^{d}$ | 3.05$^{b}$ | 0.67$^{d}$ | 1.35$^{c}$ | 1.32$^{c}$ | 3.77$^{a}$ |
| Shoot proline contents (µmol$g^{-1}$FM) | 25.54$^{f}$ | 39.57$^{b}$ | 27.93$^{e}$ | 47.01$^{a}$ | 34.37$^{c}$ | 30.40$^{d}$ |
| Essential oil yield (v/w) % | 1.59$^{b}$ | 1.69$^{b}$ | 1.89$^{a}$ | 1.98$^{a}$ | 1.02$^{c}$ | 0.85$^{d}$ |

Values with the same lower case letters in a row within subspanner heading are not significantly different at $P < 0.05$.

In the current study, plants treated with 10 ppm $TiO_2$ interestingly produced more essential oils than that of control plants (Table 1, 2). Essential/volatile oil produced in dragonhead is valuable and applicable secondary metabolite since it possesses antibacterial, antimicrobial, and antioxidant activities (Dastmalchi et al., 2007). To the best of our knowledge, this study is the first report demonstrating $TiO_2$ NPs *per se* have stimulating effect on essential oils production. This promising influence may be due to the fact that improved essential oils synthesis may contribute to prevent damages caused by free radicals triggered to some extend by $TiO_2$. In the other hand, increase in SDM of plants treated with 10 ppm $TiO_2$ (explained above) can be the other reason underling improved plant essential oil content. In general, drought stress can also induce essential oil production in the aromatic plants. But the point is that growth and biomass of the most plants would

decline concomitantly, and consequently no essential oil content may increase generally in the plants exposed to drought stress (Selmar and Kleinwachter, 2013). Therefore, present result may be the first step for future investigations on the use of $TiO_2$ NPs as exogenous stimuli for improvement of essential oil production in dragonhead and possibly the other medicinal plants as well.

Data analysis showed that MDA and $H_2O_2$ content were increased very slightly in response to 10 ppm application, showing small production of ROSs (Table 2). However, application of 40 ppm $TiO_2$ were increased leaf MDA and $H_2O_2$ content as classical markers of oxidative stress more than 2-fold in comparison with untreated plants (Table 1, 2). Genotoxicity and cytotoxicity of high concentration of $TiO_2$ NPs have been approved in plants. Formation of MDA as a consequence of lipid peroxidation is reported to be as a possible reason for the genotoxic potential of $TiO_2$ NPs (Ghosh et al., 2010). The current findings indicate that the effects of $TiO_2$ NPs are highly concentration dependent and can be adverse and cytotoxic to the plant if the concentration exceeded the special threshold.

In the present study, leaf chlorophyll content (*a* and *b*) and carotenoid pigments were significantly reduced in response to 10 ppm $TiO_2$ application (Table 2). Despite of photosynthetic pigment degradation, SDM did not decrease and even increased in response to 10 ppm $TiO_2$, as previously discussed. Our data suggest that chlorophyll may be associated with growth, but that other factors could also be important. One of the explanations for this observation is that $TiO_2$ NPs probably enhanced rate of light independent reactions of photosynthesis, since these NPs can significantly promote Rubisco activase activity and its mRNA expression (Ma et al., 2008; Gao et al., 2008). Rubisco activase plays an important role in the regulation of photosynthesis and over-expression of this enzyme increased $CO_2$ assimilation (Yamori et al., 2012). In the other hand, $TiO_2$ can significantly improve photochemical activity of photosystem II and promote energy transfer within this photosystem (Su et al., 207). Other positive effects of $TiO_2$ NPs on various growth aspects of plants (described above) can also be attributed to this observation. Negative effect of $TiO_2$ NPs on chlorophyll *a* concentration during the early growth stages of algae (*Picochlorum* sp.) has been also reported (Hazeem et al., 2016). However, there are other diverse reports showing $TiO_2$ NPs have no effect on chlorophyll and carotenoid content in bread wheat (Larue et al., 2012) and even promoted chlorophyll formation in spinach (Gao et al., 2008) and mung bean (Raliya et al., 2015). These different observations may be derived from the notion that NPs have different effects on different plants. Also, the size of used NPs and even application condition may be the source of various observations in different research (Larue et al., 2012).

### 3.2 Foliar application of $TiO_2$ NP under water-deficit stress

Water-deficit stress *per se* was the only factor which influenced on the measured traits when no NPs were applied under water-deficit stress. In this condition, morphological and physiological traits were affected (Table 1). Under water-deficit stress, SDM, RDM and leaf number of 10 ppm treated plants compared to untreated ones were less negatively affected by drought stress (Table 2). Also, water-deficit stressed-10 ppm treated plants still retained more photosynthetic pigments in comparison to that of counterpart treated plants under normal irrigation (Table 2). All together, these evidence shows that $TiO_2$ NPs probably triggers diverse reactions in different compartments within the cell.

Leaf RWC is a good indicator of plant water-status and even is a relevant screening tool for drought-tolerance (Teulat et al., 2003). In the present research, RWC has been reduced significantly in water deficit stressed-untreated dragonhead plants (Table 1, 2). It shows that transpiration rate exceeded water supply from roots to leaves and the plants sensed drought. It has been reported that significant reduce in leaf RWC of dragonhead was observed just under severe drought stress condition (Alaei et al., 2013). In this condition stomata closure and cell osmotic adjustment are the plants main strategies to prevent water losses and to cope with the cell dehydration. Following stomata closure, $CO_2$ as main substrate for the photosynthetic carbon reduction cycle in the chloroplast would be barely accessible to leaf mesophyll cells and the cycle would runs at the lowest rate. Therefore $NADP^+$ which is the final

acceptor of excited electrons produced from the photosynthetic electron transport chain would remain in its reduced form (i.e. NADPH-$H^+$). In this condition, excited electrons would be transferred to oxygen, generating superoxide radicals (Selmar and Kleinwachter, 2013). These radicals are very reactive and would enter numerous further reactions and thereby generate various types of ROS, which would finally destroy the entire photosynthetic apparatus. The first defense line against ROS is dismutation of two superoxide molecules to hydrogen peroxide ($H_2O_2$) and oxygen by superoxide dismutase (SOD) (Melchiorre et al., 2009).

In the present study, $H_2O_2$ content of water-deficit stressed plants has been increased more than 4-folds, indicating parts of superoxide radicals were scavenged by SOD (Table 2). However, leaf MDA content which is used as a biomarker to measure the level of oxidative stress has also tremendously increased indicating that lipid peroxidation has occurred in water-deficit stressed-untreated plants. Crucial site of cellular injury due to drought stress is lipid peroxidation of the membranes as a result of ROS production. There is a close relation between ROS formation and oxidative stress-induced damages to cell membranes (Nazari et al., 2012). ROS degrades polyunsaturated membrane lipids, forming MDA. The degree of lipid peroxidation can be estimated by the amount of MDA in tissues. Less MDA production, more cell membrane integrity. In the present study, MDA and also $H_2O_2$ content of the plants treated with 10 ppm $TiO_2$ was interestingly much less than that of untreated plants under water-deficit condition (Table 2). It demonstrates that stress-induced damages were ameliorated as a result of 10 ppm $TiO_2$ application. This finding is in line with the work of Mohammadi et al. (2013) who have reported that 5 ppm $TiO_2$ reduced MDA production and membrane electrolyte leakage index in the leaves of chickpea under cold stress condition. Decline in MDA content of the plants treated with low concentration of $TiO_2$ has been attributed to stabilized composition and improved physical properties of their membranes (Mohammadi et al. 2013). $TiO_2$ NPs can also improve the activities of antioxidant enzyme systems such as superoxide dismutase, catalase, and ascorbate peroxidase (Lei et al., 2008). This may be the other reason behind reduced lipid peroxidation and improved membrane integrity under drought stress. However, 40 ppm treated and water-deficit stressed plants had maximum amount of $H_2O_2$ and MDA content (Table 2). This suggests that 40 ppm $TiO_2$ NPs indirectly caused to excessive generation of superoxide radicals resulting in increased lipid peroxidation and oxidative stress. This evidence can be attributed to both toxic concentration and photocatalitic properties of $TiO_2$. That was probably why chlorophyll and carotenoid pigments along with proline and even essential oil contents were also reduced severely (Table 2). It is not clear that $TiO_2$ caused to degrade pre-existing pigments or inhibited their production at transcriptom level. Generally, 40 ppm $TiO_2$ could not alleviate adverse effects of water-deficit stress on plant growth parameters.

The other strategy that plants take to cope with cell dehydration due to drought stress is accumulation of compatible organic solutes such as proline. In addition to its role as an osmolyte for osmotic adjustment, proline contributes to stabilizing sub-cellular structures in cell cytosol (Ashraf and Foolad, 2007). In the present research, leaf proline content of drought stressed dragonhead plants increased in comparison with control plants under normal irrigation (Table 2). In the other hand, 10 ppm treated plants had significantly much more leaf proline and higher relative water content compared with untreated plants under water-deficit stress (Table 2). This shows that $TiO_2$ not only can improve activity of antioxidant enzymes but also can induce synthesis of proline which is one of the osmolytes responsible for maintaining cell turgor under drought stress condition.

In the current research essential oils of untreated plants were not increased significantly in response to water-deficit stress (Table 1, 2). That was probably because their shoot dry mass has been severely decreased in this condition. In this case, no significant changes in oil content of German chamomile (*Matricaria recutita* L.) in response to various drought intensities were reported (Baghalian et al., 2011). Furthermore, overall content of terpenoids in *Melissa officinalis* L., *Nepeta cataria* L. and *Salvia officinalis* L. were decreased under drought stress condition (Manukyan, 2011). Therefore, drought stress may have no overall positive effect on escalating

essential oil content in some plants. In this study, we found that application of nano $TiO_2$ at specific concentrations can increase essential oil content of dragonhead plants during normal and water-deficit stress conditions.

According to our data, leaf photosynthetic pigments such as chlorophyll (*a* and *b*) and carotenoids were reduced significantly in water-deficit stressed plants in comparison with counterpart plants under normal irrigation (Table 2). Degradation of these light receiving pigments would reduce photosynthetic rate and subsequently biomass production. That was probably why stem dry mass (SDM) and root dry mass (RDM) in the present study were severely declined in response to water-deficit stress. Morphological traits such as plant leaf number (PLN) and stem branch number (SBN) were also reduced. $TiO_2$ NPs is a photocatalist which can strongly absorb photosynthetically active radiation (PAR) and is capable of undergoing electron transfer reactions. When located in thylakoid membrane, proper concentration of $TiO_2$ can accelerate energy transfer from chlorophyll *b* and carotenoid to D1/D2/Cyt b559 complex within photosystem II reaction center. This would obviously lead to enhancement of oxygen evolution and generation of additional excited electrons (Su et al., 2007). Under normal environmental condition, it may proliferate photosynthetic capacity and hence promote growth of some plants (Hong et al., 2005). However, under drought stress condition when stomata are tightened and the most electron acceptors are in reduced form, production of these excessive excited electrons due to presence of $TiO_2$ can result in huge production of free radicals and photo oxidative damages.

## 4 CONCLUSIONS

Since inward flows of $CO_2$ to leaf mesophyll cells would be diminished upon stomata closure, drought stress-related increase in essential oil content is usually concomitant with decrease in growth and biomass production resulting in no overall changes of essential oils content in aromatic herbs. In the present research, 10 ppm foliar application of $TiO_2$ NPs significantly increased shoot dry mass and essential oil content of dragonhead plants under normal irrigation and water-deficit stress. If further improved, it seems that proper concentration of $TiO_2$ NPs can be a good candidate to be used as exogenous stimuli for boosting essential oils content in aromatic dragonhead plants without decrease in shoot biomass under both watering condition. However, the effect of $TiO_2$ NPs on quality or components of essential oils needs to be more clarified. MDA and $H_2O_2$ content of the dragonhead plants treated with 10 ppm $TiO_2$ were significantly much less than that of untreated plants under drought stress condition. Moreover, the treated plants not only had much more proline content but also maintained more shoot and root dry mass, leaf relative water content, leaf number and stem branches compared with untreated plants under these conditions. These evidences indicate that proper concentration of $TiO_2$ NPs can alleviate drought stress-induced oxidative damages and have potential to stabilize morphological characteristics of the plants under drought stress condition. In the other hand, from the results obtained, it can be concluded that foliar treatment of dragonhead plant with nano $TiO_2$ at high concentrations could increase deleterious effects of drought stress on physiological processes through changing the levels of MDA and $H_2O_2$ and stability of plastid pigments. Therefore, extensive use of $TiO_2$ NPs in order to lessen adverse effects of drought stress even in the case of food crops worth to be more investigated in detail. Besides, the influence of $TiO_2$ NPs residues on the environment and human health should also be considered and elucidated.

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# Assessment of morphological and molecular variation in local olive (*Olea europaea* L.) in the Northern part of Iran

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**ABSTRACT**

Iran is known as one of the origins of olive in the world with many different olive cultivars, mainly in the north. Eighty eight accessions belong to 4 main olive cultivars were investigated by 21 morphological characters and 11 ISSR markers. Analyses of morphological characters revealed the existence of high genetic variability among cultivars. Based on both morphological and ISSR cluster analyses, 88 accessions were grouped in five distinct clusters. The ISSR primers produced 77 polymorphic bands. AMOVA showed significant difference in both between and within olive cultivars. The highest and lowest coefficient of Nei's genetic distance was observed in 'Mari' and 'Shengeh' (0.105) and 'Zard' and 'Rowghani' (0.061), respectively. In both morphological and ISSR data analyses, 'Mari' showed the highest homogeneity. The olive cultivars were not clustered based on their geographical origin.

**Key words:** genetic diversity, ISSR, PIC, polymorphism

**IZVLEČEK**

**OVREDNOTENJE MORFOLOŠKE IN MOLEKULARNE VARIABILNOSTI LOKALNE OLJKE (*Olea europaea* L.) V SEVERNEM DELU IRANA**

Iran je prepoznan kot eden izmed svetovnih izvorov oljke z mnogimi sortami, predvsem v njegovem severnem delu. Raziskano je bilo 88 akcesij oljke, ki so pripadale 4 glavnim sortam na osnovi polimorfizma 21 morfoloških znakov in 11 ISSR molekulskih markerjev. Analiza morfoloških znakov je odkrila veliko genetsko variabilnost med sortami. Na osnovi morfoloških znakov in ISSR molekulskih markerjev se je 88 akcesij združilo v pet skupin. Z ISSR markerji so pomnožili 77 polimorfnih fragmentov. AMOVA je pokazala značilne razlike znotraj sort in med sortami. Največji vrednosti Neiovega koeficienta kot kazalnika genetske oddaljenosti sta bili ugotovljeni med sortami Mari in Shengeh (0.105) in Zard in Rowghani (0.061). Sorta Mari je pokazala največjo homogenost na osnovi analize morfoloIoških znakov in ISSR molekulskih markerjev. Sorte oljke se niso združevale na osnovi geografskega izvora.

**Ključne besede:** genetska raznolikost, ISSR, PIC, polimorfizem

## 1 INTRODUCTION

The olive (*Olea europaea* L.) is an economically important fruit crop of the Mediterranean basin (Rao et al., 2009). Archaelogical findings revealed that olive cultivation in Iran dates back 2000 years ago (Sadeghi, 1992). At present, the old commercial olive orchards are located mainly in

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the north of Iran and more than 85 % of olive production belongs to these regions (Noormohammadi et al., 2007). A large number of olive accessions are growing in Iran; therefore, many workers have reported on morphological or molecular characteristics of these accessions (Samaee et al., 2003; Hosseini-Mazinani et al., 2004, Omrani-Sabbaghi et al., 2007; Sheidai et al., 2007; Noormohammadi et al., 2012; Dastkar et al., 2013). The genetic diversity could be an important resource for the development of modern olive culture towards typical olive oil and fresh products (Hegazi et al., 2012).

The ability to discriminate olive cultivars to estimate genetic variability is an important factor for a better management of genetic resources and successful breeding programs (Milotić et al., 2005). Morphological descriptors of International Olive Council (IOC, 1993) are usually applied for characterization and identification of olive cultivars. Although the morphological characters are strongly affected by environmental conditions, the age of trees, the training systems, and the phenological stage of plants, the morphological approach continues to be the initial main step for description and classification of olive germplasm (Rotondi and Magli, 1999). Therefore, more comprehensive studies using reliable markers are needed to gain a better understanding of the level and distribution of genetic diversity in olive cultivars. In the last years, molecular markers, such as RAPD (Belaj et al., 2001; Besnard et al., 2001; Mekuria et al., 1999; Wiesman et al., 1998), AFLP (Angiolillo et al., 1999; Sanz-Cortes et al., 2003; Sensi et al., 2003; Owen et al., 2005) and SSR (Bandelj et al., 2002; Belaj et al., 2004; Cipriani et al., 2002; Diaz et al., 2006; Khadari et al., 2003; Rallo et al., 2000; Sefc et al., 2001), have been used to characterize olive germplasm. Also, ISSRs methods have been used (Hess et al., 2000; Pasqualone et al., 2001; Gemas et al., 2004; Terzopoulos et al., 2005). Little information is available on the genetic background of Iranian domestic olive genotypes (Dastkar et al., 2013). The cross-pollination between domesticated and wild varieties caused the significant proportion of genetic diversity. Sadeghi (1992) reported that some cultivars appeared by selection of superior trees and by selection of mutation in over the years. The goal of this study is characterizing main Iranian olive cultivar in two provinces of Gilan and Zanjan by the use of ISSR markers and morphological characteristics.

## 2 MATERIALS AND METHODS

### 2.1 Plant material

Eighty-eight trees belonging to the 4 endemic cultivars including ‘Mari’, ‘Zard’, ‘Shengeh’ and ‘Rowghani’ were used in the morphological and molecular study. Trees were sampled randomly from five different locations of two provinces of Gilan and Zanjan including Gilvan, Tarom, Aliabad, Manjil and Jamalabad. Observations were made on samples of 40 healthy adult leaves and fruits for each tree. Morphological characters were measured manually and recorded for all 21 characters including leaf characters (3 characters) and fruit characters (8 characters). Then, ten characters were recorded on free stone fruits. The morphological characters were coded as binary or multistate characters (Table 1).

### 2.2 DNA extraction

Total genomic DNA was extracted from fresh leaves using CTAB method with some modifications (Doyle and Doyle, 1990). Leaf tissue (0.5 g) is ground in liquid nitrogen and incubated at 65 °C for 30 min in 1ml extraction buffer (100 mM Tris-HCl, pH 8.0; 20 mM EDTA, pH 8.0; 1.4 M NaCl, 2 % CTAB, 1 % PVP). An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and 2 µl β-mercaptoethanol were added to the supernatant and the whole mixture was centrifuged at 12000 rpm for 10 min. The precipitation of the upper phase was obtained by adding 1 volume of 2-isopropanol at -20 °C for 20 min and then followed by centrifugation at 13000 rpm for 15 min. The DNA pellet was washed with 1 ml 75 % ethanol. The DNA pellet was resuspended in 50 µl TE (10 mM Tris-HCl, pH 8.0; 0.5 M EDTA, pH 8.0).

ISSR analysis was performed using 11 primers (Table 2). PCR reactions were performed in a 25 µl volume containing 1× PCR reaction buffer

(10 mM Tris- HCl; 50 mM KCl) 1.5 mM $MgCl_2$ ; 0.2 mM of each dNTP; 0.3 μM of a single primer; 20 ng genomic DNA and 1.0 U *Taq* DNA polymerase. The amplifications were performed in Applied Biosystems thermocycler under the following conditions: 94 °C, 5 min; 94 °C, 30 s; specific annealing temperature (Table 2), 45 s; 72 °C, 2 min; repeat to step 2, 45 times; 72 °C, 5 min.

### 2.3 Statistical analysis

Canonical discriminant, cluster analysis among cultivars by within group linkage analysis and correlation analysis were conducted using SPSS-V. 20. Molecular analysis of variance (AMOVA) was performed using GeneAlex 6.4 (Peakall and Smouse, 2006) to divide the total variation to between and within olive cultivars variation. Genetic distances between all pairwise combinations of the accessions were calculated using Nei's coefficients. Genetic diversity parameters including Ne (number of effective alleles), H (Nei's gene diversity) and I (Shannon's information index) were calculated by GeneAlex 6.4. PIC was estimated using the Excel software. ISSR polymorphism was scored for the presence (1) or absence (0) of amplified bands and was used to estimate the dissimilarity coefficients between cultivars using simple matching's coefficient method. The dissimilarity matrix was used to construct a dendrogram using the complete linkage method. These analyses were carried out using NTSYS pc ver. 2.01 (Rohlf., 1998). The agreement between dendrograms derived from morphological characters and ISSR markers, were compared using the Mantel (1967) matrix correspondence test.

**Table 1:** List of morphological characteristics and their codes and meaning (IOC)

| | Variable | Intensity |
|---|---|---|
| 1. | Leaf width (LW) | Narrow (1) Medium (2) Broad (3) |
| 2. | Leaf length (LL) | Short (1) Medium (2) Long (3) |
| 3. | Leaf shape (length/width) (LS) | Elliptic (1) Elliptic-Lanceolate (2) Lanceolate (3) |
| 4. | Fruit shape (position A) | Spherical (1) Ovoid (2) Elongated (3) |
| 5. | Fruit mass | Low (1) Medium (2) High (3) Very high (4) |
| 6. | Fruit symmetry (position A) | Symmetry(1) Slightly asymmetry (2) Asymmetry (3) |
| 7. | Position of maximum transvers diameter (position B) | Towards base (1) Central (2) Towards apex (3) |
| 8. | Fruit apex (position A) | Acute (1) Obtuse (2) Rounded (3) |
| 9. | Nipple | Absent (1) Tenuous (2) Obvious (3) |
| 10. | Presence of lenticels | Few (1) Many (2) |
| 11. | Size of lenticels | Small (1) Large (2) |
| 12. | Stone shape (position A) | Spherical (1) Ovoid (2) Elliptic (3) Elongated (4) |
| 13. | Stone mass | Low (1) Medium (2) High (3) Very high (4) |
| 14. | Stone symmetry (position A) | Symmetry (1) Slightly asymmetry (2) Asymmetry (3) |
| 15. | Stone symmetry (position B) | Symmetry (1) Slightly asymmetry (2) |
| 16. | Position of maximum transvers diameter (position B) | Towards base (1) Central (2) Towards apex (3) |
| 17. | Stone apex (position A) | Acute (1) Obtuse (2) Rounded (3) |
| 18. | Stone base (position A) | Truncate (1) Pointed (2) Rounded (3) |
| 19. | Stone surface (position B) | Smooth (1) Rugose( 2) Scabrous (3) |
| 20. | Number of grooves | Low (1) Medium (2) High (3) |
| 21. | Distribution of the grooves | Regular (1) Grouped around the surface (2) |

**Table 2:** Primers used for ISSR analysis: total number bands, polymorphic bands and % of polymorphism obtained

| | Primers | Sequence 5′-3′ | Annealing temperature | Total number of bands | Polymorphic bands | % polymorphism |
|---|---|---|---|---|---|---|
| 1 | UBC834 | $(AC)_8C$ | 48.5 | 9 | 9 | 100 |
| 2 | UBC807 | $(AG)_8T$ | 43 | 7 | 7 | 100 |
| 3 | UBC808 | $(AG)_8C$ | 45 | 10 | 10 | 100 |
| 4 | UBC809 | $(AG)_8G$ | 44.7 | 7 | 6 | 85.71 |
| 5 | UBC810 | $(GA)_8T$ | 47.74 | 10 | 6 | 60 |
| 6 | UBC822 | $(TC)_8A$ | 43.14 | 10 | 9 | 90 |
| 7 | HB12 | $(CAC)_3GC$ | 34.92 | 8 | 6 | 75 |
| 8 | UBC815 | $(CT)_8G$ | 51.33 | 6 | 3 | 50 |
| 9 | UBC816 | $(CA)_8T$ | 55.37 | 7 | 7 | 100 |
| 10 | UBC823 | $(TC)_8C$ | 52.52 | 7 | 5 | 71.43 |
| 11 | UBC825 | $(AC)_8T$ | 56.56 | 10 | 9 | 90 |
| Mean | | | | 8.27 | 7 | 70.84 |

## 3 RESULTS AND DISCUSSION

### 3.1 Morphological characterization

Correlation among morphological characteristics were worked out at phenotypic level and presented in Table 3. The significant correlation ($p$<0.01) were found between some characteristics, such as Fruit Shape and Fruit Apex (-0.592), Fruit Shape and Stone Shape (0.872), Fruit Symmetry (p A) and Fruit Shape (0.602), Fruit Symmetry (p A) and Fruit Apex (-.572), Fruit Symmetry (p A) and Stone Shape (0.587), Fruit Symmetry (p A) and Stone Symmetry (p A) (0.540), Fruit Symmetry (p A) and Stone Base (0.515), Fruit Apex and Stone Shape (-0.598), Fruit Apex and Stone Symmetry (p A) (-0.523), as well as Stone Shape and Stone Apex (-0.504).

The dendrogram obtained by within group linkage analysis grouped the 4 cultivars and 88 individuals into five clusters (Figure 1). The first cluster included the number of the individuals of ‘Rowghani’ and ‘Shengeh’. Individuals of ‘Mari’ with a limited number of ‘Rowghani’ and ‘Shengeh’ were grouped into cluster 2 and 3. All of the individuals of ‘Zard’ with a limited number of ‘Rowghani’ and ‘Shengeh’ (2 and 5 respectively) were placed into cluster 4 and 5. Among cultivars, ‘Zard’ and ‘Mari’ had the highest homogeneity. ‘Shengeh’ and ‘Rowghani’ showed high affinity. It could be due to synonymy in two cultivars. Grouping of such a mixture of accessions may be the result of the presence of synonymous/mislabeled accessions (Noormohammadi et al., 2007). These olive trees grow in the areas with close vicinity; therefore, the similarities observed among them may be due to the gene flow occurring among them.

The accuracy of the groups produced was reassessed using discriminant function analysis. The total success rate of discriminant function was 89.8 %, which indicates that it was successful in discriminating different groups. The canonical discriminant functions are described (Table 4). The first three functions had eigenvalues that are above 2 and jointly accounted for 98 % of total variance. The first two functions accounted for 82.1 % of the total variance within the individuals. Standardized discriminant function coefficient could be used to identify important characters causing variation cultivars (Table 5). In the first function, high coefficient was observed for stone shape. In the second function, stone base and leaf length had high coefficient values. In the third function, presence of lenticels and fruit mass had high coefficient values. These characters that loaded high in the three functions demonstrate their relevance in discriminating between the olive cultivars. This was further reaffirmed by the extraction of standardized canonical discriminant function coefficient.

**Table 3:** Correlation coefficients between 21 morphological characters

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1.00 | | | | | | | | | | | | | | | | | | | | |
| 2 | .317** | 1.00 | | | | | | | | | | | | | | | | | | | |
| 3 | .090 | -.283** | 1.00 | | | | | | | | | | | | | | | | | | |
| 4 | .092 | -.036 | .056 | 1.00 | | | | | | | | | | | | | | | | | |
| 5 | .060 | -.055 | .174 | -.184 | 1.00 | | | | | | | | | | | | | | | | |
| 6 | .305** | .072 | .071 | .602** | -.112 | 1.00 | | | | | | | | | | | | | | | |
| 7 | .218* | .242* | .000 | .298** | -.133 | .329** | 1.00 | | | | | | | | | | | | | | |
| 8 | -.253* | -.072 | .000 | -.592** | .082 | -.572** | -.208 | 1.00 | | | | | | | | | | | | | |
| 9 | .194 | -.117 | .113 | .188 | .007 | .348** | .106 | -.306** | 1.00 | | | | | | | | | | | | |
| 10 | .280** | .039 | .116 | .183 | .293** | .237* | .120 | -.234* | .239* | 1.00 | | | | | | | | | | | |
| 11 | .242* | .046 | .076 | .205 | -.093 | .240* | .185 | -.185 | .277** | .051 | 1.00 | | | | | | | | | | |
| 12 | .073 | -.081 | -.013 | .872** | -.096 | .587** | .186 | -.598** | .109 | .229* | .079 | 1.00 | | | | | | | | | |
| 13 | -.001 | -.083 | .000 | -.054 | .371** | -.214* | -.038 | .081 | -.330** | .043 | -.208 | .047 | 1.00 | | | | | | | | |
| 14 | .315** | .026 | .068 | .392** | -.124 | .540** | .223* | -.523** | .092 | .194 | .031 | .427** | .000 | 1.00 | | | | | | | |
| 15 | .005 | -.050 | .106 | .142 | .059 | .316** | .134 | -.190 | .121 | .091 | .088 | .094 | -.003 | .332** | 1.00 | | | | | | |
| 16 | .282** | .149 | -.123 | -.170 | .137 | -.020 | .060 | -.019 | .015 | .191 | .088 | -.115 | -.041 | .041 | .104 | 1.00 | | | | | |
| 17 | -.073 | .165 | -.198 | -.455** | -.312** | -.325** | -.032 | .430** | -.189 | -.212* | .079 | -.504** | -.070 | -.369** | -.061 | .041 | 1.00 | | | | |
| 18 | .365** | .183 | .000 | .455** | -.217* | .515** | .243* | -.495** | .179 | .047 | .479** | .376** | -.127 | .437** | .355** | .180 | -.271* | 1.00 | | | |
| 19 | -.065 | .069 | .000 | .108 | -.070 | .123 | .141 | -.246* | .009 | .212* | -.162 | .131 | -.002 | .158 | .072 | -.078 | -.163 | -.007 | 1.00 | | |
| 20 | -.091 | -.015 | -.264* | .014 | .032 | -.049 | -.194 | .120 | -.059 | -.098 | -.232* | .102 | .058 | -.038 | -.151 | -.072 | -.052 | -.289** | -.066 | 1.00 | |
| 21 | .172 | .010 | .000 | -.174 | -.115 | -.123 | -.157 | .084 | .110 | -.201 | .243* | -.280** | -.052 | -.053 | .126 | .082 | .323** | .185 | -.121 | -.050 | 1.00 |

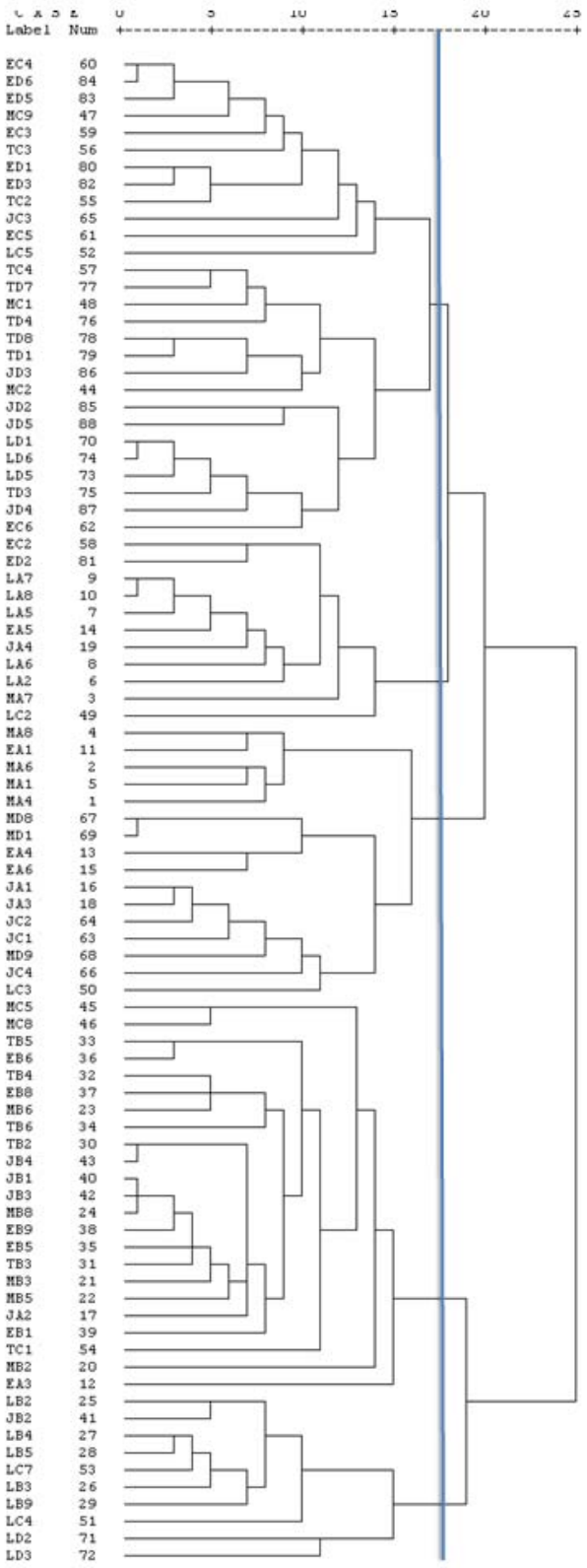


**Figure 1:** Dendrogram obtained from the 21 morphological characters, within group linkage method with squared Euclidean distance. Accessions are indicated with A, B, C and D for Mari, Zard, Shengeh and Rowghani cultivars respectively. Regions are indicated with L, M, E, T and J for Aliabad, Gilvan, Manjil, Tarom and Jamalabad respectively.

**Table 4:** Summary of canonical discriminant functions

| Function | Eigenvalue | % of variance | Cumulative % | Canonical correlation |
|---|---|---|---|---|
| 1 | 7.245 | 55.2 | 55.2 | 0.937 |
| 2 | 3.536 | 26.9 | 82.1 | 0.883 |
| 3 | 2.077 | 15.8 | 98.0 | 0.822 |
| 4 | 0.266 | 2.0 | 100.0 | 0.460 |

**Table 5:** Standardized canonical discriminant function coefficients of characters in olive cultivars

| | Function | | | |
|---|---|---|---|---|
| Characters | 1 | 2 | 3 | 4 |
| Leaf length | -0.05 | 0.461 | 0.363 | -0.064 |
| Fruit mass | -0.036 | -0.152 | 0.445 | 0.351 |
| Presence of lenticels | -0.058 | 0.006 | 0.841 | 0.151 |
| Fruit apex | -0.305 | 0.321 | -0.337 | 0.827 |
| Stone shape | 0.670 | -0.655 | -0.245 | 0.337 |
| Stone base | 0.614 | 0.740 | -0.082 | 0.215 |

### 3.2 ISSR polymorphism

The 11 ISSR primers produced 77 polymorphic bands by each primer ranged from 4-10. The highest number of polymorphic bands was obtained by UBC 808 (10 bands), while UBC 815 produced the lowest number of polymorphic bands (3 bands). PIC values ranged from 0.31 9 (UBC 810) to 0.46 (HB 12) (Table 2). The mean values of EMR, MI, effective number of alleles (Ne), Nei's genetic diversity (H) and Shannon index (I) for all the primers were 6.14, 2.37, 1.73, 0.4 and 0.6 respectively (Table 6). Among the primers, UBC 822 and UBC 816 showed that the highest number of effective alleles (1.92)

The AMOVA showed that most of the genetic diversity was attributable to differences among individuals within cultivars (91 %) rather than among cultivars (9 %) (Table 7). The calculated PhiPT for all individuals (0.086) was significant ($P< 0.01$).

The highest amount of polymorphism, Shannon's index and heterozigosity was observed in 'Zard' cultivar and the lowest polymorphism and heterozigosity in 'Mari' (Table 8). The number of effective allele varied from 1.62 to 1.67. Percent of polymorphism varied from 92.39 to 97.83. The highest polymorphism may be due to high efficiency of markers. Based on the results, the highest and lowest coefficient of Nei's genetic distance between cultivars were belonged to 'Mari' with 'Shengeh' (0.105) and 'Zard' with 'Rowghani' (0.061), respectively (Table 9).

**Table 6:** Genetic diversity parameters estimated for the ISSR primers in 88 accessions. PIC = polymorphic information content; EMR = effective multiplex ratio; MI = marker index; Ne = number of effective alleles; H = Nei's index; I = Shannon's information index.

| | Primer name | PIC | EMR | MI | Ne | H | I |
|---|---|---|---|---|---|---|---|
| 1 | UBC 834 | 0.42 | 9 | 3.78 | 1.63 | 0.36 | 0.55 |
| 2 | UBC807 | 0.37 | 7 | 2.59 | 1.63 | 0.36 | 0.53 |
| 3 | UBC 808 | 0.38 | 10 | 3.8 | 1.77 | 0.43 | 0.76 |
| 4 | UBC 809 | 0.4 | 5.14 | 2.06 | 1.84 | 0.45 | 0.65 |
| 5 | UBC 810 | 0.31 | 3.6 | 1.11 | 1.54 | 0.32 | 0.49 |
| 6 | UBC 822 | 0.37 | 8.1 | 2.99 | 1.92 | 0.48 | 0.67 |
| 7 | HB 12 | 0.46 | 4.5 | 2.07 | 1.87 | 0.46 | 0.65 |
| 8 | UBC 812 | 0.33 | 1.5 | 0.49 | 1.72 | 0.41 | 0.6 |
| 9 | UBC 816 | 0.42 | 7 | 2.94 | 1.92 | 0.47 | 0.67 |
| 10 | UBC 823 | 0.38 | 3.57 | 1.36 | 1.49 | 0.31 | 0.48 |
| 11 | UBC 825 | 0.35 | 8.1 | 2.83 | 1.67 | 0.37 | 0.55 |
| | Mean | 0.38 | 6.14 | 2.37 | 1.73 | 0.4 | 0.6 |

**Table 7:** Analysis of variance of olive cultivars based on molecular markers. Df = degree of freedom; SS = sum of squares; MS = mean squares; Est. Var = estimated variance; % = percent of diversity; PhiPT = AC/(WC+AC).

| Source | df | SS | MS | Est. Var | % |
|---|---|---|---|---|---|
| Among Cultivar(AC) | 3 | 128.739 | 42.913 | 1.319 | 9 |
| Within Cultivar(WC) | 84 | 11.72.465 | 13.958 | 13.958 | 91 |
| Total | 87 | 1301.205 | | 15.277 | 100 |
| PhiPT = 0.086 | | | P Value = 0.001 | | |

**Table 8:** Genetic diversity parameters among 4 cultivars based on ISSR loci. Na = number of different alleles; Ne = number of effective alleles; I = Shannon's information index; He = expected heterozygosity; SE = standard error

| Cultivars | Number | Na±SE | Ne±SE | I±SE | He±SE | % polymorphism |
|---|---|---|---|---|---|---|
| Mari | 19 | 1.88±0.049 | 1.65±0.036 | 0.533±0.023 | 0.365±0.018 | 92.21 |
| Zard | 24 | 1.96±0.029 | 1.67±0.033 | 0.554±0.020 | 0.379±0.016 | 97.4 |
| Shengeh | 23 | 1.95±0.025 | 1.67±0.035 | 0.547±0.021 | 0.375±0.016 | 94.81 |
| Rowghani | 22 | 1.96±0.022 | 1.66±0.036 | 0.544±0.021 | 0.371±0.016 | 96.1 |
| Mean | | 1.93±0.017 | 1.66±0.018 | 0.544±0.011 | 0.373±0.008 | 95.13 |

**Table 9:** Nei's genetic distance between pairs of 4 olive cultivars

| Cultivar | Mari | Zard | Shengeh | Rowghani |
|---|---|---|---|---|
| Mari | 0 | | | |
| Zard | 0.082 | 0 | | |
| Shengeh | 0.105 | 0.083 | 0 | |
| Rowghani | 0.073 | 0.061 | 0.081 | 0 |

### 3.3 Genetic diversity based on ISSR data

In the complete linkage dendrogram based on ISSR data, the 88 olive trees were separated in five clusters (Figure 2 and Table 10). The first cluster included all of the individuals of Manjil's 'Mari' and 'Zard' cultivars, four individuals of the Gilvan's 'Mari' and three individuals of the Aliabad's 'Mari'. The second cluster were placed all of the Aliabad's individuals of 'Shengeh' and 'Rowghani' cultivars and four individuals of the Gilvan's 'Shengeh'. Cluster 3 grouped all of the individuals of Jamalabad's 'Rowghani' and four individuals of the Aliabad's 'Zard'. All of the individuals of Tarom and Manjil's 'Shengeh' grouped in cluster 4. Claster 5 was formed by diverse individuals. Cultivars and origins couldn't form distinct cluster. Aliabad and then Manjil were able to place their individuals of each cultivar in similar cluster, in other words, these areas showed the most homogeneity. The Mantel analysis revealed a negative and significant correlation ($r = -0.164$ $p<0.001$, 1000 random permutations) between the morphological and ISSR marker-derived dissimilarity matrices.

**Table 10:** Grouping the accessions in 5 clusters. Accessions are indicated with A, B, C and D for Mari, Zard, Shengeh and Rowghani cultivars respectively. Regions are indicated with L, M, E, T and J for Aliabad, Gilvan, Manjil, Tarom and Jamalabad respectively.

| **Cluster** | number | olive accessions |
|---|---|---|
| 1 | 18 | MA4, MA6, MA7, MA8, LA2, LA5, LA6, EA1, EA3, EA4, EA5, EA6, EB5, EB6, EB8, EB9, EB10, MC10 |
| 2 | 22 | LA7, LA8, MB6, MB8, TB3, TB5, TB6, MC2, MC5, MC8, MC9, LC2, LC3, LC4, LC5, LC7, LD1, LD2, LD3, LD5, LD6, TD3 |
| 3 | 16 | JA1, JA3, LB2, LB4, LB5, LB9, TB4, JB4, JC2, JC4, JC5, ED6, JD2, JD3, JD4, JD5 |
| 4 | 21 | JA2, JA4, JB1, JB2, JB3, TC1, TC2, TC3, TC4, EC2, EC3, EC4, EC5, EC6, JC3, TD8, TD10, ED1, ED2, ED3, ED5 |
| 5 | 9 | MA10, MB2, MB3, MB5, LB3, TB2, MD8, MD9, MD10 |

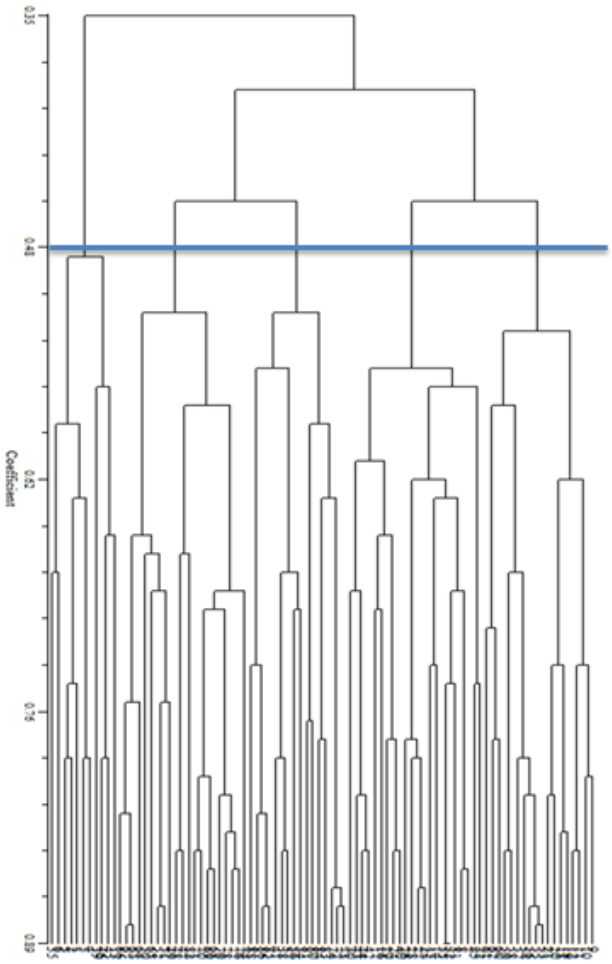


**Figure 2:** Complete linkage dendrogram based on simple matching coefficient illustrating the genetic similarities and distance among 88 olive accessions obtained by 11 ISSR primers data

## 4 DISCUSSION

Homogeneity in Aliabad trees was bigger than those of other regions. This orchard, in contrast to others, is vegetative collection. Among other cultivars, 'Zard' and 'Mary' had more homogeneity according morphological characters. Weakness of these classifications have been shown by the evidence that chemical and morphological changes in olive trees as well as other plants are influenced by domestication and agronomic selection (Sheidai et al., 2007). The high homogeneity in cultivars may reflect the selection pressure for fruit quality (Mekuria et al., 2002). Each of them was grouped in two clusters. Aliabad individuals were separated in cultivars. Cluster 2 of 'Mari' trees had acute fruit apex, rugose stone surface and few lenticels. Whereas, 'Mari' individuals in cluster 3 had obtuse fruit apex, smooth stone surface and many lenticels. 'Zard' individuals in cluster 4 had very high fruit mass, very high stone mass, spherical stone shape and many lenticels. While, 'Zard' individuals in cluster 5 had high fruit mass, high stone mass, ovoid stone shape and few lenticels.

The result showed high allelic variation in 11 ISSR markers, from which four ISSR primers had the highest amount (100 %). These four primers could be used to study genetic variation among olive genotypes. The highest level of polymorphism at ISSR loci indicates high genetic variability in olive cultivars which is in agreement with other studies (Gemas et al., 2004; Essadki et al., 2006; Terzopoulos et al., 2005; Martins-Lopez et al., 2009; Hess et al., 2000). Good discrimination

efficiency and high reproducibility of ISSRs make them particularly suitable to identify the closely related clones which are often the results of very local selection in fruit species (Essadki et al., 2006). The UBC 822 gave the highest number of effective alleles (1.92) and Nei's genetic diversity (0.48) among the ISSR primers, while the UBC 823 primer gave the lowest values for number of effective alleles (1.49), Nei's genetic diversity (0.31) and Shannon index (0.48).

The 'Mari' showed the highest homogeneity based on both data analyses and often their individuals were placed close to each other, while the highest genetic diversity compared to other cultivars was observed for the 'Shengeh'. Intra-cultivar variation has also been reported in 'Shengeh' by using morphological characters (Hosseini-Mazinani et al., 2004). 'Mari' cultivar showed the lowest mean of Shannon's index (0.533) and high genetic distance with the other cultivars, indicating little or low gene flow with other cultivars take place. 'Zard' and 'Rowghani' showed the lowest Nei's genetic distance. In the other study 'Zard', 'Rowghani' and 'Dezfool' were placed together and they showed low genetic distance, therefore, they reported probably those cultivar had the same origin (Koohi-dehkordi et al., 2006). A good correlation was not found between genetic distances estimated using ISSR markers and those based on morphological characteristics. This may be a consequence of the fact that molecular analysis probes a wider area of the genome than does morphological analysis (Rao et al., 2009). The lack of correlation between those two estimates is also influenced by the fact that a large proportion of the variation detected in trees by ISSR is, a priori, non-adaptive (Karhu et al., 1996), and hence not subject to selection, unlike phenotypic attributes (Rao et al., 2009).

Noormohammadi observed high intra-cultivar variation among North Iranian olive cultivars and without geographical separation (Noormohammadi et al., 2007; Noormohammadi et al., 2009; Noormohammadi et al., 2014), which was in agreement with the present study. Lack of a clear clustering of olive may be due to material exchanges by local gardeners and complicated in their denominations because of morphological similarity (Noormohammad et al., 2009). 'Mari' has narrow and elongated form of fruit, unlike other cultivars and identifying them is easier than others. The high intra-cultivar variation was obtained from most woody perennial outbreeding species, with most variation being within populations and existence of low gene flow (Hamrich and Godt, 1989).

## 5 MAIN CONCLUSION

In this paper, four olive cultivars have been investigated and characterized by combining morphological and molecular data. An important issue in identification of cultivars by morphological characters is the use of features receiving the least effects from environmental factors. Based on our analyses, characters of fruit and stone were more important than leaf characters. Both morphological and ISSR data analyses showed intra- and inter cultivar genetic diversity. These local cultivars must be exploited to identify individuals highly adaptive to extreme environmental conditions.

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**Agrovoc descriptors:** Weed control, cultural weed control, zea mays, maize, Abutilon theophrasti, plant competition, weed competition, weeds, noxious plants, cover plants, Phaseolus vulgaris, Glycine max, Trifolium alexandrium

**Agris category code**: H01, H60, F08

# Effect of cover crops on maize-velvet leaf competition: leaf area density and light interception

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**ABSTRACT**

Cover crops influence on canopy structure and light interception of maize (*Zea mays* L.) and velvetleaf (*Abutilon theophrasti* Medik), was studied in a field experiment. Treatments included planting of bean (*Phaseolus vulgaris* L.), soybean (*Glycine max* (L.) Merr.) and berseem clover (*Trifolium alexandrium* L.) as cover crops at the same date and 21 days after maize. Sole cropping of maize under weed- free and weedy conditions were also included in this experiment. All tested cover crops significantly reduced leaf area density and height of velvetleaf up to 50 %, while maize leaf area density increased in the presence of cover crops. Among cover crops, bean and soybean were the most effective in reducing velvetleaf leaf area density and height. Bean and soybean also strongly reduced absorbed light by velvetleaf by up to 80 % compared to clover. Maize grain yields were significantly influenced by cover crops planting in the inter row space. Compared to weeds free plots, only treatment with soybean as a cover crop resulted in similar maize grain yields, while maize intercropping with bean and clover significantly reduced maize yields. Delayed planting of cover crops, 21 day after maize, increased maize grain yield compared to cover crops and maize planting at the same time.

**Key words:** canopy structure, leaf area density, light interception, leaf area index, maize, velvetleaf, weed competition

**IZVLEČEK**

**UČINEK PODSEVKOV NA TEKMOVALNOST MED KORUZO IN BRŽUNASTIM OSLEZOM: GOSTOTA LISTNE POVRŠINE IN PRESTREZANJE SVETLOBE**

V poljskem poskusu je bil preučevan vpliv podsevkov na zgradbo sestoja in prestrezanje svetlobe koruze (Zea mays L.) in bržunastega osleza (*Abutilon theophrasti* Medik). Obravnavanja so obsegala setev nizkega fižola (*Phaseolus vulgaris* L.), soje (*Glycine max* (L.) Merr.) in aleksandrijske detelje (*Trifolium alexandrium* L.) kot podsevkov, posejane istega dne kot koruza ali 21 dni po njeni setvi. V poskus sta bili vključeni tudi 2 kontroli (čista setev koruze z zatiranjem in brez zatiranja plevelov). Vsi preizkušeni podsevki so značilno zmanjšali gostoto listne površine in višino bržunastega osleza do 50 %, medtem ko se je gostota listne površine koruze v prisotnosti vseh treh podsevkov povečala. Med podsevki sta bila fižol in soja najbolj učinkovita v zmanjševanju gostote listne površine in višine bržunastega osleza. Fižol in soja sta v primerjavi z deteljo najmočneje zmanjšala absorbirano svetlobo bržunastega osleza, do 80 %. Podsevki v vrste med koruzo so značilno vplivali na pridelek njenega zrnja. V primerjavi s kontrolo brez plevela je samo obravnavanje s sojo kot podsevkom dalo podobne pridelke, podsevka fižola in detelje sta značilno zmanjšali pridelek zrnja koruze. Odložena setev podsevkov, 21 dni po setvi koruze, je povečala pridelek zrnja koruze v primerjavi z obravnavanji, ko so bili koruza in podsevki posejani istočasno.

**Ključne besede:** zgradba sestoja, gostota listne površine, prestrezanje svetlobe, indeks listne površine, koruza, bržunasti oslez, tekmovalnost plevelov

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## 1 INTRODUCTION

Herbicide application, as one of the common weed control method in modern agricultural ecosystems, has an important role in environmental contamination (Campiglia et al., 2010). One of the environmentally friendly method in weed control is using cover crops between the rows of the main crop. The weed suppression through cover crops has been reported by many researchers (Hiltbrunner et al., 2007; Hollander et al., 2007; Campiglia et al., 2009; Ngouajio et al., 2003). In fact, a living cover crop can decrease weed growth, improve soil structure and can also improve fertility of soil through addition of organic fertilizer (Shili-Touzi et al., 2010; Abdin et al., 2000). Also, it has been shown that cover crops can reduce the frequency of weeds and increase main crops yield (Udenesi et al., 1999). Plants from Fabaceae family have been extensively used as cover crops for soil fertility and smothering weeds (Hiltbrunner et al., 2007), although a variable potential of weed control has been reported for different leguminous crops (Olorunmaiye, 2010).

Light is one of the crucial factors affecting on competition in mixed canopy (Vazin et al., 2010). The total photosynthetically active radiation (PAR) intercepted and distribution of light in plant canopy are important for evaluating the potential carbon uptake by crop (Sassenrath-Cole, 1995). Light interception and attenuation are determined by canopy structure (Maddonni et al., 2001). The leaf area index, plant height, vertical leaf area distribution and leaf angle distribution are factors that play key role in evaluating of competition for light in mixed canopies (Lindquist and Mortensen, 1999). Moreover, vertical profile of leaf area density affects the light interception and consumption, dry matter accumulation and grain yield (Ciganda et al., 2008). In a mixed canopy, vertical distribution of leaf and solar radiation can be used to study of light competition among plants (Uchino et al., 2012). Steinmaus et al. (2008) also noted that cover crops suppressed weed through reduction of light interception by weed. Utilization of broadleaf legumes as cover crops between maize rows could provide nitrogen for main plant and reduce available space for weed development. However, it is necessary to evaluate different cover crops and choose the desirable ones.

Velvetleaf is one of the most troublesome weed in maize fields in the north of Iran. The objective of this experiment was to evaluate the cover crops and their roles in reduction of velvetleaf pressure in maize through the reduction in canopy properties and light interception.

## 2 MATERIALS AND METHODS

The experiment was conducted in a research-field at the Sari Agricultural Sciences and Natural Resources University, Iran (39° 36′ N, 53° 04′ E, 12 m above sea level) in 2011. The soil type was silty clay (pH 7.52, N 23%, P 14 mg $g^{-1}$, K 278.05 mg $kg^{-1}$). Average annual precipitation and temperature were 892.4 mm, and 17.1 °C, respectively. In order to meet nutrient requirements of maize as the main crop, a nitrogen rate of 200 kg $ha^{-1}$ as urea fertilizer and 250 kg $ha^{-1}$ ammonium phosphate fertilizer were added before planting. Also, a nitrogen rate of 200 kg $ha^{-1}$ as urea fertilizer was added when maize was in 6-8 leaves stages. Irrigation was done by a drip irrigation system during the experiment.

The treatments were arranged in a randomized complete block design with three replicates. Treatments included the planting of bean (*Phaseolus vulgaris* 'Sunrise'), soybean (*Glycine max* 'Sari'), and berseem clover (*Trifolium alexandrium* 'Carmel') as cover crops at the same date (first planting date) and 21 days (second planting date) after maize sowing (*Zea mays* 'SC-704'). In addition, sole cropping of maize under weed control and weed infestation were also included. The plants were sown in plots with an area of 5×3.75 $m^2$, included 5 rows of maize (with 75×20 cm spacing) and 6 rows of cover crops (with 75×5 cm spacing). The dominant weed (up to 65 % of the total weed population densities) in this study was velvetleaf as the highest density was recorded (on average, 150 plant $m^{-2}$) and the other

weeds were removed by hand during the growing season.

For evaluation of leaf area density and absorbed light in canopy, leaf area index of plants was measured by a LAI meter (Li-COR, Model LI-3100A, USA), at 77 days after sowing (maize silking stage, soybean and bean were at early flowering stage but clover was at complete flowering stage and velvetleaf was at flowering stage). In addition, height of plants was measured at the same time as measuring leaf area.

Leaf area density (LAD) and light interception were recorded and analyzed with the INTERCOM model (Kropff et al., 1993). This model was chosen as it was possible to calculate profile and light interception in mixed stands of species (Kropff et al., 1993). Solar radiation per day was obtained from Mazandaran meteorological station. The daily PAR was evaluated to be half of the global radiation. The light interception by plants in mixed and sole treatments was calculated at 77 day after maize planting using equations 1 to 3 (Kropff et al., 1993):

Equation 1

$$L_{maize} = \frac{K_m \cdot LAI_m}{K_m \cdot LAI_m + K_c \cdot LAI_c + K_v \cdot LAI_v} \times [1 - \exp[-K_m \cdot LAI_m - (K_c \cdot LAI_c + K_v \cdot LAI_v)]]$$

Equation 2

$$L_{covercrop} = \frac{K_c \cdot LAI_c}{K_c \cdot LAI_c + K_m \cdot LAI_m + K_v \cdot LAI_v} \times [1 - \exp[-K_c \cdot LAI_c - (K_m \cdot LAI_m + K_v \cdot LAI_v)]]$$

Equation 3

$$L_{velvetleaf} = \frac{K_v \cdot LAI_v}{K_v \cdot LAI_v + K_c \cdot LAI_c + K_m \cdot LAI_m} \times [1 - \exp[-K_v \cdot LAI_v - (K_c \cdot LAI_c + K_m \cdot LAI_m)]]$$

where $L_{maize}$, $L_{covercrop}$ and $L_{velvetleaf}$ are data for light captured by maize, cover crops and velvetleaf, respectively. $k_m$, $k_c$, $k_v$ are light extinction coefficients of maize, cover crops and velvetleaf, respectively. $LAI_m$, $LAI_c$, $LAI_v$ are leaf area indexes of maize, cover crops and velvetleaf, respectively.

The light extinction coefficient ($k$) for each plant in this trial was an average of what has been reported previously by other researchers (Table 1).

**Table 1:** Light extinction coefficients ($k$) for plants

| Plant | *K* | References | *k* Average |
|---|---|---|---|
| Maize | 0.65 | Maddonni et al., 2001 | 0.59 |
| | 0.67 | Lindquist et al., 2005 | |
| | 0.6 | Flenetet al., 1996 | |
| | 0.47 | Liu et al., 2012 | |
| Soybean | 0.62 | Flenet et al., 1996 | 0.69 |
| | 0.81 | Wang et al., 2001 | |
| | 0.81 | Dermodyet al., 2008 | |
| | 0.54 | Arkebauer et al., 2009 | |
| Bean | 0.7 | BergaminFilho et al., 1997 | 0.66 |
| | 0.62 | Tsubo et al., 2005 | |
| Berseem clover | 0.41 | Soleymani and Shahrajabian, 2012 | 0.41 |
| Velvetleaf | 0.6, 0.75 | Lindquist, 2001 | 0.68 |
| | 0.51, 0.87 | Lindquist and Mortensen, 1999 | |

Vertical distribution of light in canopy was calculated according to the model described by Nasiri and Kropff (1997). The LAI, dry mass and height data were analyzed using an analysis of variance (ANOVA) using the SAS software (Ver. 9.2). Means were separated using a least significant difference (LSD) at the 5 % level of probability.

## 3 RESULTS AND DISCUSSION

### 3.1 Dry matter, height and leaf area index

The highest dry matter of maize was obtained from soybean cover crop when planted at the same date with the maize. There were no significant differences between treatments where soybean planted 21 days after maize sowing and sole cropping of maize under weed infestation in dry matter of maize (Table 2). Maize in treatments when soybean as the cover crop was planted at the same date of maize sowing, produced the highest dry matter compared to other cover crops. The lowest maize dry matter was recorded in treatments where clover was the cover crop (Table 2). The highest velvetleaf dry matter was observed in sole cropping of maize under weed infestation. The dry matter of velvetleaf was not significantly different among the other treatments (Table 2).

The highest value of maize LAI was observed in treatments where bean was planted 21 days after maize sowing (second planting date) (Table 2). Clover planted 21 days after maize sowing reduced the LAI of maize and the lowest maize LAI was recorded in this treatment (Table 2). Unayet al. (2005) also reported that the presence of cover crops increased the leaf area index of cotton. The LAI of bean and soybean was higher than the clover (Table 2). Hollander et al. (2007) showed that LAI of berseem clover was lower compared to other clover species used as a cover crop. There was no significant difference in LAI between both planting dates of bean and soybean, nevertheless the highest leaf area index of cover crops including soybean and clover was observed in the second sowing date (Table 2).

**Table 2:** Effect of experimental treatments on dry matter, LAI and height of maize, cover crops and velvetleaf

| Treatments | Dry matter (g plant$^{-1}$) | | | Leaf area index (LAI) | | | Height (cm) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Maize | Cover crops | Velvetleaf | Maize | Cover crops | Velvetleaf | Maize | Cover crops | Velvetleaf |
| Bean (T1) | 179.58abc | 3.33bc | 0.77b | 2.98bc | 2.62a | 0.13c | 197.5 | 73.02b | 69.33c |
| Bean (T2) | 221.65ab | 2.88bc | 0.19b | 3.71a | 3a | 0.11c | 202.4 | 61.72bc | 32.67d |
| Soybean (T1) | 245.06a | 9.97a | 1.17b | 3.3ab | 3.63a | 0.08c | 202.5 | 101.2a | 71.67c |
| Soybean (T2) | 232.1a | 5.65b | 0.17b | 3.2b | 4.18a | 0.17c | 211.6 | 94.82a | 31.33d |
| Clover (T1) | 127.85c | 0.78c | 2.08b | 2.5cd | 0.43b | 1.07b | 196.87 | 55.18cd | 75c |
| Clover (T2) | 120.89c | 0.31c | 1.91b | 2.43d | 0.14b | 4.84a | 196.9 | 41.83d | 101.67b |
| Maize (W) | 209.87ab | - | 70.5a | 2.93bc | - | 5.53a | 195.2 | - | 230a |
| Maize (WF) | 148.93bc | - | - | 3.04b | - | - | 192.6 | - | - |
| LSD (5%) | 79.12 | 3.49 | 12.23 | 0.49 | 0.68 | 0.82 | 12.85 | 17.25 | 10.18 |
| SE (±) | 17.16 | 1.45 | 9.92 | 0.1473 | 1.69 | 0.91 | 2.13 | 9.43 | 25.54 |
| Significance level | * | ** | *** | ** | ** | *** | N.S. | *** | *** |
| Coefficient of variation (%) | 24.31 | 50.35 | 62.71 | 9.39 | 39.73 | 27.19 | 3.68 | 13.3 | 6.55 |

T1: The first sowing date of cover crops (same date planting with maize), T2: The second sowing date of cover crops (21 days after planting of maize), W: Weedy, WF: Weed-free.
*, **, *** indicated significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively. N.S., indicates no significant difference. Means in the same column bearing the same letter(s) are not significantly different from each other at the 5 % level of probability.

The presence of bean and soybean reduced the LAI of velvetleaf compared to maize sole crop under weedy condition. The highest leaf area index of velvetleaf was recorded in maize sole crop under weedy condition and in the treatment where clover was planted 21 days after maize sowing. Furthermore, delayed planting of clover resulted in significantly lower clover plant height and LAI compared to the other cover crops (Table 2). Intercropping with bean and soybean decreased leaf area density of velvetleaf on average for 97.83 % and 97.74 %, respectively (Table 2). Hiltbrunner et al. (2007) stated that enhancement of cover crops development and dry matter, decreased the weed dry matter. Also, it was reported that planting legume cover crops resulted in reduction of weed

establishment as live legume cover is the best known to smother weeds (Akobundu, 1982). The greatest plant height of maize was recorded in the second sowing date of soybean treatments, and the shortest plants of maize were observed in maize sole crop under weed-free condition (Table 2). Among cover crops, treatment with soybean had the greatest maize plant height. The absence of cover crops in maize increased the height of velvetleaf. The velvetleaf height was significantly reduced in treatments where soybean and bean were planted 21 days after maize planting (Table 2). Barker et al. (2006) reported that competition of maize strongly reduced velvetleaf $LAI_{max}$ and height, but velvetleaf only reduced maize height by up to 2 %.

### 3.2 Leaf area density

The assessment of leaf area density showed that bean and soybean at both planting dates decreased the LAD of weed more significantly than clover (Figures 1a, 1b, 1d, 1e). In addition, the maximum leaf area density ($LAD_{max}$) of velvetleaf was distributed at higher layer of canopy compared to $LAD_{max}$ of clover (both planting date) (Figures 1c, 1f). In contrast, $LAD_{max}$ of bean and soybean was observed to be positioned at upper canopy layer than that of velvetleaf $LAD_{max}$ (Figures 1a, 1b, 1d, 1e). The low LAD of clover increased the LAD of weed, especially at the second sowing date (Figures 1c, 1f). However, leaf area density of weed in presence of clover was lower than sole planting of maize under weedy conditions (Figures 1c, 1f, 1h). In general, the $LAD_{max}$ of velvetleaf was lower than $LAD_{max}$ of bean and soybean (Figures 1a, 1b, 1 d, 1e), which indicated greater competitive ability of this two plants compare to velvetleaf. Our findings was in according to Vazin et al. (2010) results. Uchino et al. (2012) reported that LAI of weed decreased due to increasing of leaf area and growth of main crops and cover crops.

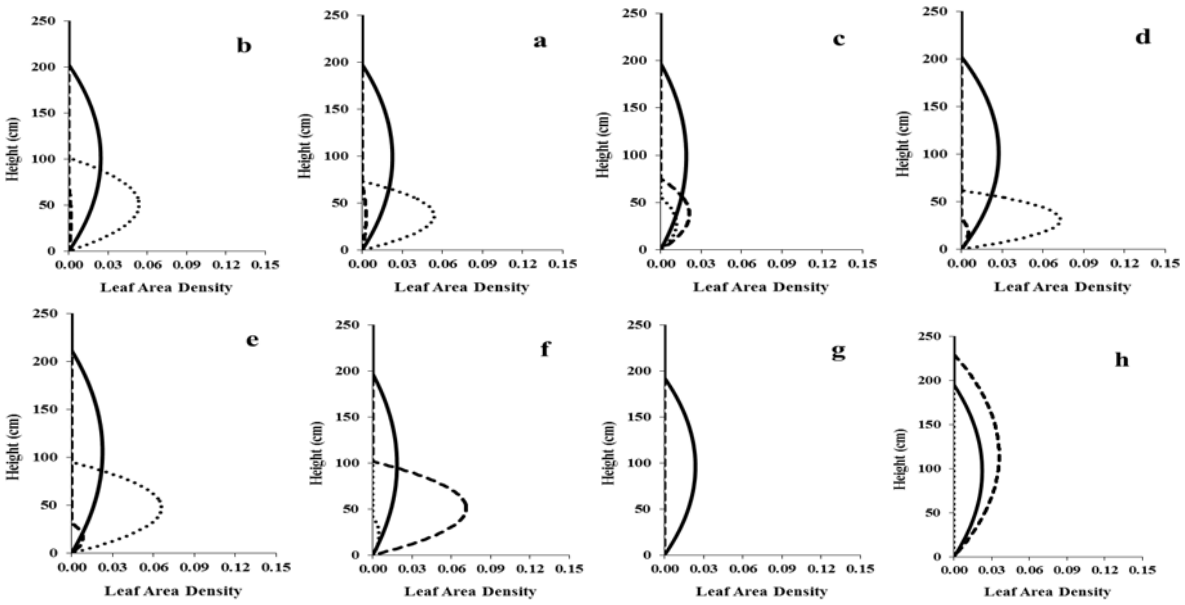


**Figure 1:** Leaf area density ($m^2$ $m^{-3}$) of maize (**—**), cover crops (**….**), and velvetleaf (- - -) in the presence of bean (a, d), soybean (b, e), and berseem clover (c, f) same date planting with maize (a, b, c) and 21 days after planting of maize (d, e, f). Sole cropping of maize under weed control (g) and weed infestation (h).

The LAD of bean was higher than velvetleaf LAD at both planting dates (Figures 1a, 1d). The $LAD_{max}$ of soybean was positioned at higher canopy layer than those of the other cover crops at both planting dates (Figures 1b, 1e). In cover crops treatments, maize $LAD_{max}$ was distributed at higher canopy layer than that of velvetleaf (Figure 1). The $LAD_{max}$ of maize was recorded at higher layer of canopy (106 cm) in treatments with soybean planted 21 days after maize (Figure 1). LAD of

velvetleaf was higher in sole cropping of maize under weedy condition (Figure 1h). $LAD_{max}$ of velvetleaf was observed at the height of 115 cm, whereas maize $LAD_{max}$ was recorded at the height of 98 cm (Figure 1h). The $LAD_{max}$ of maize was observed at the height of 96 cm in weed-free treatments (Figure 1g).

### 3.3 Absorbed light density

Maize light interception decreased by velvetleaf interference under weedy conditions (Figure 2h). In this treatment, the maximum intercepted light by maize was recorded at upper canopy layers. Uchino et al. (2009) reported that intercepted light by main crop canopy reduced because most of the solar radiation was absorbed by upper canopy layers of weeds with high plant height. In addition, weed density and biomass decreased by reduction in available light (Bilalis et al., 2009). In the presence of bean and soybean, intercepted light by velvetleaf reduced significantly. Uchino et al. (2012) noted that light competition of main and cover crops with weeds affected the weed growth. The high leaf area index of maize, bean and soybean enhanced the ability of intercepting solar radiation by these plants and therefore decreased the absorbed light by velvetleaf (Poggio, 2005).

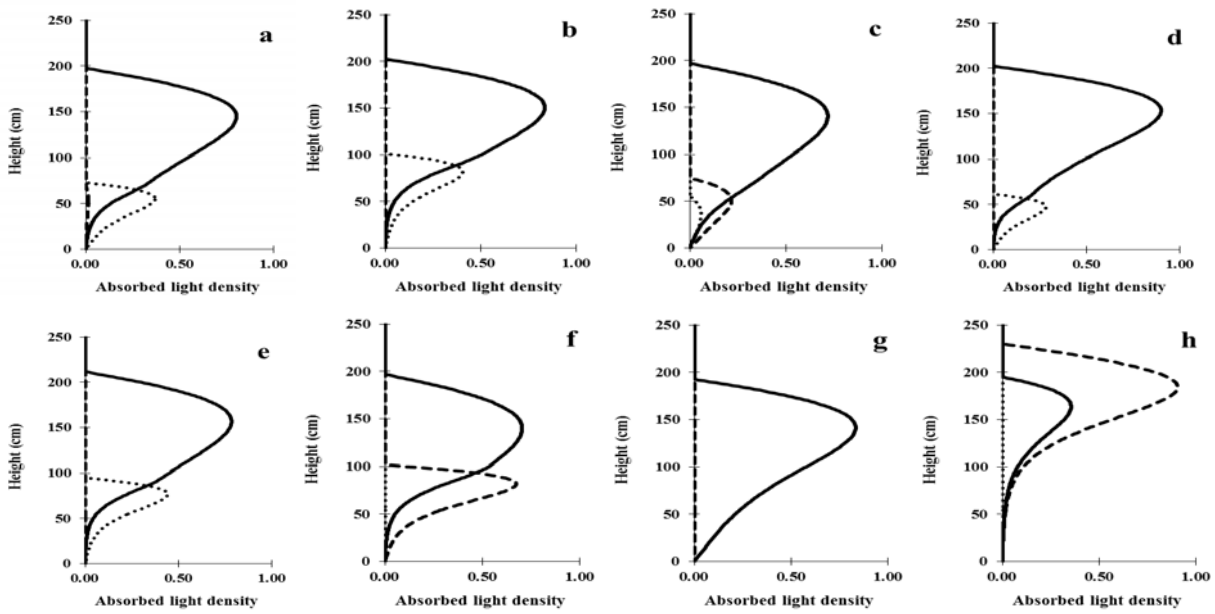


**Figure 2:** Absorbed light density percentage of maize (—), cover crops (….), and velvetleaf (- - -) in the presence of bean (a, d), soybean (b, e), and berseem clover (c, f) same date planting with maize (a, b, c) and 21 days after planting of maize (d, e, f). Sole cropping of maize under weed control (g) and weed infestation (h).

Soybean decreased the absorbed light by lower layers of maize (Figures 2b, 2e). It was because of the greater height for soybean comparing to the other studied cover crops (Table 2). In contrast to soybean, most of the light intercepted at lower layers of maize canopy in treatments where bean was planted as a cover crop. (Figures 2a, 2b, 2d, 2e). Compared to the presence of another two cover crops, lower canopy layers of maize absorbed more light in the presence of clover due to poor light interception by clover (Figures 2c, 2f).

### 3.4 Cumulative absorbed light

The highest cumulative light interception by maize was observed in maize sole cropping under weed-free condition (Figure 3g). Velvetleaf reduced maize cumulative absorbed light, so cumulative absorbed light by maize dropped to below 50 % (Figure 3h). Mondani et al. (2011) stated that weed competition decreased light interception of potato due to reduction in LAI and lodging of potato canopy. Bean and soybean decreased the cumulative absorbed light by velvetleaf, therefore light interception by maize increased compared to

pure stand of maize under weedy conditions (Figures 3a, 3b, 3d, 3e, 3h). The reduction in light interception by velvetleaf in the presence of cover crops was generally due to the wider canopy architecture of broadleaf crops (such as bean and soybean) than the cereal crops (Borger et al., 2010).The bigger height and LAI of bean and soybean compared to clover also could be another reason for the reduction in light interception by velvetleaf (Table 2). In addition, when two or more plants existed in the same area, the competition for environmental resources among plants is inevitable (Zimdahl, 2004). Therefore, cover crops suppressed the velvetleaf growth through reduction in space for velvetleaf and enhancing inter-competition (Workayehu et al., 2011). Angiras and Sharma (1996) stated that reduction of row spacing between wheat plants increased the intercepted light by crops and reduced weed biomass. The cumulative absorbed light of velvetleaf was higher in clover treatments than other cover crops (Figures 3c, 3f). Bilalis et al. (2009) showed a significant reduction in available light for weeds caused by the vetch (*Vicia sativa* L.) rather than red clover (*Trifolium pratense* L.).

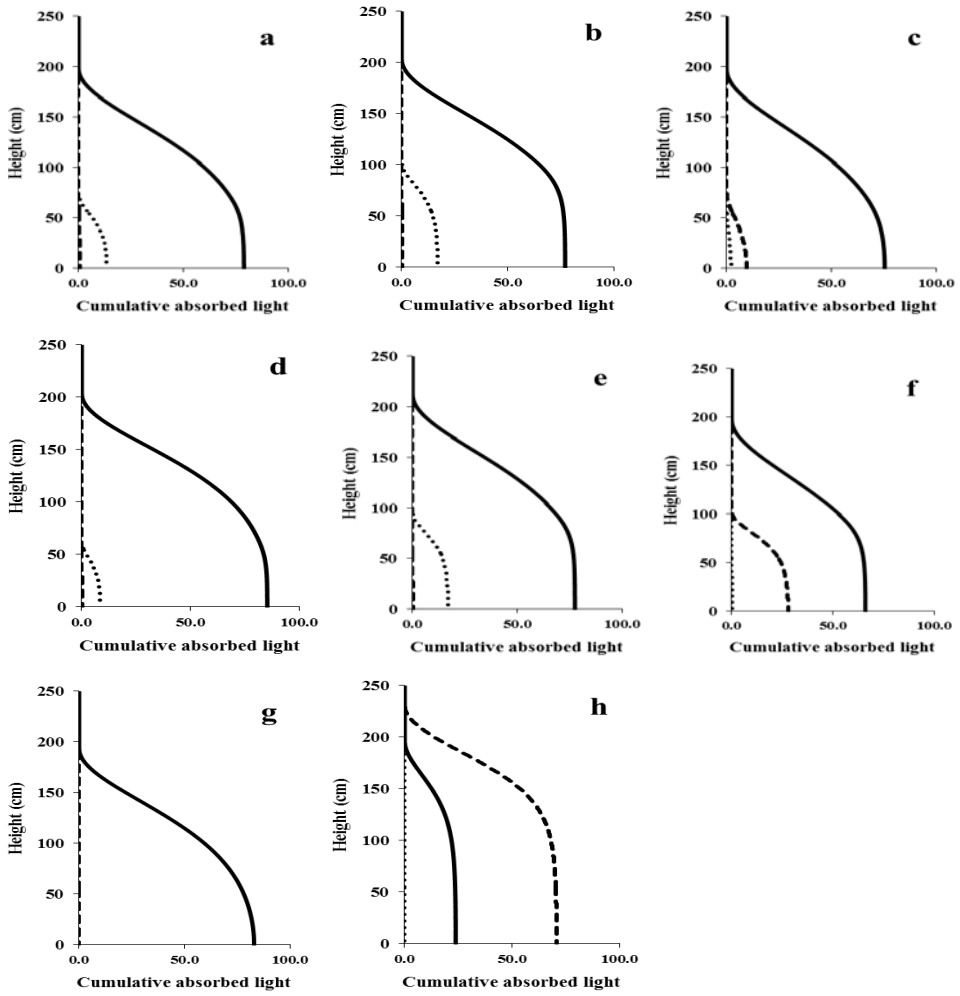


**Figure 3.** Cumulative absorbed light percentage of maize (—), cover crops (….), and velvetleaf (- - -) in the presence of bean (a, d), soybean (b, e), and berseem clover (c, f) same date planting with maize (a, b, c) and 21 days after planting of maize (d, e, f). Sole cropping of maize under weed control (g) and weed infestation (h).

### 3.5 Yield of maize

The highest yield of maize (10,741 kg ha$^{-1}$) was recorded in the sole cropping of maize under weed control (Figure 4). Olorunmaiye (2010) stated that maize grain yield under the various cover crops was similar, but cassava (*Manihote sculentus* Crantz) tuber yield was significantly higher in *Mucuna pruriens* 'Preta' than other cover crops and no cover crop treatment. The maize yield was higher when cover crops were planted 21 days after maize sowing. Bean, soybean and clover that planted 21 days after maize increased maize yield by 24.07 %, 39.66 %, and 14.75 %, respectively, compared to pure stand of maize under weedy condition (Figure 4). In cover crop treatments, the highest yield of maize was observed in soybean cover crop treatments (Fig. 4). The highest yield of soybean and maize was observed in treatment sowing of cover crops planted 21 days after sowing main crops (Uchino et al., 2009). Abdin et al. (2000) also cited that maize grain yield in inter-seeded cover crop treatments was higher than maize pure stand under weedy conditions. Moreover, Ngouajio et al. (2003) reported that the highest yield of lettuce was obtained when summer cowpea was planted as a cover crop.

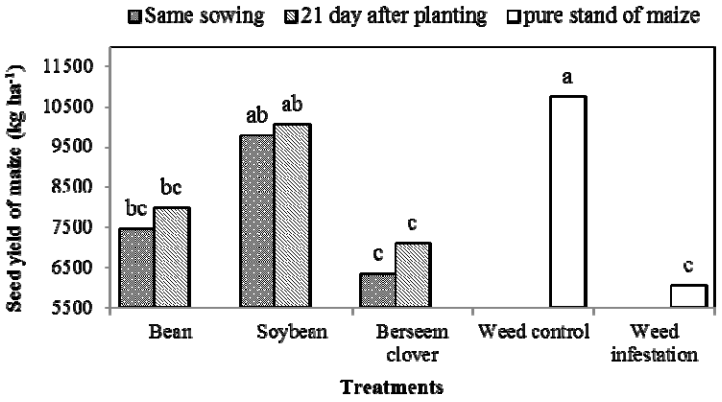


**Figure 4.** Effect of treatments on seed yield of maize. Columns with the same letter are not significantly different at the 5 % level of probability.

Maize yield was the lowest in clover cover crop treatment. This yield reduction is probably result of clover's weak suppression effect on velvetleaf performance, in fact, clover presence also decreased the leaf area index of maize (Table 1) and increased the leaf area density of velvetleaf compared to bean and soybean (Figures 1c, 1f). One of the most important aims of crop management, especially in the presence of weeds, is the better capture of solar radiation by crops (Mondani et al., 2011). In general, soybean improved the yield of maize through increasing in the light interception by maize and reduction of light interception by velvetleaf compared to maize sole cropping under weed infestation (Figure 2).

## 4 CONCLUSIONS

The experimental data showed that bean and soybean as cover crops efficiently suppressed velvetleaf through reduction in velvetleaf leaf area index, plant height and leaf area density. Clover used as a cover crop was less effective in velvetleaf suppression probably due to the lower value of clover plant height, dry matter and LAI. An evaluation of maize yield and dry matter suggested that soybean successfully reduced velvetleaf growth and absorbed light. Therefore, utilizing soybean as a cover crop could be recommended as a method for weed control program in maize. This study provided more insight into the efficiency of three Fabaceae cover crops in weed suppression ability.

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**Agrovoc descriptors:** Aphididae, Aphis gossypii, greenhouse crops, protected cultivation, cucumbers, pest control, insect control, insecticides, ecological control

**Agris category code**: H01, H10

# Bioefficacy of some biorational insecticides for the control of *Aphis gossypii* Glover, 1877, (Hemiptera: Aphididae) on greenhouse grown cucumber

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**ABSTRACT**

*Aphis gossypii* Glover, 1877 is a serious pest of cucumber in greenhouse plantings. Biorational insecticides are an alternative of broad spectrum insecticides for aphid suppression in greenhouse. In this regards, the efficiency of some biorational insecticides including soap based on coconut oil, surfactant based on sodium sulfosuccinate and antifeeding based on potassium nicotinate were assayed on *A. gossypii* in the cucumber greenhouse. The trials were set up in a randomized complete block design with four replications. Samplings were carried out one day before spraying and 3, 7, 14 and 21 days after spraying. The data were submitted to ANOVA and the means comparison was performed using Duncan's test. The results indicated that the highest mortality in insecticidal soap, surfactant and antifeeding treatments occurred after 3 days, with 78.47 %, 67.16 % and 60.48 % mortality, respectively. The results of the trials are discussed in terms of improving management of the populations of *A. gossypii*.

**Key words:** *A. gossypii,* biorational insecticides, antifeeding, soap, surfactant, cucumber

**IZVLEČEK**

**UČINKOVITOST OKOLJU PRIJAZNIH INSEKTICIDOV ZA ZATIRANJE BOMBAŽEVČEVE UŠI (*Aphis gossypii* Glover, 1877, Hemiptera: Aphididae) NA KUMARAH GOJENIH V RASTLINJAKU**

Bombaževčeva uš (*Aphis gossypii* Glover, 1877) je pomemben škodljivec kumar, gojenih v rastlinjaku. Okolju prijazni insekticidi so dobra alternativa insekticidom širokega spectra za zatiranje uši v rastlinjakih. V ta namen so bili za zatiranje omenjene uši na kumarah, gojenih v rastlinjaku, uporabljeni trije okolju prijazni insekticidi, in sicer insekticidno milo na osnovi kokosovega olja, snov za zmanjševanje površinske napetosti na osnovi natrijevega sulfosukcinata in zaviralec prehranjevanja na osnovi kalijevega nikotinata. Poskus je bil zastavljen kot naključni bločni poskus s štirimi ponovitvami. Vzorčenja so bila opravljena dan pred in 3, 7, 14 in 21 dni po škropljenju. Podatki so bili obdelani z ANOVA, primerjava povprečij pa z Duncanovim preizkusom. Rezultati so pokazali, da je bila največja smrtnost zaradi insekticidnega mila, snovi za zmanjševanje površinske napetosti in zaviralca prehranjevanja ugotovljena tri dni po uporabi z 78.47 %, 67.16 % in 60.48 % smrtnostjo. Rezultati poskusa so ovrednoteni v smeri učinkovitejšega zatiranja uši *A. gossypii*.

**Ključne besede:** *A. gossypii,* okolju prijazna sredstva za varstvo rastlin, zaviralci prehranjevanja, insekticidno milo, površinska napetost, kumare

## 1 INTRODUCTION

Cucumber is one of the major important greenhouse crops which is annually grown extensively. The area of cucumber culture in Iran consists of 70,000 hectares with production of about 1,600,000 tonnes (Faostat, 2013). At the present time, cucumber cultivation in greenhouses has been expanded in many areas of Iran, and *Aphis gossypii* Glover, 1877 is observed in a large number of cucumber greenhouses. It has a worldwide distribution and a wide range of host

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plants (Kresting et al., 1999). *A. gossypii* can quickly build up a large population on greenhouse grown cucumber and causes considerable economic damage by sucking sap directly from the phloem, producing honeydew and transmission of plant viruses (Perng, 2002). Generally, the control of *A. gossypii* has relied on a wide array of chemical insecticides which adversely affect non target organisms, are environmentally dangerous and insects frequently build up resistance to them (Wang et al., 2002, Cao et al., 2008). A considerable challenge versus greenhouse cucumber consumers is the subject of food security and healthiness. Therefore there is a real need to assay biorational insecticides which are environmentally safe, non-toxic to man, quickly decomposable and pests do not become resistant after long apply period (Khater, 2012). Biorational insecticides are synthetic and/or natural materials that are more choosey and environmentally friendly and proper to be combined in pest management programs founded on integration of biological and chemical control methods (Horowitz and Ishaaya, 2004). Soaps, surfactant and antifeedings are some biorational substances (Schuster and Stansly, 2009). Insecticidal soaps are founded on potassium fatty acids and are applied to control many crop pests (Miller and Uetz, 1998; Trdan et al., 2006). Applying insecticidal soap exhibited significant suppression of rosy apple aphid, *Dysaphis plantaginea* (Passerini, 1860), green aphid, *Aphis pomi* (DeGeer, 1773) and the spirea aphid, *Aphis spiraecola* Patch, 1914 (Lawson and Weires, 1991). Karagounis et al. (2006) demonstrated that insecticidal soap as replacement for chemical insecticide (imidacloprid) was satisfactory for controlling of *Myzus persicae* (Sulzer, 1776) on organically grown peaches. Fournier and Brodeur (2000) demonstrated that insecticidal soap meaningfully reduced the population of the potato aphid, *Macrosiphum euphorbiae* Thomas, 1878, the green peach aphid, and the lettuce aphid *Nasonovia ribisnigri* (Mosley, 1841) in greenhouse experiments. Dioctyl sodium sulfosuccinate is an anionic surfactant and is used as an emulsifier, dispersant, wetting agent and an adjuvant in insecticide formulations (Pesticides Database, 2014). Field experiments on the pear psyllid, *Psylla pyri* (L., 1758) and the strawberry mite, *Tarsonemus pallidus* (Zimmermann, 1905) and glasshouse experiments on the two-spotted spider mite, *Tetranychus urticae* Koch, 1836 demonstrated that dioctyl sulfosuccinate exhibited significant control of these pests (Bylemans and Van Goethem, 1996). Nicotine is a strong antifeeding agent, which leads to decrease consumption even pests adapted to nicotine and it also prevents pests from feeding (Steppuhn and Baldwin, 2007). Antifeeding is a product containing of potassium nicotinate that is applied as an antifeedant to efficient control of sucking pests such as aphids and aleurodids (Grabi, 2015). Pymetrozine choicely prevent feeding in aphids which therefore causes starvation (Fluckiger et al., 1992, Kayser et al., 1994). Pymetrozine is presently utilized in aphids IPM program in agricultural crops (Smagghe et al., 2010). The present study aimed to assay the efficacy of biorational insecticides including insecticidal soap, surfactant and antifeeding for using in cucumber greenhouse plantings in connection with their potential to control *A. gossypii*.

## 2 MATERIALS AND METHODS

### 2.1 Site, plants and insecticides

Field studies were performed in 1000 $m^2$ greenhouses cucumber located at Isfahan (Central Iran), during the 2012-13 growing seasons under environmental conditions of 27 ± 3 °C, 70 ± 5 % RH and L:D 16:8 h. The plants were 45 days old, *Cucumis sativus* L. of the variety 'Storm', which is the common cucumber variety in the study area. Management operations including control of other pests, fertilization and irrigation were carried out similarly on all trial plants. Three biorational insecticides including soap based on coconut oil (Palizin® 65 % SL, Kimia Sabzavar) at a dose of 3 g/l, surfactant based on sodium sulfosuccinate (D-octil® 70 % SL, A.M.C.) at a dose of 0.3 g/l and antifeeding based on potassium nicotinate (Antifeeding® 10 % SL, Grabi) at a dose of 3 g/l and the insecticide pymetrozine (50 % WG, Golsam) at a dose of 1 g/l were used.

### 2.2 Experimental design

The experiment was laid out in a randomized complete block design with four replications. Each replication included 20 plants separated from the adjacent treatment by a border plant. Treatments were four insecticides and untreated check which were randomly placed in any of the blocks. All treatments were performed by a motorized sprayer (Zeeba Co.) at a pressure of 30 kg/ $cm^2$ using a cone nozzle. The experimental plants were sprayed at a volume rate of 400 l $ha^{-1}$. A single application of each treatment was applied. The sprayer was washed with water and surfactant before applying each treatment. Check plants were sprayed with drink water. Spraying was carried out when most of the plants have been infested by the pest (an average of 15.16 and 12.49 aphids per leaf in 2012 and in 2013, respectively). Spraying was started at 9:00 a.m. and continued up to 11:00 a.m.

### 2.3 Sampling

The sampling was done in five steps including a day before treatment, 3, 7, 14 and 21 days post-treatment. For sampling, fifteen plants were randomly selected and the numbers of live aphids on both surfaces of the third leaf from the top of the plant were carefully enumerated with a 4x magnification hand lens. All nymphal stages and adults were enumerated.

### 2.4 Data analyses

Mean population density of pest was computed at any experiment unit and pest mortality percentage for any of the treatments was achieved in every sampling step according to the following model (Henderson and Tilton, 1955):

$$M = 100\times (1-(Ta\times Cb)/(Tb\times Ca)$$

where, *M*, is the mortality percentage, *Ta*, *Cb*, *Tb*, and *Ca* are the number of aphids live in treated plot after treatment, in check plot before treatment, in treated plot before treatment, and in check plot after treatment, respectively. The data of mortality percentages were transferred to arc-sine where, *M*, is the mortality percentage, *Ta*, *Cb*, *Tb*, and *Ca* are the number of aphids live in treated plot after treatment, in check plot before treatment, in treated plot before treatment, and in check plot after treatment, respectively. The data of mortality percentages were transferred to arc-sine $\sqrt{x/100}$ before analysis. All data were submitted to a one-way ANOVA analysis to compare the effect of treatments on aphid population. The comparison of means was done using Duncan's multiple range test (DMRT) ($P < 0.05$). Data were analysed by using SAS statistical software version 9.1. (SAS Institute Inc. 2004).

## 3 RESULTS

### 3.1 Aphid population before spraying

There was not a significant difference among treatments in aphid density before spraying ($F_{4,12}$ = 1.53; $p$ = 0.26, in 2012 and $F_{4,12}$ = 1.45; $p$ = 0.28, in 2013), indicating uniformity in population of the aphid in all the trial plots. Overall mean of *A. gossypii* in treatments was 15.16 and 12.49 aphids per leaf in 2012 and 2013, respectively (Tables 1 and 2). No signs of phytotoxicity at recommended field rates were seen on the experimental plants during the trial.

**Table 1:** Mean number of *Aphis gossypii* per leaf in various treatments at days before and after spraying in 2012

| Treatments | Mean ± SE | | | | |
|---|---|---|---|---|---|
| | 1 DBT | 3 DAT | 7 DAT | 14 DAT | 21 DAT |
| Pymetrozine | 14.68 ± 0.86 | 0.00 ± 0.00 | 0.03 ± 0.02 | 0.18 ± 0.02 | 0.60 ± 0.07 |
| Soap | 16.82 ± 1.01 | 0.72 ± 0.23 | 1.70 ± 0.04 | 3.35 ± 0.11 | 7.58 ± 0.33 |
| Detergent | 14.28 ± 1.47 | 2.22 ± 0.18 | 3.70 ± 0.21 | 5.83 ± 0.07 | 7.82 ± 0.59 |
| Anti-Feeding | 14.85 ± 1.07 | 3.88 ± 0.16 | 4.23 ± 0.18 | 5.88 ± 0.21 | 7.27 ± 0.20 |
| Mean | 15.16 ± 0.57 | - | - | - | - |

DBT is a day before treatment and DAT is days after treatment.

**Table 2:** Mean number of *Aphis gossypii* per leaf in various treatments at days before and after spraying in 2013

| Treatments | Mean ± SE | | | | |
|---|---|---|---|---|---|
| | 1 DBT | 3 DAT | 7 DAT | 14 DAT | 21 DAT |
| Pymetrozine | 12.98 ± 0.40 | 0.03 ± 0.02 | 0.05 ± 0.02 | 0.12 ± 0.02 | 1.33 ± 0.06 |
| Soap | 12.65 ± 0.11 | 0.88 ± 0.09 | 1.70 ± 0.04 | 2.68 ± 0.16 | 6.47 ± 0.19 |
| Detergent | 12.87 ± 0.37 | 2.08 ± 0.11 | 3.42 ± 0.20 | 5.70 ± 0.11 | 7.48 ± 0.20 |
| Anti-Feeding | 12.25 ± 0.23 | 3.42 ± 0.16 | 3.88 ± 0.13 | 5.48 ± 0.22 | 6.12 ± 0.24 |
| Mean | 12.49 ± 0.21 | - | - | - | - |

DBT is a day before treatment and DAT is days after treatment.

### 3.2 Effect of the treatments three days after spraying

There was a significant difference among treatments in terms of percent mortality after three days ($F_{3, 9}$= 148.32, $P < 0.0001$, in 2012 and $F_{3, 9}$= 186.63, $P < 0.0001$, in 2013). The mean of *A. gossypii* per leaf for each treatment is given in Tables 1 and 2. The comparison of the mortality percentage means showed that the treatments categorized into four groups (Figures 1 and 2). The highest mortality percentage among biorational insecticides was obtained in soap treatment with an average of 78.47 % and 75.78 % in 2012 and 2013, respectively (Figures 1 and 2).

### 3.3 Effect of the treatments seven days after spraying

There was a significant difference among treatments in percent mortality after seven days ($F_{3, 9}$= 104.7, $P < 0.0001$ in 2012 and $F_{3, 9}$= 296.24, $P < 0.0001$, in 2013). The mean of *A. gossypii* per leaf for each treatment is given in Tables 1 and 2. The comparison of the mortality percentage means showed that the treatments categorized into three groups (Figures 1 and 2). The highest mortality percentage among biorational insecticides was obtained in soap treatment with an average of 71.93 % and 70.43 % in 2012 and 2013, respectively (Figures 1 and 2). There was not a significant difference between two other biorational insecticides, surfactant and antifeeding, so they were categorized to the same group with the least mortality percentage (Figures 1 and 2).

### 3.4 Effect of the treatments 14 days after spraying

There was a significant difference among treatments in percent mortality after 14 days ($F_{3, 9}$= 147.97, $P < 0.0001$, in 2012 and $F_{3, 9}$= 271.94, $P < 0.0001$ in 2013). The mean of *A. gossypii* per leaf for each treatment is given in Tables 1 and 2. The comparison of the mortality percentage means showed that the highest mortality percentage among biorational insecticides was obtained in soap treatment with an average of 64.23 % and 65.12 % in 2012 and 2013, respectively (Figures 1 and 2). Two other biorational insecticides had the same mortality percentage (Figures 1 and 2).

### 3.5 Effect of the treatments 21 days after spraying

A significant difference was found in the mortality percentages among different treatments ($F_{3,\ 9}$= 156.58, $P < 0.0001$, in 2012 and $F_{3,\ 9}$= 190.52, $P <$ 0.0001, in 2013). The mean of *A. gossypii* per leaf for each treatment is given in Tables 1 and 2. The comparison of the mortality percentage means showed that the highest mortality percentage among biorational insecticides was obtained in soap treatment with an average of 48.77 % in 2012 (Figure 1). There was not a significant difference between two biorational insecticides, soap and antifeeding, in the mortality percentage in 2013 (Figure 2).

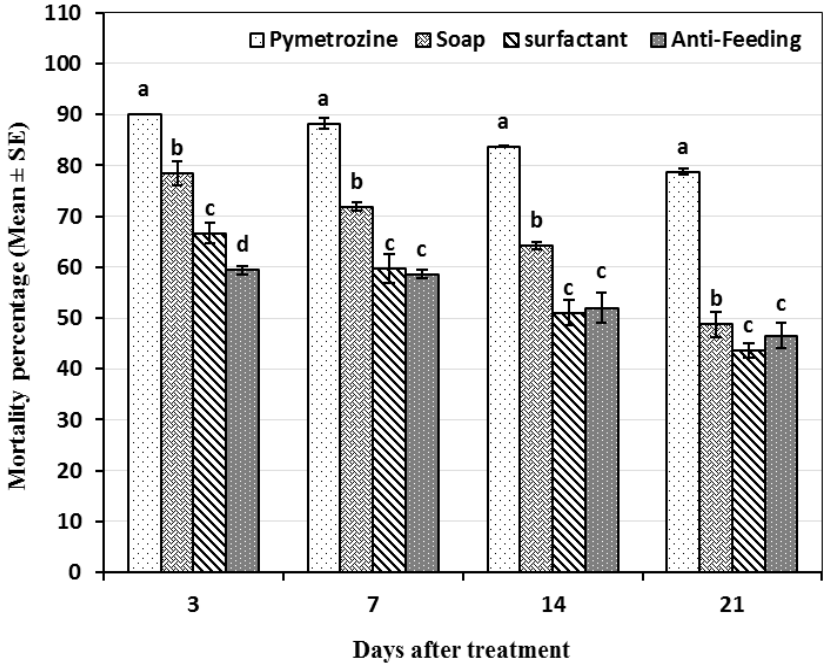


**Figure 1:** The mortality percentage of *Aphis gossypii* at different days after spraying in the greenhouse in 2012. Means followed by the same letters in each day after spraying was not significantly different ($P < 0.05$).

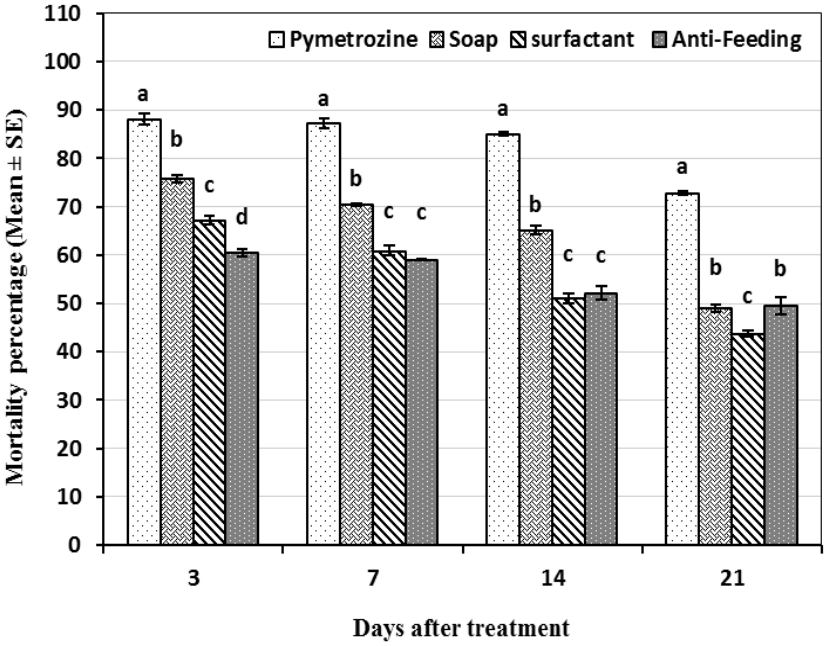


**Figure 2:** The mortality percentage of *Aphis gossypii* at different days after spraying in the greenhouse in 2013. Means followed by the same letters in each day after spraying was not significantly different ($P < 0.05$).

## 4 DISCUSSION

Insecticides differ in their toxicity and in their potential ecological effect. Biorational insecticides are relatively non-toxic to consumers with little environmental contamination. According to the US-Environmental Protection Agency biorational insecticides display minimal risk to the environment, break down quickly, have minimal residue, are safe to applier and relatively small amounts are needed for successful control (Sadeghi et al., 2009). The findings of the present study revealed that pymetrozine had the highest effect on *A. gossypii* (Figures 1 and 2). It gave promising results in control of *A. gossypii* up to 21 days after spraying (Figures 1 and 2). Mojeni and Rezwani (2000) explained that pymetrozine had the highest efficacy for suppression of *A. gossypii* in field trials. The low effect of pymetrozine on natural enemies shows it a desirable insecticide for IPM programs (Fluckiger et al., 1992, Follas and Blanc, 1995). Insecticidal soaps based on fatty acid salts are contact insecticides with minimal adverse effects that suppress small pests through suffocation (by blocking the spiracles) or disruption of cuticular waxes and membranes in the integument, causing to desiccation (Isman, 2006). Here, the greenhouse experiments in both years revealed that the insecticidal soap significantly reduced the population of *A. gossypii*, three days after spraying (Figures 1 and 2). Regarding to mean aphid mortality, spraying the insecticidal soap on leaves reduced the aphid populations in cucumber plants comparably with surfactant and antifeeding. It gave promising results in suppression of *A. gossypii* up to 14 days after spraying (Figures 1 and 2). Other studies demonstrated that the insecticidal soaps are efficient against the aphid pest populations (Pinnock et al., 1974, Moore et al., 1979, Koehler et al., 1983, Lawson and Weires, 1991, Lee et al., 2005, Karagounis et al., 2006, Baniameri, 2008). McKenzie and Puterka (2004) and Hall and Richardson (2013) showed that the use of insecticidal soap provided significant control of the psyllid pest population, *Diaphorina citri* Kuwayama, 1908. Insecticidal soaps usually caused meaningful, but short-period suppression of some important pests such as wheat aphid, *Schizaphis graminum* (Rondanni, 1852) (Nuessly and Nagata, 2005), the thrips of weeping ficus, *Gynaikothrips uzeli* Zimmerman, 1900 (Held and

Wheeler, 2007), aphid of the crepe myrtle, *Tinocallis kahawaluokalani* Kirkaldy, 1906 (Layton and Gu, 2009a), whitefly of sweet potato, *Bemisia tabaci* (Gennadius, 1889) (Layton and Gu, 2009b) and the psyllid of pistachio, *Agonoscena pistaciae* Burckhardt and Lauterer, 1989 (Kabiri and Amiri-Besheli, 2012). The maximal mortality percentage in surfactant treatment, dioctyl sodium sulfosuccinate, occurred in three days after spraying (Figures 1 and 2). The present results are consistent with finding reported by Ranaei et al., (2012) that showed the mortality of aphids was 67.13 % by deioctyl sodium sulfosuccinate. Dioctyl sodium sulfosuccinate, apart from its wetting activity, has also ovicidal, larvicidal and insecticidal effect upon mites, psyllids, aphids and scale insects (Dincsoy, 2013). However, the greenhouse experiments indicated that the use of soaps, surfactants and antifeedings can give some level of aphid suppression under commercial greenhouse conditions (Figures 1 and 2). Given the results, it would be more desirable to utilize these biorational compounds at preliminary phases of aphid infestation in joining with an aphid monitoring plan. In conclusion, according to the findings of the present research, little or no harmful impacts on lady beetles (Tremblay et al., 2008, Hall and Richardson, 2013) has been observed, using in organic and sustainable agriculture (Karagounis et al., 2006). Increasing market value for insecticide residue free products, usable around the harvest, action to slow down or minimize pest resistance to synthetic insecticides (Fournier and Brodeur, 2000) and no residual insecticidal activity (Baldwin, 2008), the insecticidal soap as a biorational insecticide could be suggested for the suppression of *A. gossypii* in cucumber greenhouse plantings in an IPM program.

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DOI: 10.14720/aas.2016.107.2.15
**Agrovoc descriptors:** Essential oil crops, new technology, fertilizers, inorganic fertilizers, micronutrient fertilizers, iron, spacing, crop performance, yield components

**Agris category code**: F01,F04

# Nano-iron fertilizer effects on some plant traits of dragonhead (*Dracocephalum moldavica* L.) under different sowing densities

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**ABSTRACT**

A field experiment was conducted with dragonhead to evaluate the effects of iron nano-fertilizer rates (0, 1, 2 and 3 g $l^{-1}$) and planting density levels (10, 15, 20 and 40 cm) on the fresh herb, essential oil content and other traits under the natural conditions. Traits such as number of flowering branches (NFB), height of first flowering branch (HFB), number of secondary branches (NSB), stem diameter (SD), essential oil content (EOC), dry mass (DM), essential oil yield (EOY), total anthocyanins (TA), chlorophyll a (CA), chlorophyll b (CB), flavonoid 270 nm (F270), flavonoid 300 nm (F300), and total flavonoid (TF) were measured. Results showed that the nano Fe treatment × trait (TT) biplot accounted 39 % and 25 % of total variation, respectively. The vertex treatments in polygon biplot were D2-N2 (15 cm density and 1 g $l^{-1}$ nano-fertilizer) was the best in the EOC, DM and EOY, while D4-N3 (40 cm density and 2 g $l^{-1}$ nano-fertilizer) was the best for TA, F270, F300 and TF. Sowing densities (10, 15 and 20 cm) with iron nano-fertilizer treatments (1 and 2 g $l^{-1}$) were the best combinations of evaluated factors for all the measured traits of the dragonhead.

**Key words:** dragonhead, essential oil, nano-fertilizer, planting distances

**IZVLEČEK**

**ANALIZA NEKATERIH LASTNOSTI MOLDAVSKE KAČJEGLAVKE (*Dracocephalum moldavica* L.) PRI RAZLIČNIH ODMERKIH GNOJENJA Z NANO-ŽELEZOVIMI GNOJILI IN RAZLIČNIH GOSTOTAH SETVE**

Poljski poskus z moldavsko kačjeglavko je bil izveden za ovrednotenje učinka različnih odmerkov nano železovih gnojil (0, 1, 2 in 3 g $l^{-1}$) in gostote setve (10, 15, 20 in 40 cm) na pridelek sveže mase, vsebnost eteričnih olj in na nekatere druge lastnosti v naravnih razmerah. Izmerjene so bile lastnosti, kot je število cvetočih poganjkov (NFB), višina prvega cvetočega poganjka (HFB), število sekundarnih poganjkov (NSB), premer stebla (SD), vsebnost eteričnih olj (EOC), suha masa (DM), pridelek eteričnih olj (EOY), celokupni antocianini (TA), klorofil a (CA), klorofil b (CB), flavonoidi 270 nm (F270), flavonoidi 300 nm (F300) in celokupni flavonoidi (TF). Rezultati "biplot" analize obravnavanje x lastnost (TT) so pojasnili 39 % in 25 % celokupne variabilnosti. Najboljši obravnavanji v poligonalnem biplotu sta bili D2-N2 (15 cm gostota setve in 1 g $l^{-1}$ nano-gnojila), ki je dalo najboljše vrednosti za lastnosti EOC, DM in EOY, in obravnavanje D4-N3 (40 cm gostota setve in 2 g $l^{-1}$ nano-gnojila), ki je dalo najboljše vrednosti za lastnosti TA, F270, F300 in TF. Za vse merjene lastnosti moldavse kačjeglavke so se kot najboljše kombinacije proučevanih dejavnikov izkazale pri gostotah setve 10, 15 in 20 cm ter pri gnojenju z 1 in 2 g $l^{-1}$ nano železovih gnojil.

**Ključne besede:** moldavska kačjeglavka, eterična olja, nano-gnojila, gostota setve

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## 1 INTRODUCTION

The Dragonhead (*Dracocephalum moldavica* L.) is a perennial herbaceous plant up to 80 cm tall and grows at altitudes of up to 2700–3100 m above sea level; it also is grown for its essential oil and for bees (Popova et al., 2008). It is native to central Asia and is domesticated in Europe and is used as a food ingredient, as a tea, as the herbal drug for its reputed medicinal properties (Dastmalchi et al., 2007). The leaves of the dragonhead are opposed, long and oval in shape on the long petioles and its flowers, on short pedicles, are blue-violet in color. It contains terpenoids and flavonoids and its extracts from the raw material have a multitude of pharmacological actions and the used material consisted of dragonhead herb are prepared at different growth phases of vegetative growth, flowering, and maturity (Nikitina et al., 2008).

Environmental factors affect the content and composition of secondary metabolites of medicinal plants and it is generally assumed that the material is best collected when the plants have reached their optimal growth (Aziz et al., 2010). Plant density is one of the important factor which determines growth, development and yield of medicinal plants and its selection to allow for expression of maximum yield performance is a management practice that would make medicinal plants production more economical (Hussein et al., 2006). Cultivation of crops with desirable density has positive effect on yield components, so this factor should be suitable to achieve optimum plant density and maintenance of optimum growth. Low plant density results in high weeds population and low yield performance, while dense plant population on the other hand causes lodging, reduces photosynthetic production and drastically reduces the yield performance (Soroori et al., 2014).

Nutrients have a great effect on yield performance and chemical composition of medicinal plants (Heidari et al., 2011), and microelements have an important role in their production (Hopkins and Huner, 2004). A balanced fertilization with micronutrients is very important issue in the high yield performance as well as high quality essential oil (Sawan et al., 2001), and for sufficient plant growth, some micronutrients are required in small amounts; however, their deficiencies cause a great negative effect in the biochemical reactions (Bacha et al., 1997). Plants absorb nutrients from soils via roots although nutrients can be supplied to plants as fertilizers by foliar sprays. There is little information on the response of dragonhead to applied nutrients, particularly of micronutrients and iron (Fe) which is a cofactor for approximately more than hundred enzymes that catalyze physiological processes (Brittenham, 1994). The alkaline nature of most soils of semi-arid environments predisposes to Fe deficiency, so that crops usually suffer from short supply of this nutrient and it has been reported that iron foliar application increased the yield performance of medicinal plants (Ebrahimzadeh et al., 2009; Nasiri et al., 2010; Jabbari et al., 2011).

Nanotechnology is a novel science that atracts researchers from different disciplines such as biologists across the globe and nanoparticles are aggregates with at least one dimension less than 100 nm, which can modify their properties compared to the bulk material (Tarafdar et al., 2014). Owing to its high ratio of surface area to volume they exhibit novel and improved properties and functions and the nano-fertilizers have a slower release compared to the conventional fertilizer application, which release heavily early and in low non-uniform amounts (Raliya and Tarafdar, 2013). They will prevent undesirable nutrient losses to environment (soil, water and air) via direct delivery to plants, and avoiding the interaction with soil, microorganisms, water, and air (De Rosa et al., 2010). The aim of the present study was to investigate the effects of different concentrations of iron nano-chelate fertilizer and various sowing densities of *Dracocephalum moldavica* L. on yield and yield components, as well as the content of essential oils obtained from this plant.

## 2 MATERIALS AND METHODS

This research was conducted in the 2014/2015 growing season at the experiment field of the college of Agriculture, Payam Noor University of Marand (latitude: 38◦25`N, longitude: 45◦46`E: 1334 m) in the northwest part of Iran. Precipitation falls in winter as snow on the mountains of the north and west and rainfall mainly occurs between November to May and the soil of the field was silty loam with pH 7.8, and the other physical and chemical properties of soil (Table 1) were determined using the methods of Chapman and Pratt (1978). A factorial experiment based on a randomized complete block design with three replications was used with four levels of iron nano-fertilizer (0, 1, 2 and 3 g $l^{-1}$) and four plant spacing (10, 15, 20 and 40 cm). The morphological characterization of iron nano-chelate particles were determined by scanning electron microscope (Fig. 1).

The seeds of *Dracocephalum moldavica* L. were sown directly in field on 23 May and four levels of iron nano-fertilizer were applied in 23 June. After two weeks from sowing, the plants were thinned twice, leaving one plant in hills. Irrigation and manually weeds' control were performed when needed. The plants were collected at full flowering stage in 24 July, and the following data were recorded for plant growth characters: number of flowering branches (NFB), height of first flowering branch (HFB), number of secondary branches (NSB), stem diameter (SD), essential oil content (EOC), dry mass (DM), essential oil yield (EOY), total anthocyanins (TA), chlorophyll a (CA), chlorophyll b (CB), flavonoid 270 nm (F270), flavonoid 300 nm (F300), and total flavonoid (TF). Chlorophyll a and b (mg $g^{-1}$) of the leaves were determined by AOAC (1990) and the resulted essential oil from each treatment was dehydrated over anhydrous sodium sulfate and then subjected to GLC analysis with Varian VISTA 6000 FID model. The two-way matrix of treatment × trait interaction is consisted of 16 treatment combinations (4 iron nano-fertilizer × 4 sowing density) which is analyzed by the treatment × trait (TT) biplot model (Yan and Rajcan, 2002). Visual analysis of dataset via TT biplot was performed using GGEbiplot software (Yan, 2001) and all biplots presented in this paper are direct outputs of this statistical software. Up-to-date information on GGE biplot is available at http://www.ggebiplot.com.

**Table 1.** Pearson's simple correlation coefficients among the Dragonhead traits

| | NFB | HFB | NSB | SD | EOC | DW | EOY | TA | CA | CB | F270 | F300 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HFB | -0.27 | | | | | | | | | | | |
| NSB | 0.11 | -0.05 | | | | | | | | | | |
| SD | 0.24 | -0.37 | 0.70 | | | | | | | | | |
| EOC | -0.52 | 0.27 | -0.08 | -0.14 | | | | | | | | |
| DW | -0.45 | 0.35 | -0.34 | -0.55 | 0.37 | | | | | | | |
| EOY | -0.53 | 0.39 | -0.23 | -0.45 | 0.71 | 0.91 | | | | | | |
| TA | -0.34 | -0.28 | -0.32 | -0.16 | 0.04 | 0.04 | 0.00 | | | | | |
| CA | 0.15 | -0.50 | 0.18 | -0.03 | 0.12 | -0.01 | 0.07 | 0.15 | | | | |
| CB | 0.17 | -0.38 | 0.02 | -0.25 | -0.05 | 0.00 | 0.01 | 0.19 | 0.92 | | | |
| F270 | -0.20 | -0.13 | -0.14 | -0.32 | 0.39 | 0.17 | 0.32 | 0.21 | 0.71 | 0.76 | | |
| F300 | -0.30 | -0.10 | -0.28 | -0.38 | 0.57 | 0.25 | 0.44 | 0.16 | 0.67 | 0.65 | 0.86 | |
| TF | -0.28 | -0.16 | -0.31 | -0.43 | 0.45 | 0.30 | 0.43 | 0.37 | 0.72 | 0.77 | 0.93 | 0.92 |

Traits are: number of flowering branches (NFB), height of first flowering branch (HFB), number of secondary branches (NSB), stem diameter (SD), essential oil content (EOC), dry mass kg $ha^{-1}$ (DM), essential oil yield (EOY), total anthocyanins (TA), chlorophyll a (CA), chlorophyll b (CB), flavonoid 270 nm (F270), flavonoid 300 nm (F300), total flavonoid (TF).

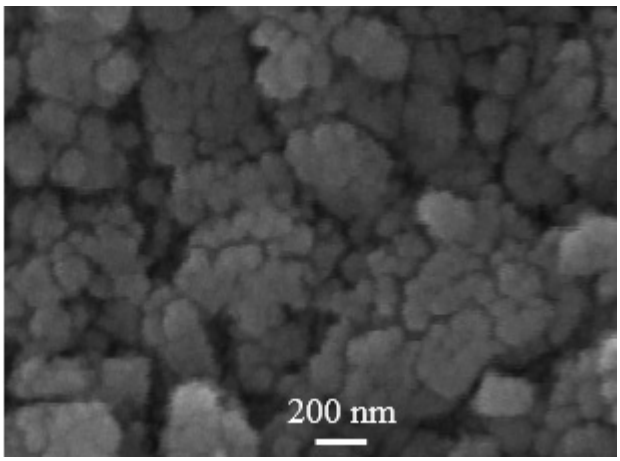


**Figure 1:** Scanning Electron Microscope (SEM) image of synthesized nanoparticles ferric oxide for iron nano-fertilizer

## 3 RESULTS AND DISCUSSION

The TT biplot of mean performance of dragonhead treatment combinations (nano-iron fertilizer × sowing density) explained 64 % of the total variation of the standardized data (Fig. 2A) and this relatively moderate percentage variation reflects the accuracy of interrelationships among the measured traits across different treatment combinations. According to Kroonenberg (1995), the fundamental patterns of complicated relations among the traits and treatment combinations should be captured by the biplot analysis. Fig. 2A is biplot showing the polygon view of the treatment × trait analysis on the morphological and other measured traits based on principal component (PC) axes (PC1 and PC2). The traits were considered as the tester and the treatment combinations as entries. The first two axes explained 39 and 25 % of the total variation among the treatment due to morphological traits measured, respectively. This figure shows which treatment(s) were best at what trait. The treatment (s) at each vertex (vertex treatment) of the polygon were the best in terms of the trait(s) found within the sector demarcated by any two lines that meet at the origin. From Fig. 2A, D2-N2 (15 cm planting density and 1 g $l^{-1}$ iron nano-fertilizer) was the best in terms of essential oil content (EOC), dry mass (DM) and essential oil yield (EOY), indicating that it can be used in the high production of dragonhead plants that are outstanding in these traits.

The vertex treatment combination D4-N3 (40 cm planting density and 2 g $l^{-1}$ iron nano-fertilizer) was the best treatment for obtaining of the total anthocyanins (TA), flavonoid 270 nm (F270), flavonoid 300 nm (F300) and total flavonoid (TF) while the vertex treatment combination D4-N1 (40 cm planting density and 0 g $l^{-1}$ iron nano-fertilizer) was the best treatment for obtaining of the number of secondary branches (NSB) and stem diameter (SD). Even though D4-N3 was identified for good performance in anthocyanins and flavonoid traits, it was not the best for EOC, DW and EOY traits, indicating that the content of the anthocyanins and flavonoid traits might not be a good trait-indicators for yield and essential oil of dragonhead. The vertex treatment combination D3-N1 (20 cm planting density and 0 g $l^{-1}$ iron nano-fertilizer) was identified for good performance in height of first flowering branch (HFB) trait while the vertex treatment combination D4-N2 (40 cm planting density and 1 g $l^{-1}$ iron nano-fertilizer) was identified for good performance in number of flowering branches (NFB) trait (Fig. 2A). The enhancing effect of iron nano-fertilizer on chlorophyll content and yield performance could be attributed to the favorable effect of iron nano-fertilizer treatments to increase biosynthesis of chlorophylls which are involved in chloroplast biosynthesis, which might be expected as a reason for chlorophyll increases in dragonhead leaves (Marschner, 2012).

On this premise, two traits are positively correlated if the angle between their vectors is an acute angle (< 90°) while they are negatively correlated if their vectors is an obtuse angle (> 90°) (Yan and Kang, 2003). Across the 16 tested treatments combinations, DM, EOY and EOC were positively associated (an acute angle) as shown in Fig. 2B. These traits were negatively correlated with SD, NSB and NFB traits (obtuse angles), and they were independent of the CA and CB traits (near right angles).These relationships suggest that it is possible to combine higher dry yield, higher essential oil content and higher essential oil in a single treatments combination (Fig. 2B). Traits F300, TF, F270 and TA were positively associated (an acute angle), and they were independent of the HFB trait (near right angles), while CA and CB traits were positively associated (an acute angle), and they were independent of the SD, NSB and NFB traits (near right angles). Finally, SD, NSB and NFB traits were positively associated (an acute angle) as shown in Fig. 2B. According to Rahbarian and Salehi-Sardoei (2014) there is strong positive correlation between dry mass of dragonhead with number of branches per plant, plant height while it was or non-significantly correlated with diameter and length of stem internode. Although most of the above predictions can be verified from the Pearson's correlation coefficients (Table 1), but some others are not consistent with the original coefficients of correlation because such discrepancies are seen because the TT biplot method explained lower than 100 % (in present study, 64 %) of the total variation. In other word, some information were ignored by using TT biplot method, but major pattern of dataset is interpreted easily via visual grasp.

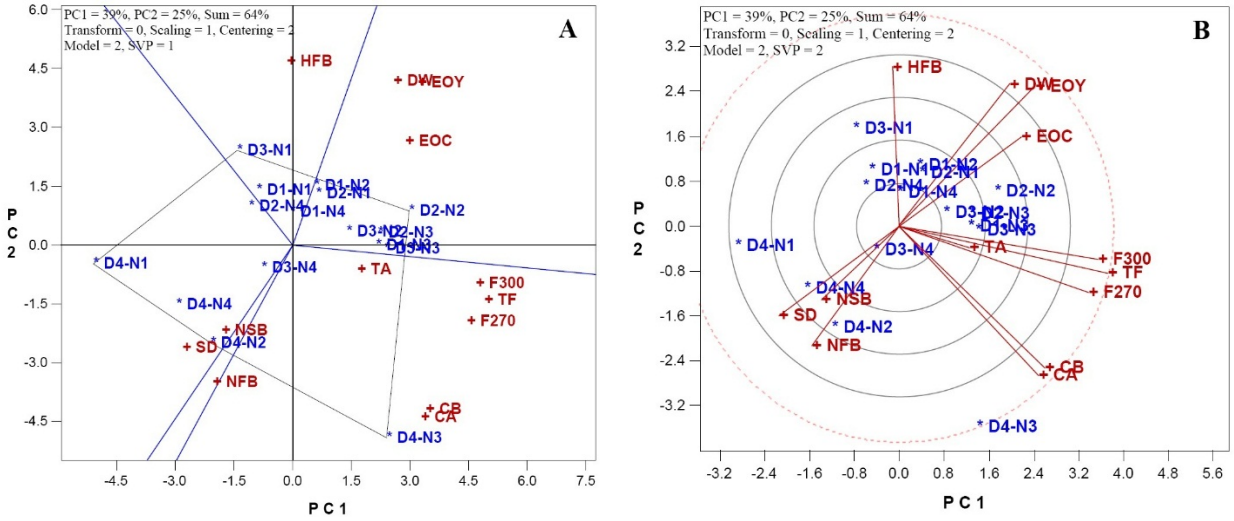


**Figure 2:** (A) Polygon view of the genotype-by-trait (TT) biplot on traits of the Dragonhead showing the which-won-where pattern and (B) Vector view of the TT biplot showing the interrelationships among all traits.

In the context of treatment-by-trait analysis, an ideal treatment has been defined as the treatment that combines several good traits in its property and it should possess the highest mean performance across traits (i.e., longest projection onto the average tester coordinate (ATC) axis and shortest entry-vector, thus, it should be close to the ideal treatment represented by the innermost concentric circle with an arrow pointing to it (Yan and Kang, 2003). Such ideal treatment can, therefore, be used as a reference check in subsequent trials where the set of traits will be measured, therefore, according to the biplot displayed in Fig. 1A, the single-arrow line that passes through the biplot origin is referred to as ATC abscissa, and on this line is ranked the treatments in terms of their performance. The ATC ordinate divides the ATC abscissa into two at the middle (Yan et al., 2001), and the portion of the ATC towards the right displays the above average treatments and towards the left shows those treatments below average. Based on this biplot (Fig. 3A), the treatment combinations that performed above average were D1-N2, D1-N3,

D2-N1, D2-N2, D2-N3, D3-N2, D3-N3, and D4-N3, while the other treatment combinations (D1-N1, D1-N4, D2-N4, D3-N1, D3-N4, D4-N1, D4-N2 and) performed below average in terms of measured parameters (Fig. 3A). It seems that moderate or small sowing densities (10, 15 and 20 cm) and moderate iron nano-fertilizer treatments (1 and 2 g $l^{-1}$) had the most favorable impact on the measured traits of the dragonhead. In other word, high concentration of iron nano-fertilizer as well as wide sowing spaces had negative effect on dragonhead. Five treatment combinations (D1-N3, D2-N2, D2-N3, D3-N2 and D3-N3) were the closest to the position of an ideal treatment which is ranked the highest in term of traits' performance because it is desirable in terms of most of the traits (Fig. 3A).

In the context of TT biplot analysis, an ideal tester of trait has been defined as the tester that combines several good treatments in its composition (Baljani et al., 2015) and according to Fig. 3B, the ideal trait was strongly related to flavonoid related traits (F270, F300 and TF) followed by TA, CA and CB traits. Flavonoids have been reported in the flowering parts of dragonhead and its extracts could be due to phenolic compounds that are present in this plant and it is well established that flavonoids are remarkable for their antioxidant activities (Povilaityee and Venskutonis, 2000).

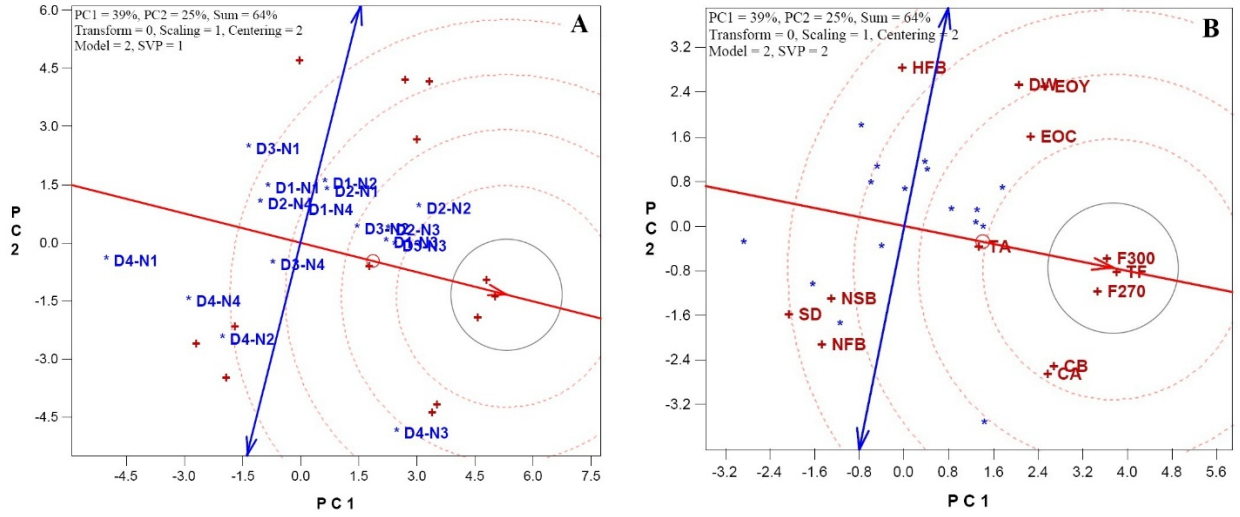


**Figure 3:** (A) Ideal entry view of TT biplot, showing the relationships of different treatment combinations with ideal entry and (B) ideal tester view of the TT biplot, showing the relationships of different traits with ideal tester.

Due to large importance of yield performance in dragonhead production, this trait was compared visually under the studied treatments and displayed in Fig. 4A and in the context of TT biplot, the best treatment combination for obtaining of dry mass (DM) could be find. A vector-view function shows treatments that have close association with a target trait among other traits and according to this biplot of (Fig. 4A), D2-N2 (15 cm planting density with 1 g $l^{-1}$ iron plus nano-fertilizer) treatment was the best treatment suitable for obtaining of high DM. Thus, application of this treatment combination is expected to lead to improved target trait (DM) under studied growing conditions. Similarly, due to importance of essential oil yield (EOY), it was compared in the studied via above biplot tool and declared that the best treatment combination for obtaining of high magnitudes of EOY is D2-N2 (15 cm planting density with 1 g $l^{-1}$ iron plus nano-fertilizer) as the best treatment (Fig. 4B). This suggests that using iron nano-sized micronutrient fertilizer plus 15 cm sowing space will not only result in the development of high dry mass of the dragonhead but also causes to obtain the other desirable traits like essential oil yield. Amirnia et al. (2014), have emphasized the positive effects of iron micronutrient nano-fertilizer on saffron production. Several investigators mentioned similar results on different crops such as El-Desuki et al. (2001) on sweet fennel, Khalil and El-Sherbeny (2003) on mint, and Hussein et al. (2006) on dragonhead, who observed that application of different macro and micro-nutrients significantly improved plant growth characters. This result may

be due to effect of iron nano-fertilizer on accelerating metabolism reactions as well as stimulating enzymes. This increment may be due to the effect of iron nano-fertilizer on mass production or/and oil content. Concerning the effect of plant sowing density on essential oil of dragonhead, wider spaces offer ample quantity of nutrients, light and other environmental factors which in turn was reflected on the high amounts of morphological traits and essential oil content.

For all 38, or 25.5% of regenerants that were transferred to the non-selective medium, molecular Results of present investigation indicate that the polygon-view as well as vector-view of TT biplot are the best tools for visualizing the interaction pattern among treatments and traits. One of the most important applications of nanotechnology in agriculture is using nano-fertilizers for plant nutrition while comparing it to the conventional application of fertilizers there is huge difference in the accuracy, smart nature, effectiveness, cost for operation, ease of construction and many others.

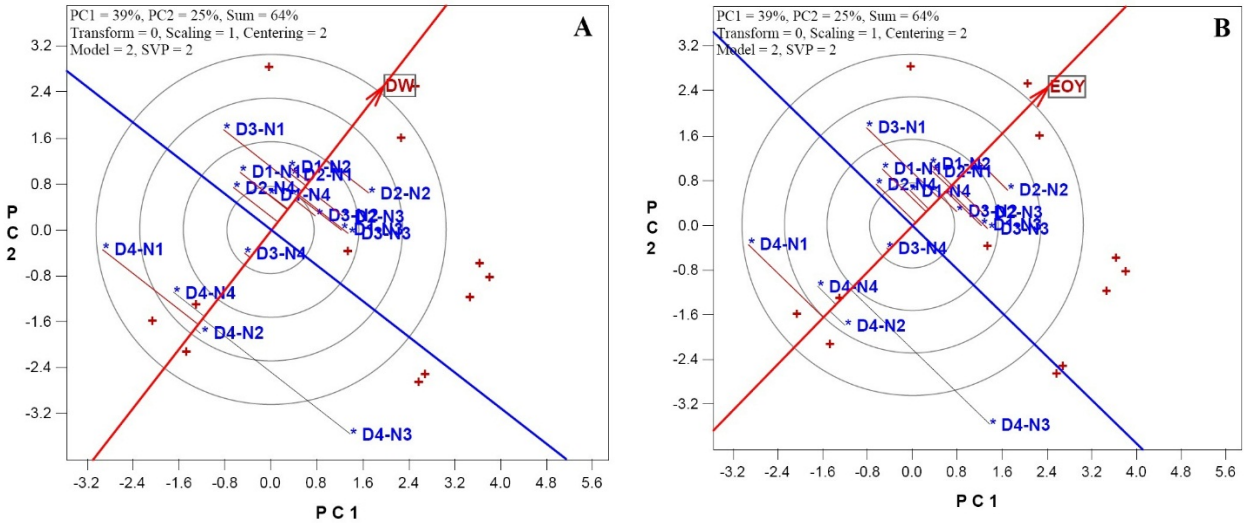


**Figure 4:** (A) Vector view of treatment by trait (TT) biplot, showing the relationships of different treatment combustions with target trait (DM, dry mass), and (A) Vector view of treatment by trait (TT) biplot, showing the relationships of different treatment combustions with target trait (EOY, essential oil yield).

## 4 CONCLUSIONS

This study indicated that application of 1 g $l^{-1}$ iron nano-fertilizer plus 15 cm planting space increase the dragonhead's dry mass yield and essential oil yield cultivated in semiarid region conditions. Also, treatment by trait (TT) biplot is an excellent statistical tool for interpreting the interaction pattern of agriculture studies.

## 5 ACKNOWLEDGEMENT

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**Agrovoc descriptors:** Ghana, farms, mechanization, farm equipment, rice, plant production, input-output analysis, productivity, farm structure, agricultural structure

**Agris category code**: f01, e14,e16

# Effect of mechanisation use intensity on the productivity of rice farms in southern Ghana

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**ABSTRACT**

This study analysed the effect of farm mechanisation on productivity of rice farms in southern Ghana. The empirical results of the stochastic frontier model of primary data solicited from 360 rice farmers in southern Ghana revealed that land size cultivated, agrochemical expenditure, tillage intensity, threshing intensity, education and transportation intensity were significant factors that positively influenced partial factor productivity with respect to mechanisation. On the other hand, reaping intensity, over use of fertilizers, and age of farmers negatively influenced partial factor productivity with respect to mechanisation. These results have implications for capacity building and government support to increase productivity on rice farms.

**Key words:** mechanisation, productivity, rice farms, southern Ghana, stochastic frontier model

**IZVLEČEK**

**UČINEK INTENZIVNOSTI UPORABE MEHANIZACIJE NA PRODUKTIVNOST PRIDELAVE RIŽA V JUŽNI GANI**

Raziskava analizira učinek uporabe mehanizacije na produktivnost pridelave riža na izbranih kmetijah v južni Gani. Rezultati modela stohastične analize mejne funkcije podatkov anketiranja 360 pridelovalcev riža v južni Gani so pokazali, da imajo na produktivnost v povezavi z mehanizacijo značilen pozitiven vpliv naslednji dejavniki: velikost obdelovalne površine, obseg uporabe zaščitnih sredstev in gnojil, intenzivnost obdelave zemljišča, intenzivnost mlačve, izobrazba in intenzivnost transporta. Po drugi strani so intenzivnost žetve, prekomerna uporaba gnojil in starost kmetovalcev negativno vplivali na produktivnost v povezavi z mehanizacijo. Dobljeni rezultati so pomembni za ustvarjanje dodatnih kompetenc kmetov in pri vladni podpori za povečanje pridelave na kmetijah riža.

**Ključne besede:** mehanizacija, produktivnost, riževe farme, južna Gana, stohastični model analize mejne funkcije

## 1 INTRODUCTION

Rice has been consumed in Ghana for a long time. Rice consumption in Ghana is dated far back in the 17th and 18th century. Before 1920, rice was grown mainly by women in Western region and the Volta region, and was used for performing rituals during festivals. In terms of being a major staple in the Ghanaian diet, it gained prominence since 1960. Over the past years, the per capita consumption of rice has increased steadily to 24 kg in 2010. It is expected to hit 63 kg by 2018 due to rapid

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population growth, urbanization and change in consumer habits (Ministry of Food and Agriculture, 2009). Annual rice production fluctuation ranges from 130,000 to 182,000 metric tons, the difference largely due to area cultivated per year. In Ghana, rice production systems are grouped into three major environments in accordance with the International Rice Research Institute: irrigated rice, rain fed low land rice and upland rice. Irrigated rice is grown on fully irrigated land in the form of flood in all seasons or partially irrigated during the wet season. These are usually capital intensive schemes developed by government. They are usually found on heavy clay soils like the vertisol which can hold surface water for a long time. Currently, cropping intensity for this system is two crops per year. Controlled flooding system is the practice on most government irrigation schemes and private commercial rice farms. High yielding exotic aromatic varieties such as 'Togo Marshall', 'Jasmine' and 'Get Three' are the common varieties grown. Rain fed lowland rice is usually at the valley bottoms with bunded fields. Such fields may have farmwater conservation facilities that traps run off whenever it rains. It may have two crops per year but mostly one crop per year. Rice output from these fields are sold primarily to households and also consumed during festivals, very little output is sold in the general open market. This system is common in the Northern Region of Ghana where most of the red rice is produced. Upland rice is grown on soils with good natural drainage on unbunded fields. Production depends on sufficient continuous natural rain during the wet season. This system has only one crop per year. It is prominent in forest zone, mountainous areas as well as on flatlands. It is practised in the Western Region and Northern parts of the Volta Region. Rice production and development on commercial basis received a major boost of government subsidy in the 1970s and 1980s. However, much of the anticipated results did not materialize due to political conditions, for example, the partial abolishing of controlled prices and the removal of subsidies during adoption of the Structural Adjustment Programme (SAP) in 1983 to revive Ghana's Economy (Kranjack-Berisavljevic et al., 2003).

One major way to improve yearly output of rice given the climatic conditions in Ghana is to increase cropping intensity. With available constant irrigation for 365 days per year and a 120 days variety, it is possible to increase crop intensity from two crops per year to five crops within two years, making it consistent with fully irrigated areas in Thailand (Tinsley, 2009). Increasing the level of mechanization is the key to reduce crop conversion time and thereby increasing crop intensity. The term "mechanisation" in agriculture is used to describe tools, implements and machinery applied to improving the productivity of farm labour and of land; it may use either human, animal or motorized power, or a combination of these (Sims & Kienzle, 2006). In practice, it involves the provision and use of all form of mechanical assistance for agricultural production. Agricultural mechanisation leads to reduction in drudgery, increased input usage as result of increased cropping intensity, expansion in area cultivated as a result of higher labour productivity, efficient utilization of inputs, and timeliness of operations leading to higher productivity and income, and improved livelihood (Sims & Kienzle, 2006).

Available data on rice productivity in Ghana revealed that between 2002 and 2010 average yield of rice was 1.7-2.4 Mt/Ha compared with achievable yield of 6.5Mt/Ha (Ministry of Food and Agriculture, 2011). The objectives of this study were twofold. First, the study determined intensity of mechanisation on rice farms. Second, the study estimated the effect of mechanisation intensity on productivity of rice farms.

## 2 MATERIALS AND METHODS

### 2.1 The study area, sampling and data

The study covered two major rice growing districts in Southern Ghana; communities in and around Asutsuare in the Shai-Osudoku District in the Greater Accra Region, and communities in and around Weta in the Ketu North District in the Volta Region of Ghana. Asutsuare has about 2,786 hectares of land under cultivation while Weta has about 880 hectares of land under rice cultivation.

These districts were selected because government through the Irrigations Development Authority (IDA) has made substantial investment in developing irrigation infrastructure in these areas for rice production. Based on the farmer population in the respective districts, 254 farmers from Asutsuare area and 106 farmers from Weta area were interviewed, to give a total sample size of 360 farmers. The rice growing communities were selected purposively and respondents were randomly selected within each community. Cross-sectional data for the 2012 major season at farm level were solicited from rice farmers using structured questionnaires.

### 2.2 Sources of mechanisation services accessed by farmers

Farmers were asked to identify sources of mechanisation whether farmer owned, private service providers, or government mechanisation centers. Descriptive statistics was used to describe the sources of access to mechanisation.

### 2.3 Level of mechanisation accessed by farmers

All the possible production activities from land preparation through to harvesting were listed and presented to the farmer. The procedure used by (Ghosh, 2010) was adopted for the analysis. An index based on farmers ownership pattern and use of modern rice cultivating implements like power tiller, planters, sprayers, and harvesters, for respective activities was used. The farmers' response to the use of these implements was coded into scores, 1 for ownership or hired usage of implement for an activity, and 0 otherwise. The total score calculated ranged from 0 % to 100 % depending on the number of production activities for which mechanisation was used in 2012 major season of production. If a farmer had a total calculated score of 50 % and above then it means half or more of his/her production activities was based on mechanisation and a value of 1 is assigned to the farmer and 0 otherwise. Descriptive statistics was used to describe the levels observed.

### 2.4 Determinants of mechanisation access and intensity on rice farms

In addressing this objective, first an index of mechanisation was calculated for each farmer. The simple proportion as used by Owombo et al., (2012) for measuring adoption index was used. The mechanisation index ($I_m$) is given as:

$$I_m = \frac{nA_m}{TA} \quad (1)$$

Where $nA_m$ is number of activities mechanized; $TA$ is total number of activities to be mechanized on the farm.

Taking into account varying number of plots and their sizes for each farmer, $I_m$ can further be specified as:

$$I_m = \frac{nP_m}{TP} \times \frac{nA_m}{TA} \times \frac{Z_m}{TZ} \quad (2)$$

Where: $I_m$ is mechanisation intensity index; $nP_m$ is number of plots mechanized by a farmer; $TP$ is total number of plots; $nA_m$ is number of activities mechanized on each plot; $TA$ is total number of activities on the farm; $Z_m$ is size of plot mechanized (each plot); $TZ$ is total size of plots (overall).

Since farmer's access to farm mechanisation could be censored, double hurdle model was employed to examine determinants of mechanisation access and intensity (Wooldridge, 2002).

### 2.5 Effect of mechanisation intensity on the productivity of rice farms

Productivity can be explained as the amount of output that can be produced with a given amount of input over time (Perloff, 2004). Productivity may be measured in terms of single input (Single Factor Productivity or Partial Factor Productivity) or in terms of multiple inputs (Multiple Factor or Total Factor Productivity (Mbam & Edeh, 2011). This study adopts the partial factor productivity approach to measure productivity of rice farms. According to Tinsley (2009), Ghana has a hot humid tropical climate with just about 12 hours of sunshine, but with warm night temperatures that promote respiration losses. Therefore, a frontier yield of 6-7 metric tonnes per hectare is optimum for rice production in Ghana.

Partial factor productivity contributes to total factor productivity (Greg & Greene, 2007). Also, improved partial factor productivity leads to higher

aggregate output and at constant prices translate into higher income for farmers and improved livelihoods. These imply that indicators of partial factor productivity provide a useful insight into the success of policy reforms.

### 2.5.1 Empirical specification of the stochastic frontier model

The stochastic frontier model for estimating farm level technical efficiency was applied but with a particular focus on the contribution of intensity of mechanisation to rice productivity.

Kumbhakar (2002), Hang & Liu (1994) and Reifscheider & Stevenson (1991) have suggested stochastic production models capable of estimating the stochastic production function and the inefficiency function at the same time while using cross-sectional data. Battese & Coelli (1995) proposed a model that is similar to Hang and Liu but specified for panel data. In recent empirical studies, the model formulated by Battese & Coelli (1995) is specified in the cross-sectional analysis.

Following Obi & Chiasngo (2011) the functional form used in this paper is the Cobb-Douglas specification because it is flexible and very convenient for estimating technical efficiency. In this paper, mechanisation is represented by capital. The generalized Cobb-Douglas model is given as:

$$Q = AL^{\alpha}K^{\beta} \tag{3}$$

Where Q is output; $L$ is labour; K is capital; $A$, $\alpha$, $\beta$ are constants. Land and capital could be interchanged and Q will be unaffected. Alternatively, the model can be stated as:

$$P(L,K) = bL^{\alpha}K^{\beta} \tag{4}$$

Where $P$ denotes total production (monetary value of all output produced within a period); $L$ denotes labor input (total man-hours within a period quantified in monetary terms); $K$ denotes capital input (monetary value of machinery, equipment, and buildings); $b$ denotes productivity (total factor or partial factor); the terms $\alpha$ and $\beta$ are the output elasticities of labor and capital, respectively. These values are constants determined by intensity of mechanisation. The output elasticities $\alpha$ and $\beta$ measure the degree of responsiveness of output to a change in levels of either labor or capital used in production, ceteris paribus.

The stochastic frontier function is specified in equation (5) as follows:

$$LnY_i = Lnf(x_i;\beta) + \exp(v_i - u_i) \tag{5}$$

$Y_i$ is the production (output) of the $i^{th}$ farmer; $X$ is a vector of input quantities from the $i^{th}$ farmer; $\beta$ is a vector of parameters to be estimated; $V_i$-$U_i$ constitutes the disturbance (error) term. Given the above, the next step is to estimate the partial factor productivity due to mechanisation. Partial Factor Productivity is the ratio of output to a single input. The logarithmic Cobb Douglas specification of the partial factor productivity ($PFP_M$) due to mechanisation is specified as:

$$\begin{aligned}\ln PFM_i = {} & \beta_0 + \beta_1 \ln Till_i + \beta_3 \ln Irrig + \beta_4 \ln Reap_i + \beta_5 \ln Thresh_i + \beta_5 \ln Transp + \\ & \beta_6 \ln Winow_i + \beta_7 \ln Land_i + \beta_8 Lab_i + \beta_9 \ln Seed_i + \beta_{10} \ln Fert_i + \beta_{11} \ln Agrochem_i + \\ & \beta_{12} \ln Creditam_i + \beta_{13} \ln NFincome_i + \beta_{14} \ln Extcont_i + \beta_{15} \ln Age_i + \beta_{16} \ln Exp_i + \\ & \beta_{17} Gend_i + \beta_{18} \ln Edu_i + \beta_{19} Loc_i + (V_i - U_i)\end{aligned} \tag{6}$$

Where $PFP_i$ is Partial factor productivity (value of output/value of mechanisation input) in 2012 major season, $\beta_i$'s are elasticities, $i$ refer to the $i^{th}$ farmer and $Ln$ is the natural logarithm operator; $U_i$ is errors due to farmer inefficiency and $V_i$ are errors due to factors outside farmers control. It is worthy to note that the gender and location variables were not log transformed because they were dummy variables. This is a hybrid form of the log-linear transformation of the Cobb Douglas production function.

### 2.5.2 Description of variables for the stochastic frontier model

The descriptions, measurements and hypothesized relationships of the independent variables with the dependent variable (i.e. partial factor productivity) are presented in Table 1.

**Table 1:** Description of variables, measurements and hypothesized relationships for the stochastic frontier model

| Variable | Description | Measurement | Hypothesized relationship |
|---|---|---|---|
| **Dependent** | | | |
| $PFP_M$ | Value of output/value of mechanisation used | Ghana Cedis (GHS) | |
| **Independent** | | | |
| Till | Tillage | Intensity Index | + |
| Irrig | Irrigation | Intensity Index | + |
| Reap | Reaping (harvesting) | Intensity Index | + |
| Transp | Transportation | Intensity Index | + |
| Winow | Winnowing | Intensity index | + |
| Land | Cultivated land area | Hectares | + |
| Lab | Labour expenditure | GHS | + |
| Seed | Improved seed expenditure | GHS | + |
| Fert | Fertilizer expenditure | GHS | + |
| Agro | Agro chemicals expenditure | GHS | + |
| Credit | Amount of credit | GHS | + |
| NF income | Non-farm income | GHS | + |
| Ext | Extension visit per year | Number of contacts | + |
| Age | Age of farmer | Completed Years | + |
| Gend | Gender of farmer | Dummy (Male =1 , Female =0) | +/- |
| Educ | Formal education | Years | + |
| Exp | Years in rice farming | Years | + |
| Loc | Location | Dummy (Asutsuare =1, otherwise =0) | +/- |

**Tillage intensity index:** This variable indicates the proportion of total land size cultivated that was tilled using either tractor, power tiller or both. It is a continuous variable. It is hypothesized that increased tillage intensity results in higher productivity (Nandal & Rai, 1986; Tinsley, 2009).

**Irrigation intensity index:** This variable indicates proportion of cultivated land that was irrigated because irrigation is a mechanisation process (Ghosh, 2010). It is a continuous variable. It is hypothesized that the size of land irrigated contributes positively to physical output of yield and hence productivity. Water is retained within root zones of plant on irrigated farms. This enables plant to utilize the water in time of need. Irrigation intensity improves cropping intensity and contributes positively to the productivity of available land (Reardon et al., 1996; Bhattarai et al., 2002).

**Reaping (harvesting) intensity index**: This is variable indicates the proportion of rice farm that was harvested using motorized rice reaper (cutter) or combined harvester. Under good soil conditions using machines to harvest rice reduces drudgery, improves efficiency of labour, reduce harvesting wastage, and hence, positively influences rice productivity (Mahrouf & Rafeek, 2003; Tinsely, 2009).

**Threshing intensity index**: This variable represents the proportion of the rice farms that was threshed using combine thresher or stationary motor powered thresher, just as mechanized reaping, using machine to thresh harvested paddy contributes positively to productivity (Mahrouf & Rafeek, 2003; Tinsely, 2009).

**Transportation intensity index**: This variable measures the proportion of the cultivated farm produce that was transported using automobile vehicle for example, tractor, power tiller, or any motor powered truck. The mode of transporting the harvested produce from the field to the drying floor could cause wastage of the paddy, or delays on the field which may cause paddy to deteriorate. It is hypothesized that increase intensity of

transportation is positively related to the quality and quantity of rice output and hence, productivity.

**Winnowing intensity**: This variable measures the extent to which motorized equipment is used to clean the harvested rice paddy on the drying floor. It is a continuous variable and it is hypothesized that increased intensity of winnowing will influence productivity of rice positively.

**Land**: This variable indicates the size of farm land cultivated in hectares. Larger land sizes may improve rice yield per hectare and hence, productivity of the land is effectively utilized (Binswinger 1978ab; Bagyo & Lingard, 1983; Foster & Rosenzweig, 2011).

**Labour**: This variable indicates expenditure on labour used (GHS). Expenditure on labour influences productivity with respect to mechanisation. Studies indicate that increases in intensity of mechanisation is associated with increase in labour expenditure due to larger area cultivated and also employment of skilled labour to operate the machinery (Balishter & Singh, 1991; Verma, 2008).

**Fertilizer expenditure**: This variable represents fertilizer use intensity. It is established that the use of fertilize is positively related to productivity (Reardon *et al.,* 1996). It is expected that fertilizer usage to the optimum recommended level is positively related to productivity and vice versa.

**Amount of institutional credit**: Access to institutional credit enables the farmer to purchase mechanisation equipment with ease or afford available mechanized services which translate into higher productivity. Nakano & Kajisa (2011) suggest that access to credit improves timely purchase of fertilizer and agrochemical hence, positively related to productivity. It is therefore intuitive that increase in the amount of credit received will increase productivity.

**Nonfarm income**: This variable represents the amount of non-farm income received by the farmer during the season under study. Mechanisation equipment is capital intensive and requires substantial cash resources. Reardon et al*., (*1996) suggest that non-farm income contributes positively to acquisition of farm machinery and positively relates to productivity of farms.

**Improved Seed Expenditure**: This variable indicates hybrid seeds or improved variety and the amount of expenditure made on improved seed. It is hypothesized that farmers who use adequate quantities of improved varieties are more likely to achieve higher levels of productivity. The optimum quantity of seed for transplanting and broadcasting is 75 kg per hectare and 100 kg per hectare, respectively. Inadequate seed input is less likely to achieve higher productivity (Obi & Chisango, 2011).

**Total physical output of rice**: At the same input level and land size, the higher the physical output the higher the productivity and the income, all things being equal.

**Age**: This variable measures age of household head in years. Older farmers may have enough wealth due to longer periods of saving and better network. These enable easy access to resources compared to younger farmers. It is hypothesized that age is positively correlated to machinery access, and higher productivity (Mushunje et al., 2003).

**Chemicals Expenditure**: This variable measures the intensity of herbicides and pesticide usage (value of total amount spent in (GHS). It is hypothesized that farmers with efficient and controlled usage of herbicides and pesticides will attain higher yield. Agrochemicals are complimentary input and hence influence productivity positively (Nakano & Kajisa, 2011).

**Number of contacts with extension agent**: This is measured by frequency of contacts made with extension agent either by visitation or during training sections. Frequent contact with extension agents may provide the farmer with information on new technologies for farming and how to access the technologies. It is hypothesized that higher number of contacts with the extension agents will correlate positively with access to improved technology which will translate into higher productivity.

**Gender of farmer**: This variable indicates the sex of the respondent. This is a dummy variable

(1 = Male, 0 = Female). Gender could be positively or negatively related to productivity.

**Education level of farmer**: This is measured by the number of years in formal education. It is expected that higher years of formal education will positively correlate with mechanisation and hence productivity.

**Experience of farmer**: Number of years in farming could influence farmers to employ more labour saving technologies in carrying out activities on the farm to improve productivity.

**Location**: This variable indicates the location of the farmer. It is represented by a dummy (1 = Asutsuare, and 0 otherwise). There could be differences in productivity due to geographical location as a result of soil characteristics or precipitation. This may influence productivity positively or negatively.

## 3 RESULTS AND DISCUSSION

### 3.1 Level of mechanisation use intensity by farmers in the production cycle

Eleven activities or operations in the rice paddy production process required mechanisation. The number of activities mechanised indicates the operations for which motorised equipment was used. The level of mechanisation achieved indicates the proportion of activities mechanised. The minimum number of activities mechanised was 1 (one); with a corresponding level of mechanisation achieved being 9.0 %. About 4.4 % of farmers achieved the minimum level of mechanisation. All the respondents were able to access machinery to mechanise at least one activity in the paddy production process. The maximum number of activities mechanised was six (6), with a corresponding level of mechanisation being 55 %. About 26.4 % of respondents achieved 55 % mechanisation in the rice production process. A relatively higher proportion of respondents (47.2 %) mechanised four activities in the paddy production process representing 36.0 % level of mechanisation. It appeared that this level was considerably low to cause appreciable level of increased productivity.

### 3.2 Effect of mechanisation intensity on the productivity of rice

The empirical results of partial factor productivity of rice farms with respect to mechanisation intensity are presented in Table 2. Since both side of the partial factor productivity equation are logged, the results could be discussed in terms of percentages (elasticities). The results of the stochastic frontier estimates indicated that tillage intensity, threshing intensity, transportation intensity, land size cultivated, agrochemical expenditure, and gender were positively related to partial factor productivity of rice with respect to mechanization. In this regard, land size had the greatest positive influence on rice productivity, followed by tillage intensity, transportation intensity, threshing intensity, agrochemical usage and experience.

**Table 2:** The effect of activity specific mechanisation intensity on partial factor productivity

| **Stochastic frontier normal/exponential model** | | | |
|---|---|---|---|
| **Ln_PFP** | **Coef.** | **Std. Err.** | **P>z** |
| LnTil | .3212*** | .0581 | 0.000 |
| LnIrr | -.0346 | .0237 | 0.146 |
| LnReap | -.1204** | .0480 | 0.012 |
| LnThresh | .0934** | .0487 | 0.055 |
| LnTrans | .1836*** | .0472 | 0.000 |
| LnWinow | .0126 | .0284 | 0.656 |
| LnLand | .3757*** | .0900 | 0.000 |
| LnLab | .0073 | .0466 | 0.874 |
| Lnseed | -.0088 | .0407 | 0.827 |
| Lnfert | -.1834*** | .0616 | 0.003 |
| LnAgrochem | .0822** | .0363 | 0.024 |
| Credit amt. | -.0000 | .0001 | 0.578 |
| NF Income | -.0002 | .0001 | 0.112 |
| Ext contact | -.1936 | .1294 | 0.135 |
| Age | .05180*** | .0185 | 0.005 |
| Experience | -.0081 | .0209 | 0.698 |
| Gender | -.2892 | .3554 | 0.416 |
| Edu (yrs) | -.0774** | .0410 | 0.059 |
| Loc | .1485 | .1189 | 0.212 |
| cons | -3.6689 | .9229 | 0.000 |
| Observations (N) | 360 | | |
| Prob>chi Squared | 0.0000 | | |
| Log likelihood | -142.5860 | | |
| Wald chi Squared | 77.40 | | |

Significant denoted as *** (1 %), ** (5 %) and *(10 %)
Source: Authors' computation from field data, 2012

Tillage Intensity: The coefficient of tillage intensity was positive and significant at 1 percent significance level, contributing to productivity with respect to mechanisation. An increase in the intensity index of tillage will cause productivity with respect to mechanisation to increase by 32 percent and vice versa. Mechanized tillage is labor-saving (Binswinger, 1978ab) and promotes cultivation of larger land sizes. Seedling root establishment is related to how well the soil is cultivated and this increases productivity (Tinsley, 2009).

Reaping (Harvesting) intensity: This variable was significant at 5 percent but negatively contributes to productivity. An increase in reaping (harvesting) intensity index will cause partial factor productivity with respect to mechanisation to decrease by 12 percent. The result of reaping intensity is contrary to the expected hypothesized relationship. This could be attributed to the following reasons: First, the rice reaping machines (harvesters) operate well under specific conditions. It could be that the harvesters being used were not operating well because of suboptimal field conditions. Field conditions such as soil moisture condition, weed population, plot size, maturity of rice, crop density, lodging and operator skills affect performance of harvesting (Mahrouf & Rafeek, 2003). Low performance levels of harvesting machinery as a result of suboptimal field conditions could result in low physical output of paddy rice, hence, translating into a low productivity with respect mechanisation. Secondly, the entry of rice harvesting machinery unto the irrigated rice farm depends on the wet and sticky or dry nature of the soils (Mahrouf & Rafeek, 2003). This is influenced by the predominant soil series on a particular rice farm. Soils which are light and less sticky (loamy) with relative amount of sand are quick to dry and are compatible with rice harvesting machinery better than heavy soils

(vertisol). For example, the Akuse series (vertisol), records higher physical output of paddy than the Amo series (loam). Therefore, it is possible that the soils on the farms on which mechanised reaping of rice was done have low productivity of rice due to their physical, chemical and biochemical properties.

Threshing intensity index: This variable contributes positively to productivity of rice farms and was significant at 10 percent. An increase in the intensity index of threshing will lead to an increase in the productivity of rice farm with respect to mechanisation and vice versa.

Transportation intensity index: This variable was significant at 1 percent and positively related to the productivity of rice farms.

Land size cultivated was significant at 1 percent and positively contributes to partial factor productivity of rice farms with respect to mechanisation. This is due to the fact that as cultivated land area increases, higher demand is made on the amount of labour used, hence, the need to replace labour with machinery, and this increases productivity. This finding is consistent with land size cultivated having the greatest influence on yield (Bagyo & Lingard 1983); and farm mechanisation is being most profitable where land is relatively abundant (Binswinger 1978ab).

The expenditure on fertilizer purchased was significant at 1 percent but contributes negatively to the productivity of rice farms. This result could be due to the fact that farmers are applying quantities of fertilizer above the recommended quantities on their fields. This is negatively affecting the output of rice. Gebrekidan & Seyoum (2006) revealed that overdose application of fertilizer on rice in flooded vertisols has a negative effect on rice yield.

Expenditure on agrochemical used was significant at 5 percent and contributes positively to the productivity of rice farms. A cedi increase in agrochemical expenditure will cause productivity due to mechanisation to increase by 8 percent. This could be explained by the fact that agrochemical usage complements cultivation of larger farm sizes and promotes substitution of labour with machinery, which could translate to higher productivity. The usage of agrochemical is a component of mechanisation in total (Obi & Chisango, 2011).

selective medium. Out of them, 9 or 5.4 % grew successfully, while 5 or 3 % failed (Tables 3 and 4). The age of farmers was significant at 1 % percent. Age variable contributes to inefficiency of farmers with respect to mechanisation.

Number of years of education was significant at 5 percent and negatively influences inefficiency of mechanisation in rice farms. This indicates that increased number of years in formal education reduced farmer's inefficiency, and therefore, contributes positively to productivity. This could be attributed to the fact that as farmers spend more years on formal education they turn to adopt technologies that in turn reduce inefficiency. This result is somewhat consistent with Corner-Thomas et al. (2015) that education of the farmer positively influences the use farm management tools that results in increase productivity.

The results of the study revealed that the major source of machinery service provision was private service providers for all activities that used mechanization except in the case of irrigation where government is the major service provider. This implies that rice farmers rely on private service providers for their mechanisation service on the farm because mechanisation services from government is either unavailable or inadequate. However, these private service providers are not necessarily those supported under public private partnerships. Furthermore, results revealed that government is the dominant service provider for irrigation on the rice farms. This means that prospective rice farmers who are interested in cultivating irrigated rice would have to rely on government for infrastructural support and service.

The findings suggested that farms with higher intensity of mechanisation had increased productivity compared to farms with low intensity of mechanisation.

Farmers' decision to mechanise operations on rice farms is limited by unavailability of machinery for mechanising some specific activities. Farms with available machinery service centre in their communities are more likely to mechanise

activities on the rice farms because of greater access. This means that developing the capacity of machinery service providers to establish service centers within relatively shorter distances to farms will improve mechanisation of activities on the rice farms and hence increase productivity.

The higher the access to credit, non-farm income and returns from rice farms, the more activities that will be mechanised on the rice farms. Therefore attempts to improve credit access, increase nonfarm income or returns from rice will raise rice productivity.

Land owners are less likely to intensify mechanisation use because of relatively smaller land holdings. Therefore policies aimed at developing capacity of farmers to enable farmers own larger lands and farms will promote mechanised farming and increased productivity of rice.

Increased usage of agrochemical and increased investment in skilled labour compliment mechanisation and cultivation of larger areas. These contribute to higher access to mechanised farming, increased mechanisation intensity and higher productivity of rice farms.

Planting improved seed quantities as well as applying fertilizer quantities above the recommended rate reduces productivity with respect to mechanisation. Therefore, it is important that farmers are educated on the need to avoid excessive sowing of seeds and misapplication of fertilizers.

Farmers who cultivate larger land size have greater access to mechanisation and higher productivity of rice. This implies that policies targeted to support land consolidation and development in order to expand farms cultivated will increase productivity of rice.

Farmers who intensify their use of machinery for land preparation are more likely to achieve higher productivity than farmers who do not mechanize tillage. This means that policies aimed at developing farmers' capacity in order to intensify mechanized tillage will improve rice productivity.

The results also indicated that farmers who mechanized threshing and transportation of paddy rice are more productive with respect to mechanisation than those who do manual threshing and transportation.

To ensure that mechanised reaping (harvesting) of rice improves productivity, the crops must not be logged and the soil must possess good easily drying characteristics to enable access of the machinery unto the field. This implies that soil management practices that promote productivity of soil and optimum environment for reaping machines to work will improve rice productivity.

Experienced farmers turn to intensify mechanisation to a higher extent as compared to less experienced farmers. In other words, experienced farmers are more productive in the use of mechanisation than less experienced farmers.

Rice farmers with smaller household size were more likely to intensify the use of mechanisation and hence, were more productive with respect to mechanisation as compared to farmers with bigger household sizes.

Male farmers and younger farmers have a higher chance of accessing machinery and hence were more productive with respect to mechanisation.

Reaping (harvesting) intensity is negatively related to partial factor productivity of rice with respect to mechanisation. However, this could largely be attributed to the fact that some reaping machines do not operate well in small, wet, weedy fields, muddy fields or when crops are logged. Also, it could be partly due to variations in rice yields as a result of soil series variations in the study area. For instance, the Akuse series (the soil series in Shai-Osudoku area) is observed to contribute to higher yields of rice compared to the Amo series (the soil series in Ketu North District). However, mechanisation is more difficult on the Akuse series compared to the Amo series.

## 4 CONCLUSIONS AND IMPLICATIONS

First, the study has implications for capacity building. The number of years of education negatively influences inefficiency of mechanisation in rice farms. This indicates that increased number of years of formal education reduced farmer's inefficiency, and therefore, contributes positively to productivity. This could be attributed to the fact that as farmers spend more years on formal education they turn to adopt technologies that in turn reduce inefficiency. The Agricultural Extension Agents could educate the farmers on optimum quantities of mechanized services to utilise as well as improved seed and fertilizers rate per hectare to increase productivity. Also, increasing agrochemical usage and investment in skilled labour positively influenced rice productivity. It is recommended that farmers should explore application of agrochemical and more use of skilled labour to expand area cultivated. This will improve productivity of rice farms with respect to mechanisation. Furthermore, farmers should be educated to practice good management of soils conditions to provide optimum crop environment for harvesters to work; this will ensure that increased usage of reaping (harvesting) machines will increase the productivity of rice farms.

Second, the results have implications for government support in order for the rice farmers to increase productivity. Thus, to increase rice productivity farmers should increase the use of machines for tillage, threshing and transportation of paddy in order to increase rice productivity. Government can facilitate this process by proposing ways by which the farmers can acquire power tillers, cutters, threshers, and tractors for farming. The results suggest that private service providers dominate the delivery of mechanisation service to rice farmers. It is imperative that the capacity of private service providers be developed through government assistance to enable them acquire and set up mechanisation service centres that are close to the rice farms. This will make farm machinery and equipment available to enable easy access for rice farming. Further, it is critical that government continues to fast track the implementation of massive irrigation infrastructure development such as the proposed Accra plains development project which potentially will enable farmers a bigger access to irrigated land and modernized agriculture.

Finally, businessmen interested in investing in mechanized service provision should expand their services to activities such as transplanting, insect control, weed control, fertilizer application and drying. These will increase farmers' level of mechanisation for higher productivity. Rice farmers should avoid excessive use of seed quantities and fertilizers because these practices turn to reduce access to mechanisation (through probably spending their incomes unnecessarily on these inputs), thereby negatively influencing rice productivity. Credit institutions should consider the timely need of credit by farmers, in responding to providing credit to farmers. This will enable the farmers utilize the credit to serve its intended purpose in improving productivity. Rice income and non-farm income improved mechanisation intensity. Therefore, rice farmers should expand their alternative sources of income; this will promote intensification of mechanisation on rice farms for increased productivity.

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# Volatile phenols in wine: Control measures of *Brettanomyces/Dekkera* yeasts

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**ABSTRACT**

This review focuses on the considerable amount of research regarding volatile phenols production by *Brettanomyces* and on microbiological and technological parameters that influence development of these compounds during all stages of grape processing and winemaking. Also, volatile phenols impact on wine aroma and quality and prevention methods were discussed. The yeast genus *Brettanomyces* is the major microorganism that has the ability to convert hydroxycinnamic acids into significant concentration of phenolic compounds, especially of 4-ethylphenol and 4-ethylguaiacol, in red wine. When volatile phenols reach concentrations above the sensory threshold in wine, it is then characterized as wine with fault. In order to control the growth of *Brettanomyces* and preclude volatile phenols production, it is helpful to keep good quality of grape, winery sanitation, control of oxygen and sulphite level, as well as orderly check physiochemical composition of wine.

**Key words:** wine, volatile phenols, *Brettanomyces*, growth, factors, prevention

**IZVLEČEK**

**HLAPNI FENOLI V VINU: KONTROLNI UKREPI ZA KVASOVKE *Brettanomyces/Dekkera***

Ta pregled se osredotoča na znatno število raziskav, ki preučujejo tvorbo hlapnih fenolov s kvasovkami rodu *Brettanomyces*, ter na mikrobiološke in tehnološke parametre, ki vplivajo na sintezo tovrstnih fenolov v vseh fazah predelave grozdja in predelave vina. Prav tako je obravnavan tudi vpliv hlapnih fenolov na aromo in kakovosti vina ter preventivni ukrepi. Kvasovke rodu *Brettanomyces* so glavni mikroorganizmi, ki imajo sposobnost pretvorbe hidroksicimetnih kislin v značilne vsebnosti prisotnih hlapnih fenolov v rdečem vinu, predvsem 4-etilfenola in 4-etilgvajakola. Ko hlapni fenoli dosežejo koncentracije nad senzoričnim pragom zaznave za vino, je potem le-to spoznano kot vino z napako. Za nadzor rasti kvasovk rodu *Brettanomyces* in preprečevanje nastajanja hlapnih fenolov je koristno upoštevati kontrolo kakovosti grozdja ob trgatvi, higienske razmere v vinski kleti, kontrolo količine kisika in sulfita, ter redno kontrolo fizikalno-kemijske sestave vina.

**Ključne besede:** vino, hlapni fenoli, *Brettanomyces*, rast, dejavniki, preprečevanje

## 1 INTRODUCTION

Wine is a complex mixture of hundreds of compounds and most of them contribute to sensory characteristics of wine such as the colour, mouth-feel and aroma. There is a huge interest in wine aroma and numerous components are identified as playing a role in specific sensory notes. Flavour and aroma of wine are determined by many factors including grapevine variety, viticultural and winemaking practices, wine maturation and aging conditions. Besides, a wide range of microorganisms influence wine aroma. These microorganisms come into contact with wine

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during grape processing and wine production and their metabolic activities, synthetic and degrading enzymes impact wine aroma. Volatile phenols are important group of compounds that can be formed in wine and their elevated concentrations are associated with unpleasant smelling aroma often described as "phenolic", "leather", "horse sweat", "stable" or "varnish", etc. (Chatonnet et al., 1993; Chatonnet et al., 1992; Rodrigues et al., 2001). Within this group of compounds the most widely represented are 4-vinylphenol, 4-vinylguaiacol, 4-ethylphenol and 4-ethylguaiacol. The most unpleasant odoured are 4-vinylphenol (reminiscent of pharmaceuticals, gouache paint and 'Band Aids') and 4-ethylphenol (stables and sweaty saddles). Also, 4-vinylguaiacol (carnations) and 4-vinylguaiacol, (smoky, spicy aromas) are much less unpleasant, but however they are always associated with 4-vinylphenol and 4-ethylphenol, respectively. The olfactory impact of the two vinylphenols or ethyl-phenols should therefore be considered together, in the proportions in which they are present in the wine (Ribéreau-Gayon et al., 2000).

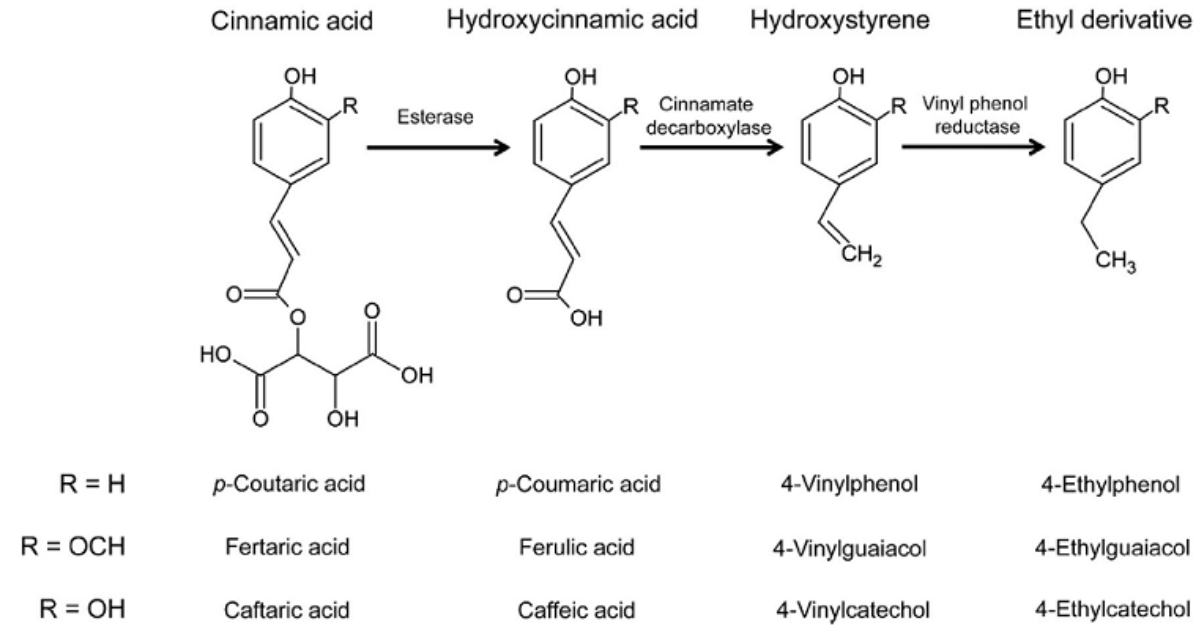
The origin of ethylphenols in wine aroma is due to different sources, but the most usual ways of formation are enzymatic processes of wine yeast and aging. Volatile phenols formation involves the sequential action of two enzymes on a hydroxycinnamic acid (ferulic, *p*-coumaric or caffeic acid) substrate. Hydroxycinnamate decarboxylase first turns these hydroxycinnamic acids into hydroxystyrenes (vinylphenols), which are then reduced to ethyl derivatives by vinylphenol reductase (Edlin et al., 1998; Dias et al., 2003). The enzyme that facilitates decarboxylation is present in a large number of bacteria, fungi, and yeasts, but it is shown that the reduction step is only performed by the species *Brettanomyces bruxellensis* Kufferath & von Laer (*Dekkera bruxellensis* van der Walt), *Dekkera anomala* Smith et van Grinsven, *Pichia guillermondii* Wickerham, *Candida versatilis* (Etchells & T.A. Bell) S.A. Mey. & Yarrow, *Candida halophila* Yarrow & S.A. Mey and *Candida mannitofaciens* (Onishi & Tom. Suzuki) S.A. Mey. & Yarrow (Chatonnet et al., 1995; Edlin et al., 1995; Chatonnet et al., 1997; Dias et al., 2003). Lactic acid bacteria may produce significate amounts of vinylphenols but produce only traces of ethylphenols under wine conditions (Chatonnet et al., 1995, 1997).The fermenting yeast *Saccharomyces cerevisiae* Meyen ex E.C. Hansen and other wine contaminants (e.g. *Pichia* sp., *Torulaspora* sp., *Zygosaccharomyces* sp.), may also produce 4-vinylphenol but are incapable of producing 4-ethylphenol (Chatonnet et al., 1993, 1995; Rodrigues et al., 2001). In *D. bruxellensis* the enzymes cinnamate decarboxylase and vinylphenol reductase are active under wine conditions and so these yeasts should be regarded as the off-flavour producers (Chatonnet et al., 1995, 1997). It is turned out that, 4-ethylguaiacol and 4-ethylphenol are formed in very small concentrations during malolactic fermentation by *Lactobacillus* able to transform phenolic acids in ethyl phenols (Baumes et al., 1986; Dubois, 1983). It has also been observed that ethyl phenols increase in wine during the aging and high levels were found in wine aged in used barrels (Chatonnet et al., 1992).

In this review we examine important group of compounds that influence wine aroma – volatile phenols, their origin in wine, emphasizing parameters that influence growth and activities of *Brettanomyces/Dekkera* yeast, as well as, the measures of prevention its development and volatile phenols production.

## 2 VOLATILE PHENOLS IN WINE

The accumulation of volatile phenols in wine has been a cause of great concern in modern oenology being now a key point in the control of wine quality. The quality of wine is considered to be mainly affected by the accumulation of 4-ethylphenol and 4-ethylguaiacol, whose presence is commonly described as responsible for sensorial notes reminiscence of leather, horse sweat, animal, and medicinal. Actually there are six compounds responsible for the phenolic flavour: 4-ethylguaiacol, 4-ethylphenol, 4-ethylcatechol and their precursors 4-vinylguiacol, 4-vinylphenol and 4-vinylcatechol (Fig. 1).Volatile phenols found in wines are microbial derived product formed from

hydroxynnamic acids naturally present in grapes (Boulton et al., 1996). Vinylphenols (4-vinylphenol and 4-vinylguaiacol) and ethylphenols (4-ethylphenol and 4-ehtylguaiacol) may be produced in wine, in a sequential pathway, due to microbial activity, imparting undesirable odours and flavours. Mainly, they are formed by metabolism of hydroxycinnamic acid (ferulic, *p*-coumaric or caffeic acid) substrate by *Brettanomyces/Dekkera* yeast which involves the sequential action of two enzymes. Hydroxycinnamate decarboxylase first turns these hydroxycinnamic acids into hydroxystyrenes (vinylphenols), which are then reduced to ethyl derivatives by vinylphenol reductase (Figure 1). The decarboxylation step is present in a large number of bacteria, fungi and yeast species (Degrassi et al., 1995; Edlin et al., 1995; Suezawa et al., 1995). The reduction step is much less frequent and has been reported as particularly effective in the species *Dekkera bruxellensis* (Chatonnet et al., 1995, 1997), *D. anomala* (Edlin et al., 1995), *Pichia guilliermondii* (Dias et al., 2003), *Candida versatilis, C. halophila* and *C. mannitofaciens* (Suezawa, 1995).




| | | | | |
|---|---|---|---|---|
| R = H | *p*-Coutaric acid | *p*-Coumaric acid | 4-Vinylphenol | 4-Ethylphenol |
| R = OCH | Fertaric acid | Ferulic acid | 4-Vinylguaiacol | 4-Ethylguaiacol |
| R = OH | Caftaric acid | Caffeic acid | 4-Vinylcatechol | 4-Ethylcatechol |

**Figure 1:** The formation of volatile phenols from their hydroxycinnamic acids precursors
**Slika 1:** Tvorba hlapnih fenolov iz prekurzorjev hidroksicimetnih kislin

The presence of this kind of aroma character can be considered either negative or positive depending on the concentration and expectation of a particular wine. At low concentration, these compounds can contribute to aroma complexity, but in concentration above threshold can create an unpleasant experiences (Chatonnet et al., 1990). Also, the judgment of brett wines is controversial and depends on individual and cultural preferences (Wedral et al., 2010).

Based on the literature, there are a lot of reports on presence of *Brettanomyces* metabolites in wine indicating a worldwide issue. Although only trace amounts are present in must, wine contains volatile phenols at concentrations between a few tens and several hundreds of µg $l^{-1}$ (Dubois, 1983; Chatonnet et al., 1988). The perception threshold of an odoriferous compound is conventionally considered to be the minimum concentration at which its presence in a model dilute alcohol solution is detectable by 50 % of trained tasters. The recognition threshold of an odoriferous compound corresponds to its perception threshold in wine. The preference threshold of a compound is the concentration above which the overall aroma of a wine is affected. In the case of vinyl- and ethylphenols, the preference thresholds have been estimated at 720 µg $l^{-1}$ for 4-vinylphenol and 4-vinylguaiacol in white wines, and at 420 µg $l^{-1}$ for 4-ethylphenol and 4-ethylguaiacol in red wines. It has been reported (Licker et al., 1999) that wines

with high, medium and no brett character have an average 4-ethylphenol concentrations of 3.00, 1.74 and 0.68 mg $l^{-1}$, respectively.

Volatile phenols concentration in red wine and its sensory descriptors are presented in Table 1 (Steensels et al., 2015; Curtin et al., 2005). White wines contain variable quantities of vinyl-phenols but comparing to red wines almost no of ethylphenols. On the contrary, reds contain only small quantities of vinyl-phenols and have variable concentrations of ethyl-phenols (Table 2). The volatile phenol composition of rose wines is between those of red and white wines (Chatonnet et al., 1992b, 1993b). The variety of grapevine used also affects the sensorial perception of ethylphenols. Phister and Mills (2004) indicated the detection thresholds to be high in mono-varietal Cabernet Sauvignon wines, and lower in Tempranillo wines. Pollnitz et al. (2000) analysed 61 bottles of different commercially available varietal Australian red wine, where a 4-ethylphenol was detected in all analysed wines. The concentrations found in the wines varied between 2 µg $l^{-1}$ in a Merlot and 2660 µg $l^{-1}$ in a Shiraz, with a mean concentration of 795 µg $l^{-1}$. 4-Ethylguaiacol was also found in every red wine analysed, varying in concentration from 1 µg $l^{-1}$ in a Pinot Noir up to 437 µg $L^{-1}$ in a Merlot with a mean concentration of 99 µg $l^{-1}$. An average ratio of 4-ethylphenol and 4-ethylguaiacol was approximately 10:1 for Cabernet Sauvignon, 9:1 for Shiraz, 8:1 for Merlot and 3.5:1 for Pinot Noir, what is in accordance with reports by Chatonnet et al. (1992; 1995).

The ratio of 4-ethylphenol to 4-ethylguaiacol also varied from wine to wine with reports varying from 3:1 to over 40:1 (Gawel et al., 2004; Steensels et al., 2015). The reason for these differences in wine are still not fully understood, even though they are likely caused by the combined effect of differing ratios between wines *p*-coumaric and ferulic acids (the precursors of 4-ethylphenol and 4- ethylguaiacol, resp.) and of different strains of *Brettanomyces/Dekkera* with some being more effective in producing one compound relative to the other (Buron et al., 2012; Gawel, 2004; Vigentini et al., 2008). Fariña et al. (2007) analysed six Tannat wines from Uruguay and results indicated that in three of the six analysed wines the 4-ethylphenol was found, and quantified in two of them with concentrations of 1120 and 170 g $l^{-1}$. 4-ethylguaiacol was quantified only in one of the six wine samples, in concentrations of 120 g $l^{-1}$. Recently Baša-Česnik et al. (2016) determined 4-ethylphenol, 4-vinylphenol, 4- ethylguaiacol and 4-vinylguaiacol in Teran PTP wines that were produced in the Kras winegrowing district (Slovenia). During the 2011-2013 periods, these authors found average concentrations: 153±193 µg $l^{-1}$ for 4-ethylphenol, 1265±682 µg $l^{-1}$ for 4-vinylphenol, 69±94 µg $l^{-1}$ for 4-ethylguaiacol and 128±106 µg $l^{-1}$ for 4-vinylguaiacol. Earlier from the same region the concentration of 4-ethylphenol in bottle-aged Teran PTP wines was reported to be 1016, 678 and 616 µg $l^{-1}$ for the 2007, 2008 and 2009 vintages, respectively (Čuš et al., 2011).

Volatile phenols are usually analysed by gas chromatography, after their extraction from the sample. Traditionally, liquid–liquid extraction methods were employed (Monje et al., 2002; Chatonnet, 1988), but now simpler and more selective extraction methods are applied, such as solid-phase extraction (SPE) (López et al., 2002; Domínguez et al., 2002) solid-phase microextraction (SPME) (Monje et al., 2002; Martorell et al., 2002) or stir bar sorptive extraction (SBSA) (Díez et al., 2004).

**Table 1:** Volatile phenols in wine (Steensels et al., 2015; Curtin et al., 2005)
**Preglednica 1:** Hlapni fenoloi v vinu (Steensels in sod., 2015; Curtin in sod., 2005)

| | Concentration in red wine (ppb) | Sensory descriptor |
|---|---|---|
| 4-vinylphenol | 8.8 – 43 | Phenolic, medicinal |
| 4-vinylguaiacol | 0.2 – 15 | Clove-like |
| 4-ethylphenol | 118 - 3696 | Medicinal, horsy |
| 4-ethylguaiacol | 1 – 432 | Spicy, clove-like |
| 4-ethylcatechol | 27 – 427 | Phenolic, medicinal |

**Table 2:** Ethyl- and vinyl-phenol concentrations in different wines (μg $l^{-1}$) (Chatonnet et al., 1992, 1993)
**Preglednica 2:** Vsebnosti etil- in vinilfenolov v različnih vinih (μg $l^{-1}$) (Chatonnet in sod., 1992, 1993)

| Volatile phenols | White wines *n* = 54 | Rose wines *n* = 12 | Red wines *n* = 83 |
|---|---|---|---|
| **Vinyl-4-phenol** | | | |
| Minimum | 73 | 3 | 0 |
| Maximum | 1150 | 215 | 111 |
| Mean | 301 | 71 | 35 |
| Standard deviation (%) | 79 | 99 | 75 |
| **Vinyl-4-guaiacol** | | | |
| Minimum | 15 | 4 | 0 |
| Maximum | 496 | 75 | 57 |
| Mean | 212 | 17.5 | 12 |
| Standard deviation (%) | 44 | 113 | 79 |
| **Ethyl-4-phenol** | | | |
| Minimum | 0 | 0 | 1 |
| Maximum | 28 | 75 | 6047 |
| Mean | 3 | 20 | 440 |
| Standard deviation (%) | 229 | 122 | 179 |
| **Ethyl-4-guaiacol** | | | |
| Minimum | 0 | 0 | 0 |
| Maximum | 7 | 15 | 1561 |
| Mean | 0.8 | 3 | 82 |
| Standard deviation (%) | 225 | 159 | 230 |

Coulter et al. (2004) concluded that the extent to which the sensory properties of a wine may be affected by 4-ethylphenol depends on the style and structure of the wine, i.e. the concentration and intensity of other wine compounds that could mask (e.g. volatile oak compounds) or accentuate (e.g. 4-ethylguaiacol) the aroma of 4-ethylphenol. For example, in a light-bodied red wine with little oak influence, the sensory perception threshold of 4-ethylphenol may be as low as concentration of 350 μg $l^{-1}$, compared with 1000 μg $l^{-1}$ in a full-bodied red wine with intense fruit and considerable oak influence.

## 3 THE PRODUCTION OF VOLATILE PHENOLS BY *BRETTANOMYCES/DEKKERA*

*Brettanomyces bruxellensis* is yeast found on surfaces of grapes as well as in barrels, but the greatest concern is its presence in wine. The yeasts of the genus *Brettanomyces*, or its teleomorph *Dekkera*, were first described by Claussen in 1903, in beer production (Gilliland, 1961). *Brettanomyces*/*Dekkera* yeasts exist in two forms: *Brettanomyces*, the asexual, non-sporulating form, and *Dekkera*, the sexual, sporulating form. These genera are particularly known as spoiling agents in beer, wine, cider and soft drinks industries (Deak and Beuchat, 1996). The current taxonomy includes five species within the genera of *Dekkera*/*Brettanomyces*. Those are the anamorphs *Brettanomyces bruxellensis*, *Brettanomyces anomalus* Custers, *Brettanomyces custersianus*van der Walt, *Brettanomyces naardenensis* Kolfsch. & Yarrow, and *Brettanomyces nanus* (M.T. Sm., Bat. Vegte & Scheffers) M.T. Sm., Boekhout, Kurtzman & O'Donnell, with teleomorphs existing for the first two species, *Dekkera bruxellensis* and *Dekkera anomala* (Cocolin et al. 2004; Oelofse et al., 2008). Different strains of *Brettanomyces* can show great differences in their production of volatile phenols (Joseph and Bisson, 2004). In wines, the metabolic products responsible for spoilage by *Brettanomyces/Dekkera* sp. are mainly volatile phenols, isovaleric (3-methylbutyric) acid, tetrahydropyridines and acetic acid (Heresztyn, 1986; Licker et al., 1999; Larue et al., 1991; Ciani and Ferraro, 1997). Coulter et al. (2004) found that 4-ethylphenol and 4-ethylguaiacol (both compounds associated with 'Band-aid', 'medicinal', 'barnyard' and 'stable' aroma characters and 'metallic' taste attributes) were the main compounds derived from *Brettanomyces* that were associated with off-odours in red wines. They found that the concentration of isovaleric acid (associated with 'sweaty', 'cheesy' and 'rancid' characters) was independent of the concentrations of these two key spoiler compounds. It therefore appears that isovaleric acid may be involved in additional sensory effects with other *Brettanomyces*-derived compounds, thereby enhancing the apparent aroma intensity of those other compounds.

Works on *D. bruxellensis* have mainly focused on its early detection to reduce economic losses (Wedral et al., 2010). Also, studies have been carried out to understand the mechanisms of 4-vinylphenol and 4-ethylphenol production (Dias et al., 2003; Godoy et al., 2008; Harris et al., 2008; Harris et al., 2009). Barata et al. (2008) concluded that the production of 4-ethylphenol in red wines is related to the presence of growing populations of *D. bruxellensis*, demonstrating that the primary management objective should not be their complete elimination but their maintenance at constant levels. If the numbers of *D. bruxellensis* increase, removal or inactivation procedures must be performed. Coulon et al. (2009) highlighted a relationship between the physiological state of *B. bruxellensis*, and its capacity to produce volatile phenols. Cultivable populations seem indeed able to synthesize higher amounts of ethyl-phenols than viable but non-cultivable cells. They also stated that sequential ethyl-phenol production could systematically be correlated to the *B. bruxellensis* physiological state and found that maximum vinyl-phenol concentrations were found with fast-multiplying cells. They then decreased, indicating that the yeast metabolism is centred on ethyl-phenol production. This was generally accompanied by a population regression or/and a non-cultivable state shift. Other authors had already noted that ethyl-phenols synthesis occurred during the late exponential phase or stationary growth phase in synthetic media (Dias et al. 2003; Harris et al. 2008) or wine (Romano et al. 2008). This indicates that the overall information about the growth physiology of *D. bruxellensis* and metabolite production appears to be sometimes contradictory. Some authors concluded that production of ethylphenols was intrinsically related to *D. bruxellensis* growth (Barata et al., 2008b; Dias et al., 2003; Vigentini et al., 2008), whereas other studies suggest the existence of a sulphite-induced viable but non-culturable subpopulation, which is able to produce vinylphenols and ethylphenols (Agnolucci et al., 2010; Laforgue and Lonvaud-Funel, 2012; Serpaggi et al., 2012).

*Brettanomyce*s spoilage of wine usually occurs when they are fermented or aged in oak barrels (Rayne et al., 2008). The yeast grows slowly so it usually imparts flavours only when the wine is aged. Since *Brettanomyces* is present at low

numbers early in the fermentation, it is outnumbered by other indigenous yeast and may go undetected (Wedral et al., 2010). Garde-Cerdán et al. (2002) investigated the behaviour of the barrels at the completion of their cycle of use (5–6 years old) on the volatile composition of a red wine. In all the wines high concentrations of 4-ethylphenol and 4-ethylguaiacol were found, and these have a negative impact on the quality of the product, probably due to the presence of contaminating microflora as the barrels were old (Garde-Cerdán et al., 2002).

The results of Silva et al. (2011) suggest that the conversion of 4VP into 4EP, catalysed by the vinylphenol reductase, may lead to the re-oxidation of NADH. An analogous statement was made by Fugelsang and Edwards (2007) for *Brettanomyces/Dekkera*: since a reduced cofactor is generally required for the enzymatic reduction activity, it is likely that the production of volatile phenols, specifically the reduction of 4-vinylphenol into 4-ethylphenol, is a source of $NAD^+$ during growth of this organism by maintaining the redox balance of its cells in red wines.

However, red wines are more susceptible to *Brettanomyces bruxellensis* due to their lower acidity, higher polyphenol concentration and barrel aging. Therefore *Vitis vinifera* red varieties with higher polyphenol concentration are the most susceptible to the *Brett* (Wedral et al., 2010). The loss of viability in white wines is largely due to the efficacy of sulphur dioxide at low pH (Loureiro et al., 2006) and due to absence of precursor compounds (Chatonnet et al., 1992).

### 3.1 Factors affecting growth of *Brettanomyces* and production of volatile phenols

#### 3.1.1. Carbon and energy sources

Bioconversion of ethylphenols precursors, like *p*-coumaric, by *B. bruxellensis* is highly dependent on the growth media (Dias et al., 2003) and cinnamic acid ratios (Romano et al., 2008). Dias et al. (2003) showed that reduction step was dependent on the carbon and energy source, i.e. high conversion rates of *p*-coumaric acid to 4-ethylphenol only occurred when glucose or ethanol were substrate. Regarding to trehalose sugar, Chatonnet et al. (1995) suggested that this residual sugar may allow synthesis of 4-ethylphenol. These results are not in agreement with Dias et al. (2003) who demonstrated that contribution of this sugar to the overall production of 4-EP is not relevant in wine.

*D. bruxellensis* grown in the presence of glucose showed relatively low growth rate compared to other wine related yeast such as *S. cerevisiae* and *Z. bailii* (Lindner) Barnet et al. (Rodrigues et al., 2001a). Vigentini et al. (2008) found that *D. bruxellensis* used fructose as a preferred carbon source in a synthetic medium with a high concentration of ethanol. Also, the amount of less than 2 g $l^{-1}$ fermentable sugar in "dry" wines is still not limitation to the production of 4-EP by *D. bruxellensis.* The production of 4-ethylphenol was detected at sugar concentrations over 0.2 g $l^{-1}$ and increased under higher sugar concentrations (Barata et al., 2008). In line with this, Sturm et al. (2015) showed that 210 mg $l^{-1}$ of sugars (glucose, fructose and trehalose) was enough to allow *B. bruxellensis* growth. Sugars were completely consumed during the first growth phase, whilst consumption of glucose and fructose was faster than trehalose.

The growth of *Brettanomyces/Dekkera* in synthetic media containing autolysed *Saccharomyces cerevisiae* has also been studied (Guilloux-Benatier et al., 2001). Under these conditions it was observed that these contaminating yeasts grew easily, even in glucose concentrations of less than 150 mg $l^{-1}$. However the quantity of ethylphenols formed was smaller than expected, probably because of the adsorption of the phenolic compounds by the cell wall fragments. More recent studies have reported the high capacity of yeast cell walls to adsorb phenolic compounds (Morata et al., 2005; Morata et al., 2003).

The inhibition of growth and 4-ethylphenol production by 13 % (v/v) of ethanol is observed in wines with high concentration of ethanol and these wines did not show high concentrations of this phenol (Rodrigues et al., 2001; Dias et al., 2003). These authors showed that 4-ethylphenol concentrations in wines were not correlated with acetic acid concentrations expect in media with high sugar concentration, as also observed by Gerós et al. (2000) in *D. anomala.* This observation indicates that these two *D. bruxellensis*

spoiling features (acetic acid and 4-ethylphenol production) are independent. However, acetic acid production is variable among strains of the genera *Dekkera/Brettanomyces* cultivated under the same conditions (Freer, 2002).

Barata et al. (2008) demonstrated that even in the presence of a carbon and energy source, the levels of volatile phenols do not increase in commercial red wines when *D. bruxellensis* is not growing. This conclusion has direct implications in the management of the phenolic taint, because it shows that the primary objective should be the prevention of actively growing populations and not the reduction of *D. bruxellensis* to the lowest possible level, as suggested by Renouf et al. (2007). In fact, complete absence of viable cells of *D. bruxellensis* is not easy to achieve under winery conditions, especially when oak aging is used due to the porous nature of the wood (as discussed by Loureiro and Malfeito-Ferreira, 2006).

Coulon et al. (2009) confirmed as it has previously been observed that *B. bruxellensis* is not very demanding from a nutritional point of view and that it can grow with other energy sources than glucose and fructose (Alguilar Uscanga et al., 2000; Conterno et al., 2006).

3.1.2. Precursors

The biosynthesis of volatile phenols is related to the sequential activity of two enzymes which decarboxylate hydroxycinnamic acids (ferulic, *p*–coumaric and caffeic acids) spontaneously present in grapes into vinylphenols, which are then reduced to ethylphenols (Steinke et al., 1964). The formation of volatile phenols in wine depends on the presence of precursors and is proportional to the size of the *Brettanomyces/Dekkera* population (Gerbeaux et al., 2000, Suárez et al., 2007). It has previously been suggested that microorganism's decarboxylate HCAs in order to produce less toxic compounds (Goody et al., 1982). Vinyl-phenol reductase and cinnamate decarboxylase, the two enzymes involved in ethyl-phenol production in *B. bruxellensis* are precursor inducible, but these precursors also have an inhibitory effect on *B. bruxellensis* growth (Harris et al., 2008). Variations in wines can be noted because of the instability, esterification and cell-adsorption of *p*-coumaric acid (Salameh et al., 2007).

Grapevine varieties differ in the quantity of phenolic acids present in the berries (Rodrigues et al., 2001; Morel-Salmi et al., 2006; Morata et al., 2007; Rentzsch et al., 2007). The presence of the three hydroxycinnamic acids (caffeic acid, ferulic acid and *p*-coumaric acid) in grapes originate from their bound form with tartaric acid known as caftaric acid, fertaric acid and coutaric acid, respectively. Goldberg et al. (1998) measured the concentration of *p*-coumaric acid, the precursor to 4-ethylphenol, in single variety red wines from various countries and found that Pinot Noir had a lowest concentration of *p*-coumaric acid, among all studied varieties and countries. General concentrations for hydroxycinnamic acids present in *Vitis vinifera* L. juice (oxidative and hydrolytic losses prevented) are about 150 mg $l^{-1}$ of caftaric acid, 20 mg $l^{-1}$ of coutaric acid and 1.0 mg $l^{-1}$ of fertaric acid (Boulton et al., 1996). Other grapevine varieties have been identified to have higher amounts of hydroxycinnamic acids for example wines from Grenache variety can contain between 270-460 mg $l^{-1}$ of caftaric acid (Morel-Salmi et al., 2006). Other wine varieties mean values for caftaric acid range from 50 to 60 mg $l^{-1}$ (Rentzsch et al., 2007). Dias et al. (2003) studied the capacity of a number of yeasts present in wine microbiota to produce 4-ethylphenol from *p*-coumaric in model media. Molar conversions of 90 % were reported for *D. bruxellensis, D. anomala* and *P. guillermondii*; other fermentative yeasts were incapable of producing 4-ethylphenol at these rates of conversion.

Recently, Cabrita et al. (2012) showed that phenolic acids concentration decreases while volatile phenols concentration increases and the proportion of caffeic acid taken up by *Dekkera bruxellensis* is lower than that for *p*-coumaric or ferulic acid, i.e. less 4-ethylcatechol is formed. Before, the presence of 4-ethylcatechol, has been reported only once, by Hesford et al. (2004), until Carrillo and Tena (2007) reported the presence of 4-ethylcatechol in some wines affected by *Brettanomyces*. Study of Cabrita et al. (2011) has shown that 4-ethylcatechol is the last one to appear in wine, and it is the least significant. In this study conversion rates greater than 90 % were obtained for the conversion of *p*-coumaric acid into 4-ethylphenol and from ferulic acid into 4-ethylguaiacol, but a rate smaller than 20 % was

ethylcathecol. These results may justify why only the presence of 4-ethylcatechol in wines was reported (Hesford et al., 2004). Although caffeic acid is present in wines in considerable amounts and has a structure similar to the other phenolic acids, these results seem to indicate that *p*-coumaric and ferulic acids are easily used by yeast metabolism.

In the study of Sturm et al. (2015), all five examined *D. bruxellensis* strains were able to simultaneously metabolise *p*-coumaric and ferulic acid with production of their respective volatile phenols, being the conversion rate of ferulic acid lower than *p*-coumaric acid. In contrast, Oelofse et al. (2009) suggested that the conversion pathway of ferulic acid as precursor was preferred to *p*-coumaric acid by different *D. bruxellensis* strains grown in wine spiked with similar amounts of both compounds (100 mg $l^{-1}$). Besides, the ratio of *p*-coumaric and ferulic acid was 8:1 (Sturm et al., 2015) and it has been proven that ferulic acid is slightly more toxic to the yeast than *p*-coumaric acid (Harris et al., 2008).

Recently, Lentz et al. (2015) showed that caffeic acid was the weakest inhibitor of the HCAs tested. This observation is in general agreement with published data for other strains that show weak or no inhibition of growth by this compound compared to other cinnamic acids (Harris et al., 2009). When a strain showed variation for inhibition by ferulic acid and *p*-coumaric acid, it's turned out that ferulic acid was always a more potent inhibitor (Lentz et al., 2015). This data supports results from similar experiments using different strains of *B. bruxellensis* and *B. anomalus* (Harris et al., 2009). It appears that *Brettanomyces* in general are only weakly inhibited by caffeic acid, and are slightly more susceptible to ferulic than *p*-coumaric acid.

Kosel et al. (2014) examined the impact of ethanol, and hidroxycinnamic and vinylphenol precursors on the production of volatile phenols in fermentations of mixed and pure cultures of yeasts *Saccharomyces cerevisiae* and *Dekkera bruxellensis*. Results showed that in mixed culture fermentations less vinylphenols and more ethylphenols were produced in comparison with *D. bruxellensis* pure culture fermentations. Vinylphenol precursors significantly inhibited the growth of *S. cerevisiae* and the production of ethylphenols. It was found that *D. bruxellensis* genes encoding for enzymes coumaric acid decarboxylase (CAD) and vinylphenol reductase (VPR) are more responsive to vinylphenol precursors in comparison with hidroxycinnamic acids. Consequently, higher concentrations of vinylphenols in the cell were found to be more cytotoxic than hidroxycinnamic acids. Also, these authors showed that 10 % of ethanol strongly reduced the growth and volatile phenol production of yeasts *D. bruxellensis* and *S. cerevisiae*.

### 3.1.3. Temperature, ethanol concentration, pH

The impact of different chemical factors and different temperatures on volatile phenol production has been well studied. Different strains of *B. bruxellensis* vary in their capacity to produce volatile phenols and it is always greater at lower alcohol concentrations (more are made at 12 % v/v than at 14 % v/v) and at higher temperatures (e.g., more is produced at 18 °C than at 13 °C) (Gerbeaux et al., 2000). Recently, study of Kosel et al. (2014) confirmed that low ethanol concentrations induced higher production of volatile phenols by *S. cerevisiae* and *D. bruxellensis.* Little significance is attributed to the pH of the wine or the presence of residual sugars in this respect. The intensity and temperature of maceration and the use of pectolytic enzymes have been studied as possible factors conditioning the formation of volatile phenols by *Brettanomyces* and *Dekkera* from hydroxycinnamic acids released from the grape skins (Gerbeaux et al., 2002). Godoy et al. (2008) demonstrated that both enzymatic activities were stable at pH 3.4, but in the presence of ethanol the coumarate decarboxylase activity decreased drastically while the vinyl reductase activity was more stable.

Dias et al. (2003) described that a 5 % ethanol concentration is adequate to obtain volatile phenols in the culture medium, since an increase in ethanol concentration is detrimental to the yeast population and consequently leads to a decrease in volatile phenols. Garde-Cerdán et al. (2008) showed that alcohol concentration was the oenological parameter that had the greatest impact on the accumulation of volatile compounds in wines and according to same author accumulation of ethylphenols in the wines diminished as the

alcohol concentration of the wines increased from 12.5 % to 13.5 %.

Results obtained by Ganga et al. (2011) are similar to that obtained by Dias et al. (2003) and Garde-Cerdán et al. (2008), where although *D. bruxellensis* presents basal coumarate decarboxylase activity, it is necessary to add ethanol to the culture medium to increase its production. Ganga et al. (2011) investigated the influence of the interaction between the concentration of *p*-coumaric acid, ferulic acid and ethanol as well as growth temperature on the production of CD activity and the expression of a putative gene that codes for this enzymatic activity. These authors concluded that the interaction of cinnamic acids with growth temperature, and growth temperature with ethanol concentration, as well as ethanol concentration, are highly important variables in the production of CD activity. Analysing the assayed growth temperatures (16 ºC to 28 ºC) shows that the increase of this parameter brings about a decrease of coumarate decarboxylase activity. This affirmation is not in accordance with reports by Benito et al. (2009), who indicated that at a temperature between 20 and 30 ºC the yeast consumes the greatest quantity of *p*-coumaric acid, which is indirectly associated to the presence of higher coumarate decarboxylase activity . At 22 ºC with 10 vol. % alcohol, the yeast on average only metabolizes 38 % of the *p*-coumaric acid in the culture medium, while with 3 % ethanol, 74 % of the initial *p*-coumaric acid was metabolized by the yeast. This result was also obtained at 16 ºC. Salameh et al. (2008) indicated that *p*-coumaric acid can react with the ethanol in the medium or be absorbed through the yeast wall, which leads to a decrease in the acid concentration in the culture medium. A slow metabolization of *p*-coumaric acid in the culture medium is closely related to the growth rate of the yeast, with yeast growth slower at 10 % than at 3 %.

3.1.4. Conditions during aging and storage of wine

The use of old wooden casks can increase the presence of *Brettanomyces* and *Dekkera* species in wine due to difficult cleaning and impossibility of their sterilization. *Brettanomyces/Dekkera* has been found at 8 mm down within the wood of barrel staves (Malfeito-Ferreira, 2006). These yeasts survive treatments where contact with $SO_2$ is limited, e.g., around bung holes, in the oak structure, and in yeast sediments (lees). Besides, *Brettanomyces custersii* and *Dekkera* intermedia metabolise cellobiose, a disaccharide, forming the basic repeating unit of cellulose (a structural polysaccharide of wood) (Freer, 1991; Park et al., 1999; Park et al., 2000).

The age of the barrel greatly influences the growth of *Brettanomyces* populations during the long aging of red wines. Old barrels favour *Brettanomyces* contamination – oak wood is extremely porous and yeasts deep in the barrel staves are difficult to eliminate – and any ethylphenols in the mass of the wood are released (Chatonnet et al., 1999). However, some authors (Lonvaud-Funel and Renauf, 2005) report that, due to their higher oxygen and sugar contributions, new barrels are even more likely to favour the maintenance of large *Brettanomyces* populations. It is probable that the wood pores become blocked as the barrels are used, so the oxygen arriving via them decreases. The frequent re-use of casks and the use of the micro-oxygenation technique to accelerate wine maturation, facilitates the polymerisation of wine pigments and the modification of the wine volatile profile (frequently associated with the use of oak chips or barrel aging), resulting in the proliferation of *Brettanomyces/Dekkera* (Aguilar-Uscanga et al., 2003; Ciani and Ferraro, 1997; Ciani et al., 2003).

Pollnitz et al. (2000) determined 4-ethylphenol and 4-ehtylguaiacol concentration in red wine aged in new and used French and American oak barrels of different ages. Wine stored in shaved and renovated with fireing oak barrels contained up to 85 % less 4-ethylphenol and 4-ethylguaiacol than wine stored in usual barrels of the same age that were not shaved. Oak barrels that become contaminated with *B. bruxellensis* cannot be effectively sterilized. Neither careful washing followed by rinsing with sulphited water, nor shaving and firing, nor ozone treatment achieves sterilization (Pollnitz et al., 2000) – a result of the large internal volume and porous nature of oak barrels. The sanitation of barrel wood requires at least 7 g of $SO_2$ gas per barrel. Filled wine barrels should receive 20–25 mg $l^{-1}$ of free $SO_2$ (30–35 mg $l^{-1}$ during hot summers) (Henick-Kling et al., 2000). Malfeito-Ferreira (2005) tested four barrel sanitation procedures: (I) cold water rinse followed

by three hot water rinses (70 °C); (II) the same as the previous plus filling with an aqueous solution of $SO_2$ (200 mg l-1, pH 3) and storing for one month; (III) cold water rinse, followed by filling the barrel with hot water (90 °C for 10 min); (IV) cold water rinse, followed by a hot water rinse (70 °C) and low pressure steam (10 min). The last one appeared the most effective and author recommended isolation of brett infectioned barrels to reduce the contamination of others during disinfection and wine pumping.

Garde-Cerdán et al. (2010) analysed 510 wines, from four different Spanish geographic zones, and aged in different oak barrels types for at least 6, 12 and 18 months. Theyconcluded that accumulation of volatile oak compounds and ethylphenols was affected mainly by the storage time of the wines in the oak barrels, while the oenological parameters, the geographic origin and the oak barrel type had smaller influences on the accumulation of these volatile compounds in the wines. The total average of the ratio 4-ethylphenol and 4-ethylguaiacol of the aged-6 wines was below that of the aged-12 and aged-18 wines. A ratio for the aged-12 and aged-18 wines was within the range found by Pollnitz et al. (2000), i.e. between 3.5 and 10.1.

However, young red wines in stainless-steel vessels or bottled wines are also prone to this type of spoilage (Rodrigues et al., 2001; Renouf et al., 2007). At the end of barrel aging, before bottling, residual population of *B. bruxellensis* can often be detected (Nisiotou and Gibson, 2005; Renouf et al., 2006; Curtin et al., 2007). Although populations are usually too low at this point to synthesize ethyl-phenols, they could further develop and spoil the wine during bottle storage, when the winemaker can no longer intervene (Coulon et al., 2010).

Recently, results obtained by Rubio et al. (2015) indicated the spoilage risk exists when *Brettanomyces* cells are present, even at a low level, in wines subjected to aging, both in the cask and the bottle. *Brettanomyces* presence and ethylphenol production during aging, is affected more by the aging conditions (aerobic/anaerobic and sulphiting) than by the origin of the oak. They had shown that aging only under aerobic conditions with racking and sulphur dioxide addition, showed lower *Brettanomyces* levels than the combined aerobic and anaerobic maturation with racking but without sulphur dioxide addition. Also, wines aged in Chinese oak revealed different behaviour to the other three in terms of the level of *Brettanomyces* and in the ethylphenol concentration, probably due to the fact that this oak has the highest porosity.

## 4 MEASURES FOR THE PREVENTION

Prevention of the growth of *Brettanomyces/Dekkera* in wine involves attention to fruit quality and winery sanitation, control of sulphite and oxygen levels, as well as to the use of uncontaminated barrels (Wedral et al., 2010). *B. bruxellensis* is found on damaged grapes and in winery equipment, so effective general sanitation is the first step in the prevention. Adequate amounts of $SO_2$ should be added to fermentation vessels and maintained during fermentation in order to inhibit growth of *B. bruxellensis.* After addition of $SO_2$, *Brettanomyces* enter in to viable but not culturable state, so the yeast may be still present after depletion of free $SO_2$ (Umiker et al., 2007). As control factors, winemakers should consider using a starter yeast culture, alcohol, acid levels, temperature and oxygen exposure. Using a starter culture can decrease indigenous fermentation, decreasing the opportunity for *Brettanomyces* to grow. Though fermenting wines to a higher alcohol level may often inhibit development of flavour precursors, ethanol tolerance is also a strain dependent character (Vigentini et al., 2008).

Apart from limiting *Dekkera* growth one way of avoiding ethylphenol production is to minimize the concentration of precursors in wine (Gerbaux et al., 2002). A common oenological technique is the addition of enzyme preparations during maceration to aid in the release of phenolic compounds from the grape berries. These preparations have been shown to be relatively effective in releasing free hydroxycinnamic acids from their esterified form, which then leaves these available for conversion into volatile phenols. Therefore it has been

recommended that enzyme preparations possessing cinnamoyl esterase not be used in winemaking as it increases the chance of spoilage by volatile phenols (Gerbaux et al., 2002).

Chatonnet et al. (1993) stated that wines are more susceptible to the phenolic taint in warmer months. In fact, the impact of air temperature on 4-ethylphenol production was related with the production rate, and not with the total amount produced. Thus, keeping wines at low cellar temperatures only delays the process, being an efficient prevention measure if cell growth is fully inhibited. Couto et al. (2005) already presented lethal heat-treatment parameters for *D. bruxellensis*, showing that significant inactivation of *D. bruxellensis* in wine began at 35 °C, stimulated by the ethanol concentration of wine. In addition, other data (Barata et al., 2008) showed that relatively mild temperatures (about 36 °C) overnight are a reasonable technological option when wines are found contaminated by viable *D. bruxellensis* and 4-ethylphenol tends to increase. According to these authors, this mild temperature has no obvious detrimental effects on wine quality and may be achieved using electric devices to heat wine in stainless-steel vessels or simply by heating bottled wine, without disgorging, if contamination could not have been avoided during bottling. Barata et al. (2008) managed to achieve stable levels of 4-ethylphenol (100 µg $l^{-1}$) with viable but non-growing *D. bruxellensis* populations of 2000 CFU $ml^{-1}$ during barrique storage at low temperatures (6–8 °C). Removal and inactivation of *D. bruxellensis* by strict process operations (e.g. heat treatment, sterile filtration) would only be advisable when there is an increase in the 4-ethylphenol levels.

Decreases of 4-ethylphenol and 4-ethylguaiacol concentrations were found in red wine containing yeast lees compared to the same wine aged without lees (Guilloux-Benatier et al., 2001). Chassagne et al. (2005) shown that yeast lees were effective in removal of 4-ethylguaiacol and 4-ethylphenol. Also the presence of other wine constituents sorbed by yeast influenced the sorption of both volatile phenols, with a greater effect in the case of 4-ethylphenol. Yeast lees provide a cost-effective and efficient approach to remove or to decrease organoleptic defects in wine due to phenols.

However, preventive methodologies have been based on the generation of conditions unfavourable to *Brettanomyces/Dekkera* (Benito et al., 2009). Sturm et al. (2015) also concluded that potential spoilage of wine by *D. bruxellensis* was more related to the ability of the strains to develop in the wine environment than the CD and VR enzymatic activity recorded in laboratory conditions and therefore, the most efficient way to prevent wine spoilage by *D. bruxellensis* should be the control of its development.

Certain additives can inhibit the growth of *Brettanomyces* (Suarez et al., 2007). The most common is sulphur dioxide ($SO_2$), although it is hard to keep the concentration stable over prolonged aging periods in casks in which the environment is mildly oxidizing. It is known that, in a red wine at pH 3.65, initial doses of free $SO_2$ of 15, 25, 30 and 35 mg $l^{-1}$ are significantly reduced after four months of aging in barrels to 6, 11, 10 and 15 mg $l^{-1}$, respectively (Chatonnet, et al., 1993).

The action of $SO_2$ on *Brettanomyces* seemed to be rapid, with cells having their viability greatly reduced and losing their culturability completely within 330 min of exposure. The uptake of free molecular $SO_2$ by *Brettanomyces* is fast, as exposure of the cells to molecular $SO_2$ for only a short period of time showed (Du Toit et al., 2005). Also, the addition of $O_2$ to wine that contains low concentrations of $SO_2$ can support the survival and growth of *Brettanomyces* and it usually happen during racking and other transfer or transport. Therefore, when it is suspected that wine has been contaminated by *Brettanomyces*, it should be avoided excessive $O_2$ exposure and molecular $SO_2$ concentrations should be checked and regularly adjusted to 25–35 mg $l^{-1}$ of free $SO_2$. Winemakers should bear in mind that at excessive concentrations, $SO_2$ might affect the aroma and colour of red wine because the reaction of $SO_2$ with the red form of anthocyanins leads to the bleaching of red wine colour (Ribéreau-Gayon et al., 2000). The use of between 0.5 and 0.8 mg $l^{-1}$ of molecular $SO_2$ is recommended and it should be remembered that the molecular $SO_2$ concentration achieved is pH-dependent; 30 mg $l^{-1}$ of free $SO_2$ releases 0.4 mg $l^{-1}$ of molecular $SO_2$ at pH 3.7, and 0.8 at pH 3.4 (Henick-Kling, et al., 2000). The antimicrobial potential of $SO_2$ against

*Brettanomyces*, and the effectiveness of physical treatment like racking to remove yeast cells from barrels have been shown also by (Oelofse et al., 2008; Suárez et al., 2007). Besides, the inhibitory effect of these two actions (sulphating and racking) overcame the stimulating effect of oxygen on *Brettanomyces* growth (Kheir et al., 2013; Aguilar-Uscanga et al., 2003). Also, lower *Brettanomyces* levels is achieved when wine is aged only in aerobic conditions (12 months cask) with racking and sulphur dioxide addition, than the combined aerobic and anaerobic maturation (6 months in cask and 6 in bottle) (Rubio et al., 2015).

Filtration can reduce the presence of contaminating yeasts, but this poses problems of reducing wine aroma and colour (Suárez et al., 2007).. In order to be effective, membranes with a pore size smaller than 0.45 μm must be used (Calderón et al., 2004) and they causes a deterioration of the wine's colloidal structure and can reduce the intensity of its colour. Dormant, elongated forms of *Brettanomyces* cells may be able to pass through a 0.45 μm filter (Suárez et al., 2007). One of the solutions is fining operation of red wines before introducing them into their barrels and in that way contaminating populations of *Brettanomyces* can be reduced by 40 to 2000-fold by treatment with fining proteins (Murat and Dumeau, 2003). There are differences in effectiveness between fining agents and sometimes fining is rejected by winemakers since it also impacts wine aroma and colour. Anyway, the greater reduction in the initial population is achieved when the more fining agents used; i.e. intense finings can almost entirely remove these yeasts (Suárez et al., 2007). Fining with casein or potassium caseinate can reduce ethylphenol levels if these are not too high (Ruiz-Hernández, 2003).

It is known that certain weak acids, such as sorbic, benzoic and fumaric have antifungal activity and can be used against *Brettanomyces/Dekkera*, but their action is not selective and they are not authorized for use in winemaking. Characteristically, weak-acid preservatives do not slay micro-organisms but rather inhibit their growth, causing extended lag phases. Benito et al., (2009) report that sorbic acid can act as inhibitor in conversion of precursor compound to 4-ethylphenol but *Brettanomyces* appear the more resistant yeast species to sorbic acid. Antioxidants such as ascorbic and erythorbic acids can be used to reduce the presence of oxygen during maturation, preventing ethylphenol formation (Suárez et al., 2007).

Other alternative inhibitors are dimethyl dicarbonate and chitosan. Effectiveness of dimethyl dicarbonate has been proven, but Delfini et al. (2002) showed that a dose of 400 mg $l^{-1}$ cannot completely inhibit the growth of *B. anomalus* and in contrast, other fermentative yeasts are inhibited by dosages of 250–400 mg $l^{-1}$. Chitosan is a polysaccharide derived from chitin and it has a selective effect on *Brettanomyces*, causing a delay in its latent phase in mixed cultures with *Saccharomyces cerevisiae* (Gómez-Rivas et al., 2004). *B. bruxellensis* and *B. intermedius* cannot grow in the presence of 3–6 g $l^{-1}$ of chitosan, while it does not affect the development of *Saccharomyces cerevisiae*. Chitosan at 0.05–0.1 % is known to delay spoilage by yeasts at 25 °C; in fact it even inactivates some other species (Kiskó et al., 2005).

Puig et al. (2003) showed that application of pressures of 400–500 MPa for 5–15 min at temperatures of 5 to 20 °C can reduce populations of certain yeasts (including *B. bruxellensis*) and lactic acid and acetic acid bacteria by more than 99.99 %, without causing major modifications to the wine's physicochemical properties, enzymatic activity, or sensorial properties. Not only is effective, it reduces the use of $SO_2$. The concentration of ethylphenols can be reduced by reverse osmosis and adsorption (Ugarte et al., 2005). These authors reduced initial concentration (900 μl $l^{-1}$ of 4-ethylphenol plus 4-ethylguiacol) by 77 % after a 3-hours treatment involving reverse osmosis with an appropriate membrane and tangential-flow filtration equipment and a hydrophobic adsorbent resin. Using this method, no significant reduction in wine colour, tannins, body (glycerol and diols) or ethanol was observed, but it was seen reduction in aromatic compounds, like methyl- and ethyl vanillate and other esters (Suarez et al., 2007).

The use of various antimicrobial agents, bacteriolytic enzymes, zymocines and yeast strains with antimicrobial activity constructed by genetic engineering represent biological control of contaminating yeasts and bacteria. However, in the

wine environment they are not very effective and other techniques are usually required as well (Du Toit and Pretorius, 2000).

Some wineries use polyvinylpolypyrrolidone and charcoal to treat wines containing volatile phenols. The recommended doses are from 0.015 to 0.24 g $l^{-1}$ charcoal for slight off-odours, and from 0.12to 0.96 g $l^{-1}$ for more intense off-odours. polyvinylpolypyrrolidone (0.06-0.48 g $l^{-1}$) is used to remove ethylphenols (Suarez et al., 2007).

Fulcrand et al. (1996) proposed the formation of the malvidin derivative from 4-vinylphenol in order to decrease the production of ethylphenols and in the same way to increase the formation of highly stable pigments during maturations. Vinyl phenolic derivatives are highly stable due to their aromatic heterocyclic ring. Other pyranoanthocyanic derivatives, such as the vitisins, function in the same way (Mateus, et al., 2001). Besides, since the formation of this ring involves carbon 4 of the anthocyanin, decolouration by $SO_2$ and reductions in colour intensity caused by high pH are less likely (Bakker and Timberlake, 1997).

Yeasts with hydroxycinnamate decarboxylase activity can also be used to decarboxylate hydroxycinnamic acids, and this forms vinylphenols that condense with grape anthocyanins to produce pyranoanthocyanin vinylphenolic adducts of great colour stability (Morata et al., 2006). This eliminates the hydroxycinnamic acid precursors of ethylphenol from wine, and forms highly stable, long-lasting pyranoanthocyanins during fermentation. During fermentation, vitisins A and B, pyranoanthocyanic molecules structurally similar to vinylphenol derivatives, are produced in significant amounts by selected yeast strains (Morata et al., 2003). These adduct formed via the condensation of vitisin A and vinylphenols are very stable molecules that provide wine a red–blue colour (Mateus et al., 2006). Benito et al. (2009) used *S. cerevisiae* strains with high HCDC in order to reduce the concentration of ethylphenol precursors and it is shown that these strains can minimize possible alterations caused by *Brettanomyces/Dekkera* in red wines. This technique has been proven successful in real musts from the Tempranillo variety and significant differences were obtained in the production of ethylphenols. Such a strategy offers natural protection of this undesirable yeast and volatile phenols concentration below the sensorial threshold can be obtained even after *Brettanomyces* contamination.

## 5 CONCLUSION

The occurrence of volatile phenols in wines has been extensively studied in last decades and there are a lot of reports on the presence of *Brettanomyces* metabolites in wine indicating that it is a worldwide issue. Volatile phenols are very important in term of wine sensory characteristics, because their elevated concentrations in wine are associated with unpleasant aroma. As wine market became very demanding and a big wine competition is present, it is very important to place on the market wine that is from sensory points of view in good condition, i.e. without any flaws. As yeast species *B. bruxellensis* is the main culprit for volatile phenols production and if their presence in wine is noticed, the primary objective should be its elimination or its maintenance at constant level. However, winemakers should consider all factors that influence growth of *Brettanomyces* and volatile phenols production beginning from viticultural practices in order to keep grape healthy, cellar sanitation, physiochemical composition of wine (energy/carbon and precursor sources, alcohol concentration and pH) and very important conditions during wine aging and its storage. Using a starter culture is a good preventive measure that can decrease indigenous fermentation, decreasing the opportunity for *Brettanomyces* to grow. Also, starter cultures can be used to decarboxylate hydroxycinnamic acid precursors of ethylphenol from wine, and forms highly stable, long-lasting pyranoanthocyanins during fermentation i.e. adducts of great colour stability. Generally, from additives the most effective turned out $SO_2$ and its concentration should be orderly checked and maintained on necessary level. Besides, there are differences among grape varieties in hydroxycinnamic acid precursors accumulation and therefore in volatile

phenols production. Further research that will consider different grapevine varieties, different starter cultures including control of wine in different phases of winemaking process should be put in a word.

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**Agris category code**: Q01, Q02, Q04

# Comparison of total polyphenols content and antioxidant potential of wines from 'Welschriesling' and 'Sauvignon Blanc' varieties during ageing on fine lees

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**ABSTRACT**

Phenolic compounds are key components of wine, since they contribute to wine characteristics such as colour, astringency and bitterness. They also act like antioxidants, with mechanisms involving free-radical scavenging that could prevent cardiovascular diseases and cancer. The aim of the present work was to compare the obtained results of total polyphenols content and antioxidant potential (AOP) of several white wines (welschriesling and sauvignon blanc) during ageing on fine lees. The total polyphenols content decreased in average for 16.1 % in welschriesling wines and for 18.7 % in sauvignon blanc wines in the period of three months of wine ageing on lees. In the same period AOP of wines decreased in average for 16.0 % in welschriesling wines and for 8.0 % in sauvignon blanc wines. Expectedly, the samples with added oak chips in grape must had higher antioxidant potential than others.

**Key words:** white wines, antioxidant potential, phenolic compounds, DPPH, yeast lees

**IZVLEČEK**

**PRIMERJAVA VSEBNOSTI CELOKUPNIH FENOLOV IN ANTIOKSIDATIVNEGA POTENCIALA VIN IZ SORT 'Welschriesling' IN 'Sauvignon Blanc' MED STARANJEM NA FINIH DROŽEH**

Fenolne spojine so ključne sestavine vina, ki prispevajo k značilnostim vina, kot so barva, trpkost in grenkoba. Delujejo kot antioksidanti z mehanizmi, ki vključujejo lovljenje prostih radikalov, kar lahko prepreči kardiovaskularne bolezni in raka. Namen dela je bil primerjati dobljene rezultate vsebnosti skupnih fenolov in antioksidativni potencial (AOP) belih sortnih vin laški rizling in sauvignon med zorenjem vina na finih drožeh. Trije meseci spremljanja so pokazali, da se je vsebnost skupnih fenolov zmanjšala v povprečju za 16,1 % v vinih sorte laški rizling in za 18,7 % v vinih sorte sauvignon. Vrednost AOP se je v tem času prav tako zmanjšala v povprečju za 16,0 % pri vinih sorte laški rizling in za 8,0 % pri vinih sorte sauvignon. Pričakovano so imeli vzorci z dodatkom trsk iz hrastovega lesa v mošt večji antioksidativni potencial kot ostali.

**Ključne besede:** bela vina, antioksidativni potencial, fenolne spojine, DPPH, droži

## 1 INTRODUCTION

Phenolic compounds are key components of wine that not only contribute to the organoleptic characteristics of wine but they also are the main cause of colour changes in wine. The light yellow colour as well as the brown colour in white wines is undesirable due to the higher content of phenolics and its oxidation. Nevertheless, phenolic compounds also act as antioxidants, with mechanisms involving free-radical scavenging that

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we are examining (Peréz-Serradilla and Luque de Castro, 2008).

White wines are characterised by lower concentrations of total polyphenols (typically 200-500 mg $l^{-1}$) than red wines because of a lack of red-coloured anthocyanins; white wines are additionally characterized by the predominance of hydroxycinnamic acids (Kilmartin, 2010). Phenolic compounds have received much attention in the prevention of human cardiovascular disorders and cancer due to their antioxidant properties (Paixão et al., 2007). Cells of aerobic organisms are constantly exposed to the effects of reactive oxygen species (ROS) – free radicals. Phenolic compounds have a functional role as they behave as antioxidants against the free radicals. We could say that they increase the antioxidant capacity in the human body after (especially red) wine consumption (Serafini et al., 1998).

We were monitoring the total polyphenols content and antioxidant potential of wine during ageing on fine lees. Yeast cells have been shown to exert a protective effect toward polyphenol oxidation during ageing on lees (Salmon et al., 2002). They have a role as a competing substrate for oxygen in wine. During alcoholic fermentation, yeast cells require oxygen for their metabolic activity and the reactive oxygen species that are produced can potentially oxidise wine polyphenols. At the end of fermentation, phenolic compounds take part in oxygen consumption. After completion of alcoholic fermentation, yeast lees can consume oxygen for up to 3 years in contact with ageing wine. The consumption of oxygen by yeast lees has been ascribed to a mild oxidation of the membrane lipids of the yeast lees that leads to lipid peroxides and further products that may add to the wine flavour (Kilmartin, 2010).

The aim of the present work was to compare the obtained results of total polyphenols content and AOP of several white wines (from 'Welschriesling' and 'Sauvignon Blanc' varieties) during wine ageing on fine lees.

## 2 MATERIALS AND METHODS

### 2.1 Samples

We collected 35 samples of Slovenian wines (18 samples of welschriesling and 17 samples of sauvignon blanc wines) and then examined the content of total polyphenols and AOP of these young wines. Investigated samples were taken one week after alcoholic fermentation was completed (at time t=0). Wine ageing was performed in the cooling room of Biotechnical Faculty at temperature 4 °C. No sulphite was added to welschriesling and sauvignon blanc wines. We were monitoring content of total polyphenols and AOP of wine at different times – at time $t_1$=0, $t_2$=14 days, $t_3$=28 days, $t_4$=48 days and $t_5$=76 days, during wine ageing on fine lees.

### 2.2 Folin-Ciocalteu assay

Total polyphenols were determined by Folin-Ciocalteu (FC) spectrofotometric method (Singleton and Rossi, 1965).

Undiluted samples of white wine and gallic acid (used as a standard) were incubated in sodium carbonate solution (20 %, w $v^{-1}$) and FC reagent for 2 hours at room temperature. The absorbance was measured at wavelength 765 nm. Measured absorbance is proportional to mass concentration of phenolic compounds and it is expressed as gallic acid equivalents (mg of gallic acid per litre of wine). The determination was performed in duplicate and the results are expressed as the mean value.

### 2.3 Determination of antioxidant potential (AOP) of wine with the 1-diphenyl-2-picrylhydrazyl radical (DPPH$^{\bullet}$) scavenging method

Antioxidant potential of wine was determined by DPPH$^{\bullet}$ radical scavenging method (Brand-Williams et al., 1996). DPPH$^{\bullet}$ solution in 99 % methanol was added to 50 µl diluted sample (R=2). After 30 min, the absorbance was measured at 517 nm. For each sample there was a blank (methanol) and reference (DPPH$^{\bullet}$ solution). When antioxidant reacts with the DPPH$^{\bullet}$ radical and radical becomes a stable molecule, a decrease in

absorbance occurs. DPPH• scavenging ability is expressed as concentration of DPPH• in mmol $l^{-1}$. The determination was performed in triplicates and the results are expressed as the mean value.

## 3 RESULTS AND DISCUSSION

### 3.1 Antioxidant potential (AOP) of wines

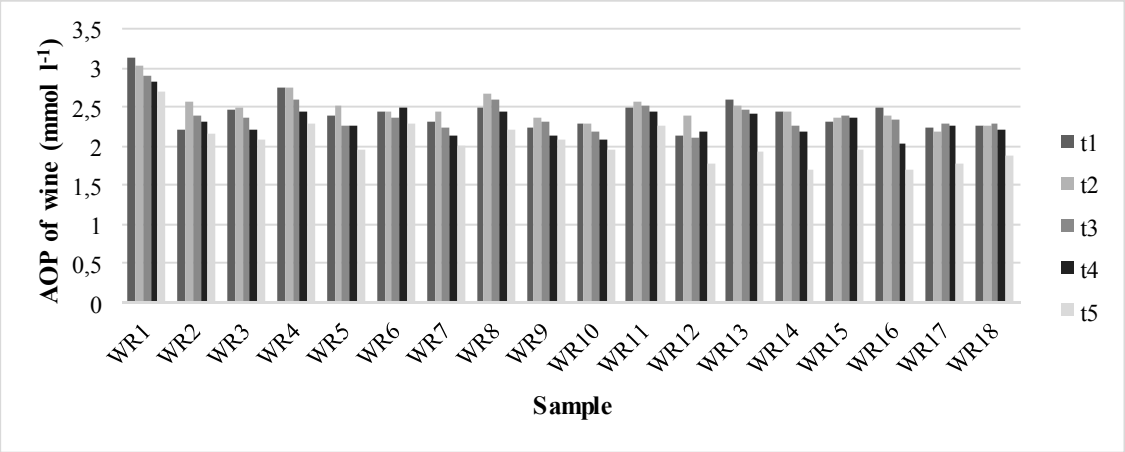


**Figure 1:** AOP of welschriesling wines at different sampling times ($t_1 = 0$, $t_2 = 14$ days, $t_3 = 28$ days, $t_4 = 48$ days and $t_5 = 76$ days) after completion of alcoholic fermentation

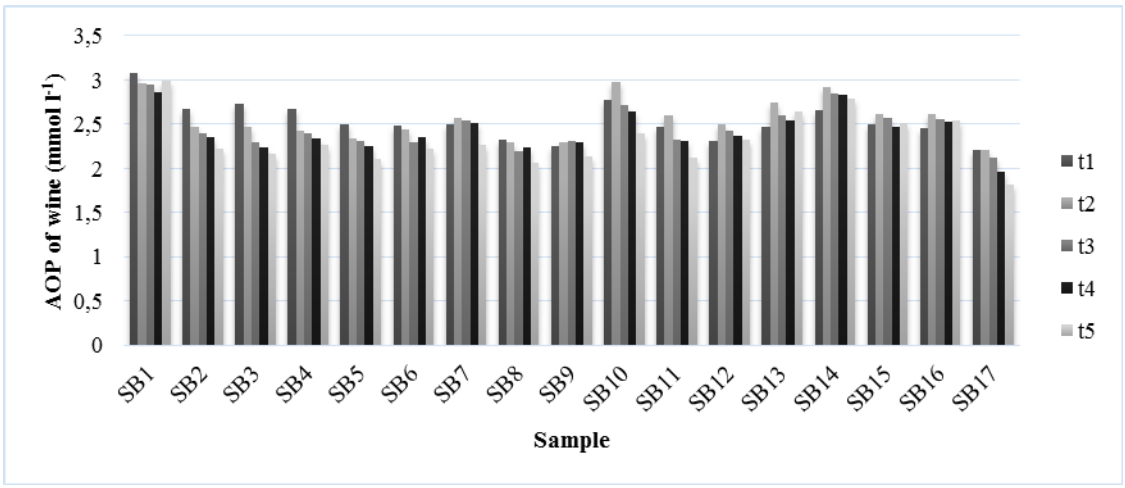


**Figure 2:** AOP of sauvignon blanc wines at different sampling times ($t_1 = 0$, $t_2 = 14$ days, $t_3 = 28$ days, $t_4 = 48$ days and $t_5 = 76$ days) after completion of alcoholic fermentation

The results of the AOP of welschriesling and sauvignon blanc wines are presented in Figure 1 and Figure 2, respectively. Both Figures show the gradually decrease of antioxidant potential of welschriesling and sauvignon blanc wines as a function of wine ageing on fine lees. We noticed the increase of AOP in some of the wine samples that decreased at the next sampling. The most obvious was this phenomenon of the AOP increasing at the second time of sampling in welschriesling wines WR2, WR8, WR11, and in sauvignon blanc wines SB7, SB10 and SB12-SB16. The increase of AOP of wine could be caused by the antioxidant defence system of yeast - the increase in antioxidant content and increase in the levels of antioxidant enzymes including superoxide dismutase and glutathione reductase on yeast entering into the stationary phase, may

constitute adaptive response to the enhanced oxidative stress (Jakubowski et al., 1999).

**Table 1:** Content of reducing sugars (RS), content of total polyphenols (PFT), FC index and antioxidant potential (AOP) after 76 days of wine ageing on fine lees.

| Sample | RS ($g\ l^{-1}$) | PFT (mg GAE $l^{-1}$) | FC index (/) | AOP (mmol $l^{-1}$) |
|---|---|---|---|---|
| welschriesling wines | | | | |
| WR1 (control) | 117.2 | 444.6 | 36 | 2.70 |
| WR2 | 3.1 | 327.6 | 6 | 2.17 |
| WR3 | 1.1 | 322.1 | 6 | 2.08 |
| WR4 | 1.5 | 331.1 | 10 | 2.28 |
| WR5 | 3.6 | 299.5 | 9 | 1.96 |
| WR6 (AM-HT[1]) | 1.9 | 353.1 | 9 | 2.28 |
| WR7 (AM-HT[1]) | 1.4 | 331.1 | 11 | 2.01 |
| WR8 (AM-HT[1]) | 1.4 | 325.8 | 10 | 2.22 |
| WR9 | 1.7 | 324.1 | 7 | 2.07 |
| WR10 | 1.2 | 305.4 | 8 | 1.95 |
| WR11 | 7.7 | 334.1 | 7 | 2.27 |
| WR12 | 3.4 | 297.6 | 7 | 1.76 |
| WR13 | 2.4 | 320.1 | 10 | 1.93 |
| WR14 | 31.4 | 328.6 | 3 | 1.70 |
| WR15 | 1.9 | 328.6 | 10 | 1.95 |
| WR16 | 1.5 | 297.1 | 10 | 1.70 |
| WR17 | 1.4 | 309.0 | 11 | 1.77 |
| WR18 | 1.7 | 322.1 | 10 | 1.87 |
| sauvignon blanc wines | | | | |
| SB1 (control) | 121.7 | 451.1 | 38 | 2.99 |
| SB2 | 2.3 | 320.6 | 7 | 2.22 |
| SB3 | 1.3 | 310.1 | 6 | 2.16 |
| SB4 | 2.1 | 315.6 | 9 | 2.27 |
| SB5 | 3.4 | 298.6 | 9 | 2.11 |
| SB6 | 5.0 | 322.2 | 9 | 2.23 |
| SB7 (AM-MT[2]) | 5.2 | 328.6 | 9 | 2.26 |
| SB8 | 1.0 | 284.1 | 4 | 2.06 |
| SB9 | 1.2 | 252.6 | 6 | 2.14 |
| SB10 | 1.3 | 340.6 | 8 | 2.39 |
| SB11 | 1.3 | 311.7 | 7 | 2.12 |
| SB12 (AM-MT[2]) | 1.1 | 294.9 | 4 | 2.33 |
| SB13 (FR-MT[3]) | 1.2 | 356.6 | 10 | 2.65 |
| SB14 (FR-MT[3]) | 1.7 | 371.6 | 9 | 2.79 |
| SB15 (AM-MT[2]) | 1.5 | 342.6 | 9 | 2.51 |
| SB16 (AM-MT[2]) | 1.7 | 342.6 | 9 | 2.54 |
| SB17 | 9.1 | 233.1 | 7 | 1.81 |

[1] Addition of American oak chips (highly toasted), [2] Addition of American oak chips (medium toast), [3] Addition of French oak chips (medium toast)

The highest antioxidant potential was measured in a control sample SB1, where spontaneous fermentation was performed, and sample SB14 with addition of French oak chips (medium toast). As observed from Table 1, the control samples have the highest amount of reducing sugars ($\gamma_{RS(SB1)}$ = 121.7 g $l^{-1}$ and $\gamma_{RS(WR1)}$ = 117.2 g $l^{-1}$; while the average amount of reducing sugars in

other samples is $\gamma_{RS}$ = 3.3 g $l^{-1}$) and they interfere with free radical DPPH•. Because of that it shows greater antioxidant potential than it should.

The lowest antioxidant potential had sample WR16 with addition of commercial yeast strain EC-1118 (40 g $hl^{-1}$) and nutrient Naturferm (40 g $hl^{-1}$).

On Figure 1 and Figure 2 we can see that wines with added oak chips (samples WR6, WR7, WR8, SB7, SB10, SB12, SB13, SB14, SB15 and SB16) express stronger ability to scavenge DPPH• radical than other samples. AOP of samples SB12-SB16 was even higher at sampling time $t_5$ than at sampling $t_0$. This could be due to extracted polyphenols from oak chips to wine and therefore increasing AOP of investigated samples.

### 3.2 Content of total polyphenols

As observed from Figure 3 and Figure 4, content of total polyphenols is constantly decreasing during wine ageing on fine lees.

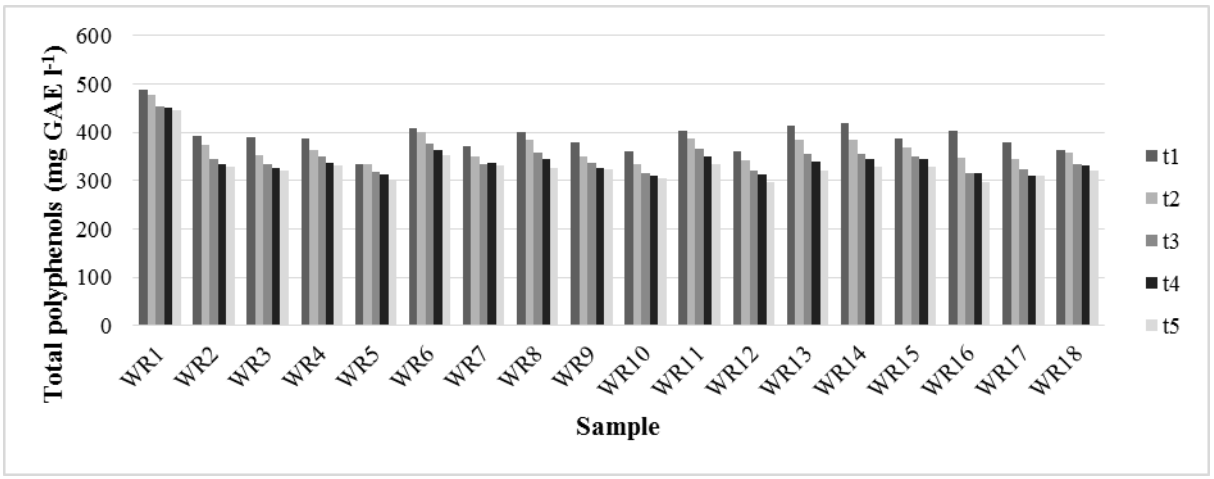


**Figure 3:** Content of total polyphenols in welschriesling wines at different sampling times ($t_1$ = 0, $t_2$ = 14 days, $t_3$ = 28 days, $t_4$ = 48 days and $t_5$ = 76 days) after completion of alcoholic fermentation

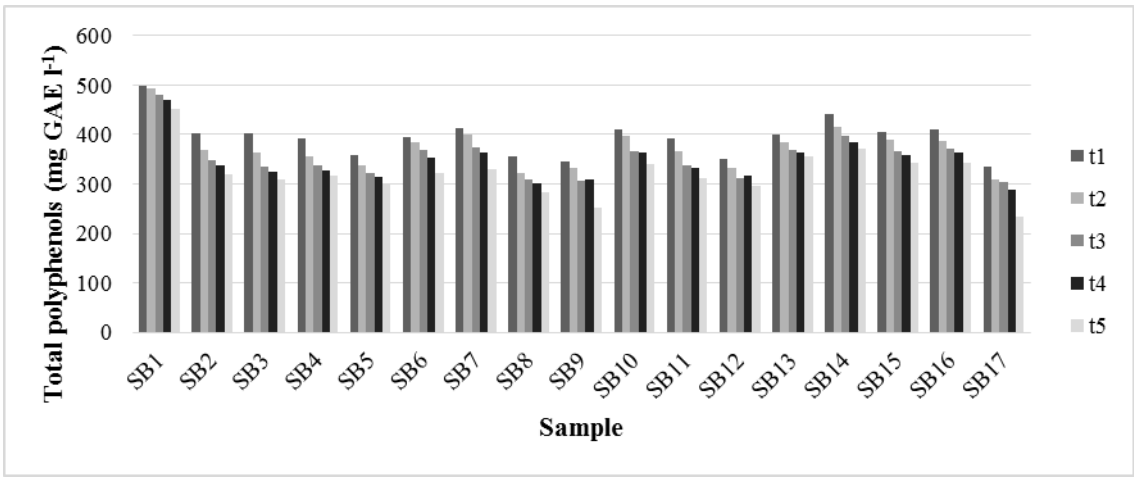


**Figure 4:** Content of total polyphenols in sauvignon blanc wines at different sampling times ($t_1$ = 0, $t_2$ = 14 days, $t_3$ = 28 days, $t_4$ = 48 days and $t_5$ = 76 days) after completion of alcoholic fermentation

The highest content of total polyphenols was determined in samples WR1 and SB1, produced by spontaneous fermentation.

Table 1 lists concentrations of total polyphenols and reducing sugars in both welschriesling and sauvignon blanc wines at sampling time $t_5$ = 76 days. It shows that samples with higher amount of

reducing sugars have considerably higher content of total polyphenols. The disadvantage of using Folin-Ciocalteu method for determination of total polyphenols is that the mentioned reagent reacts nonspecifically with all phenolic hydroxyl groups (-OH), including aromatic amino acids, ascorbic acid, reducing sugars and organic acids (Abramovič, 2011). We could clean up samples by a solid phase extraction (SPE) to remove other reducing compounds and to get more accurate results. Therefore our results indicate higher content of total polyphenols and the correction is needed.

**Table 2:** Correction of total polyphenol content (mg GAE $l^{-1}$) with FC reagent regarding the amount of reducing sugars (g $l^{-1}$) (Košmerl and Kač, 2010)

| Reducing sugars (g $l^{-1}$) | Division factor of total polyphenols concentration |
|---|---|
| 0-10 | / |
| 10-25 | 1.03 |
| 25-100 | 1.06 |
| 100-200 | 1.10 |

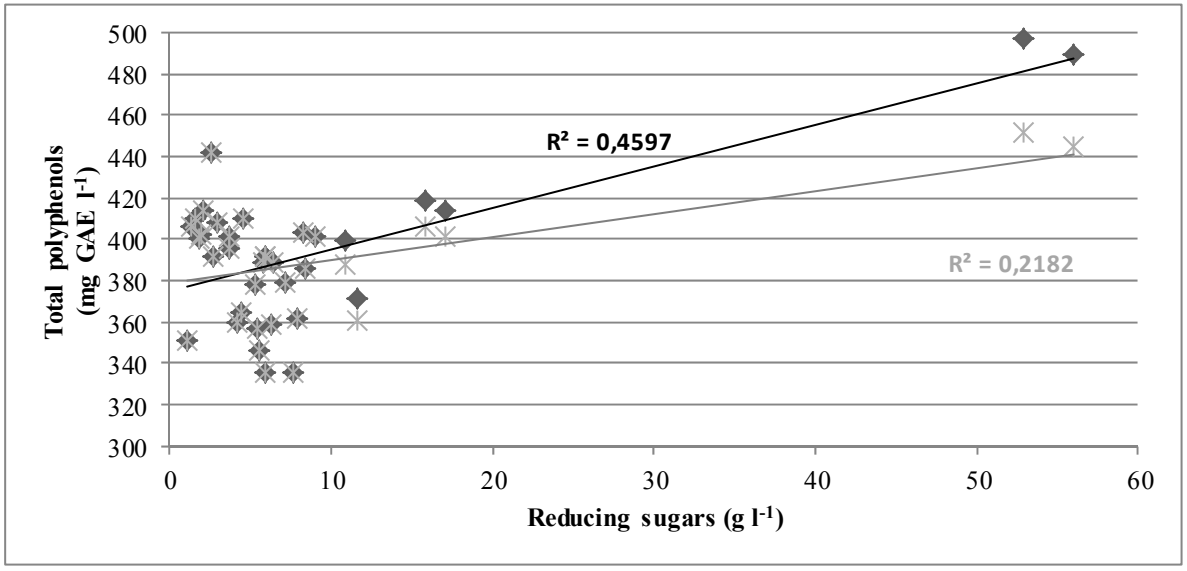


**Figure 5:** Relation between total polyphenols and reducing sugars with (*) and without correction (♦)

The correlation between reducing sugars and total polyphenols (PFT) was even worse after PFT-correction ($R^2$ = 0.2182) in comparison to non-correction ($R^2$ = 0.4597) for our investigated samples.

Expectedly, addition of oak chips in grape must of 'Welschriesling' and 'Sauvignon Blanc' varieties (samples WR6, WR7, WR8, SB7, SB10, SB12-16) resulted in much higher content of total polyphenols. We can assume that polyphenols were extracted from oak chips into wine during wine ageing on fine lees and therefore have increased content of total polyphenols.

### 3.3 Correlation between total polyphenols and AOP of wines

Figure 6 and Figure 7 presents the correlation between total polyphenols content and AOP of welschriesling and sauvignon blanc wines. It shows a strong positive dependence of AOP on the content of total polyphenols in all wines of both varieties.

The linear correlation between AOP of wine and total polyphenols concentration was better for sauvignon blanc wines than for welschriesling wines. From these obtained results we can

conclude that the correlation between AOP and total polyphenols is particularly varietal characteristic as also demonstrated Košmerl and Cigić (2008). Phenolic composition of individual variety has different influence on correlations of total polyphenols with AOP; polyphenols with higher numbers of hydroxyl groups and those having hydroxyl groups in ortho positions in the aromatic rings usually have higher antioxidant potential (Košmerl and Cigić, 2008).

It can be also summarized that the correlation between AOP of wine and total polyphenols concentration has increased during wine ageing on lees.

**Table 3:** Coefficient of determination ($R^2$) for welschriesling wines at different sampling times

| Sampling | Coefficient of determination ($R^2$) |
|---|---|
| $t_1$ | 0.6538 |
| $t_2$ | 0.5850 |
| $t_3$ | 0.7224 |
| $t_4$ | 0.7398 |
| $t_5$ | 0.6530 |

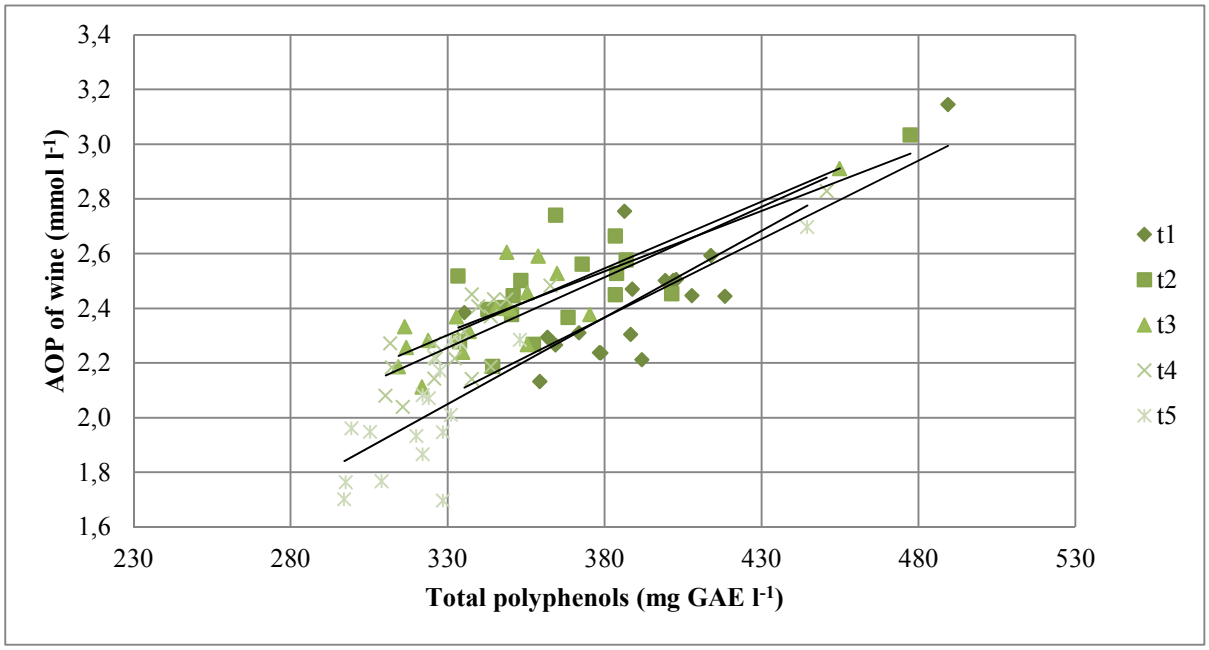


**Figure 6:** Correlation between total polyphenols and AOP of welschriesling wines at different sampling times ($t_1$=0, $t_2$=14 days, $t_3$=28 days, $t_4$=48 days and $t_5$=76 days) after completion of alcoholic fermentation

**Table 4:** Coefficient of determination ($R^2$) for sauvignon blanc wines at different sampling times

| Sampling | Coefficient of determination ($R^2$) |
|---|---|
| $t_1$ | 0.7434 |
| $t_2$ | 0.7141 |
| $t_3$ | 0.7619 |
| $t_4$ | 0.7868 |
| $t_5$ | 0.8467 |

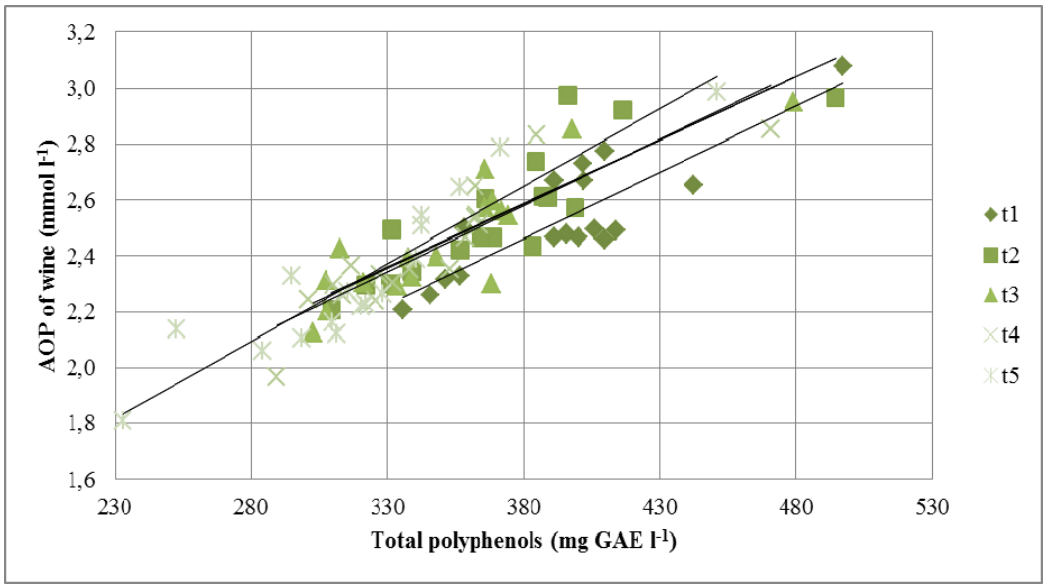


**Figure 7:** Correlation between total polyphenols and AOP of sauvignon blanc wines at different sampling times ($t_1$=0, $t_2$=14 days, $t_3$=28 days, $t_4$=48 days and $t_5$=76 days) after completion of alcoholic fermentation

### 3.3 Correlation between FC index and total polyphenols

In Table 1 are given informative values for FC index. In the first part we examined correlation between FC index and total polyphenols content and in the second part correlation between reducing sugars and FC index.

As shown in Figure 8, correlation between FC index and total polyphenols content was very weak in the case of sauvignon blanc wines and higher for welschriesling wines.

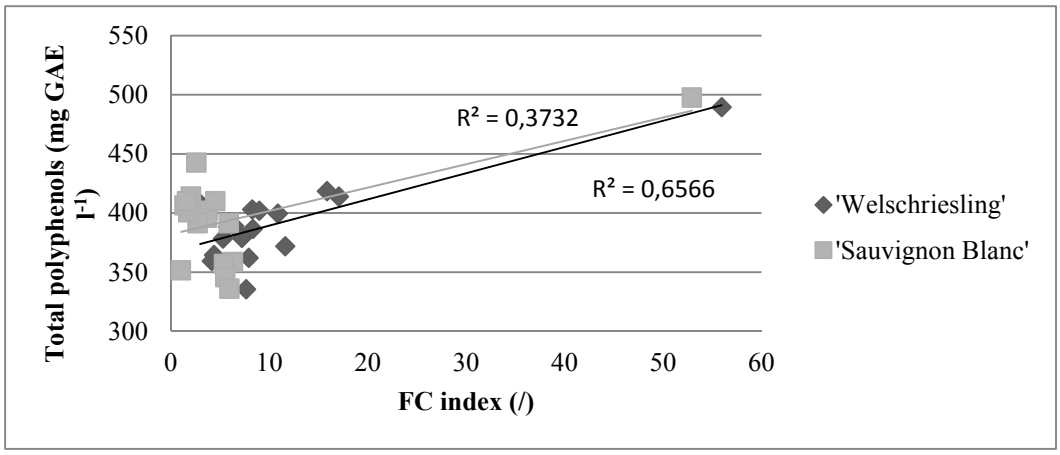


**Figure 8:** Correlation between FC index and total polyphenols content at sampling time $t_1 = 0$

On the other hand Figure 9 presents that correlation between reducing sugars and FC index at sampling time $t_1 = 0$ was strong ($R^2 = 0.6879$) of both welschriesling and sauvignon blanc wines. The correlation between reducing sugars and FC index was even stronger at sampling time $t_5 = 76$ days ($R^2 = 0.8449$). It means that the amount of reducing sugars influence on values of FC index.

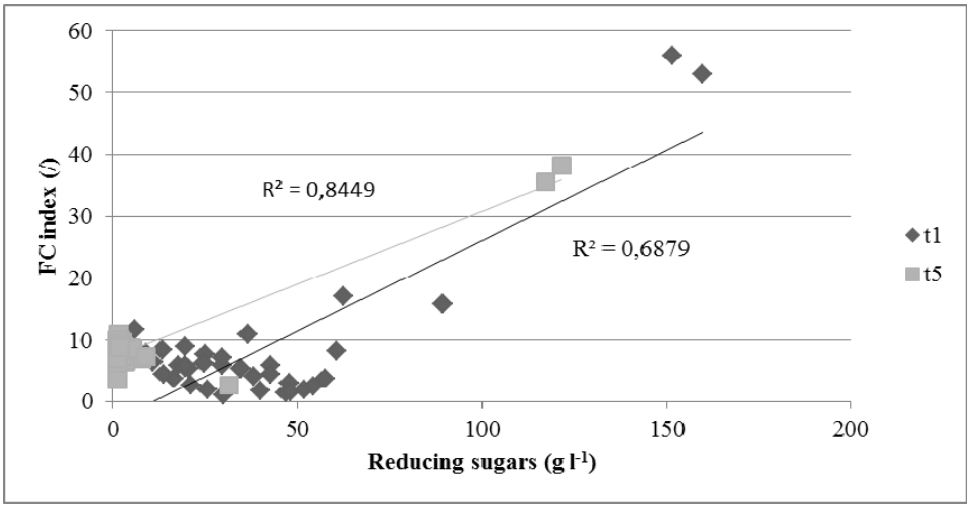


**Figure 9:** Correlation between reducing sugars and FC index at sampling times $t_1 = 0$ and $t_5 = 76$ days

## 4 CONCLUSIONS

Based on the obtained results, decrease of total polyphenols content and therefore decrease of antioxidant potential of wine during wine ageing on lees was observed. The total polyphenols content has decreased in average for 16.1 % (in the range from 36 to 105 mg GAE $l^{-1}$) in welschriesling wines and for 18.7 % (in the range from 44 to 103 mg GAE $l^{-1}$) in sauvignon blanc wines. Antioxidant potential of wines has decreased in average for 16.0 % (in the range from 0.04 to 0.80 mmol $l^{-1}$) in welschriesling wines and for 8.0 % (in the range from 0.09 to 0.57 mmol $l^{-1}$) in sauvignon blanc wines. We would like to emphasize that welschriesling and sauvignon blanc wines are not entirely comparable because oak chips are present in 3 samples of welschriesling wines, while oak chips were added to 7 samples of sauvignon blanc wines.

An increase of AOP was noticed in samples with added oak chips during wine ageing. We can assume that it is because of expected additional extraction of polyphenols from oak chips into wine. For comparison, in samples with added oak chips into grape must the total polyphenols decreased in average for 15.5 % in 76 days, while in the remaining samples they decreased for 18.4 %. AOP of wines with added oak chips has decreased in average for 3.7 %, while in other samples it decreased in average for 15.5 %. The correlation between total polyphenols content and antioxidant potential showed a strong positive dependence of AOP on the content of total polyphenols in all the wines of both varieties.

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# Clone candidates differentiation of grapevine *Vitis vinifera*'Škrlet bijeli' using aroma compounds detected by gas chromatography-mass spectrometry

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## ABSTRACT

The aim of this work was to investigate existence presence and stability of must specific aroma compounds (monoterpenes, $C_{13}$-norisoprenoids, $C_6$-alcohols, alcohols, esters and carbonyl compounds) and which can be used to establish differences among clone candidates of 'Škrlet bijeli' (*Vitis vinifera* L.) grapevine variety. The compounds responsible for the varietal aroma profile were determined by gas chromatography- mass spectrometry (GC-MS), in must samples of ten clone candidates grown on two vineyard sites for three consecutive years. Significant variation among clone candidates is shown in 22 out of the total 35 identified aroma compounds. Significant impact of the vineyard site on the clone candidate's aroma profile was identified. Differences in primary aroma compounds responsible for flavour of 'Škrlet bijeli' variety, linalool, terpinolen, nerol and α-terpineol, were not significant among clone candidates, while remarkable differences were established for β-damascenone. Contrary to expectation, monoterpene geraniol was not detected. Other identified aroma compounds (*trans*-ocimene, 2-methyl-1-butanol, myrcene, α-phelandrene, *cis*-ocimene and 3-methyl-1-butanol) noticeably less participate in total flavour description, but they still enable notable clone candidates discrimination.

**Key words:** clonal selection, must, aroma compounds, gas chromatography-mass spectrometry (GC-MS), multivariate statistical analysis

## IZVLEČEK

### RAZNOLIKOST KLONSKIH KANDIDATOV *Vitis vinifera* 'ŠKRLET BIJELI' V AROMATIČNIH SNOVEH, DOLOČENIH S PLINSKO KROMATOGRAFIJO-MASNO SPEKTROSKOPIJO

Namen tega dela je bil ugotoviti prisotnost in stabilnost specifičnih aromatičnih spojin mošta (monoterpeni C13-norizoprenoidi, C6-alkoholi, alkoholi, estri in karbonilne spojine) ter katere od teh spojin se lahko uporabljajo za razlikovanje klonskih kandidatov sorte *Vitis vinifera* 'Škrlet bijeli'. V ta namen smo spojine, ki so odgovorne za sortni aromatični profil, identificirali s plinsko kromatografijo-masno spektrometrijo (GC-MS), v vzorcih mošta desetih klonskih kandidatov, ki rastejo na dveh lokacijah, v treh zaporednih letnikih. Za določanje razlik med kloni in opredelitev spojin, ki so odgovorne za te razlike, smo uporabili multivariatne statistične metode (analizo glavnih komponent in linearno diskriminantno analizo). Značilne razlike med klonskimi kandidati so se pokazale v 22 od skupno 35 identificiranih aromatičnih spojin. Za aromatski profil klona smo ugotovili prevladujoč vpliv lokacije vinograda. Razlike v primarnih aromatičnih spojinah, odgovornih za aromo 'Škrlet Bijeli', linaloola, terpinolena, nerola in α-terpineola, niso bile statistično značilne med klonskimi kandidati, medtem ko so bile določene pomembne razlike v β-damascenonu. V nasprotju s pričakovanji, monoterpena geraniola nismo določili. Druge določene aromatične spojine (*trans*-ocimen, 2-metil-1-butanol, mircen, α-felandren, *cis*-ocimen in 3-metil-1-butanol) občutno manj sodelujejo pri skupnem opisu sortne arome, vendar še vedno omogočajo razlikovanje klonskih kandidatov.

**Ključne besede:** klonska selekcija, mošt, aromatične spojine, plinska kromatografija-masna spektrometrija (GC-MS), multivariatna statistična analiza

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## 1 INTRODUCTION

Individual clonal selection is the method most commonly used for genetic improvement of autochthonous grapevine varieties, which results by divergent clones. Usually the selection is performed on grape quality parameters such as sugars and acids content, while aroma profile is checked in late selection stages. How the wine aroma profile is important for wine identity and quality and as aroma precursors originated from must, it is very important to include monitoring of must aroma compounds in early stages of clonal selection. Specific varietal wine aroma originates from volatile compounds such as monoterpenes, norisoprenoids, aliphatic compounds, phenylpropanoids, methoxypyrazine and volatile sulfur compounds synthetized in grapes, which in numerous combinations make a unique, distinctive, typical varietal aroma (Coombe and McCarthy, 1997; Ebeler and Thorngate, 2009). Listed compounds can be used for varietal identification (Marais, 1983; Rapp and Mandery, 1986) because characteristic wine aroma of specific variety is attributed to the aroma compounds of grape.

The most important group of grape compounds with the greatest contribution to distinctiveness of aroma variety are terpenes (Marais, 1983; Câmara et al., 2007) to which belongs groups of monoterpene and $C_{13}$ norisoprenoids (Mateo and Jiménez, 2000). The biggest impact on wine primary aroma provide monoterpenes that are present in wine in free form, while they are present in grape in both free and bound glycoside forms. Monoterpens have very important contribution on white wines aroma of Muscat varieties, but also on other aromatic varieties (Mateo and Jiménez, 2000; del Caro et al., 2012). Free monoterpenes are present in less aromatic and non-aromatic varieties in significantly lower concentration (Iyer et al., 2010; Genovese et al., 2013). Aroma precursor's analysis has been used as a strategy to determine the aroma potential of grape both from aromatic and non-aromatic varieties (Loscos et al., 2009). Monoterpenes have strong impact on wine flavor character that is verified by strong correlation of linalool and α-terpineol content and floral description of wine (Komes et al., 2006; Skinkis et al., 2008; Sánchez-Palomo et al., 2012).

Aroma profile is extremely important in clonal selection procedure despite that main subject is always wine aroma profile but not grape aroma. Koch et al. (2010) highlighted a significance of aroma compound 2-methoxy-3-isobutylpyrazine in clonal selection of 'Cabernet Sauvignon' variety as characteristic compound of primary aroma, while Boidron (1995) quoteed that wine of two clones of 'Chardonnay' variety have pronounced Muscat aroma tint in comparison to other studied clones and recognizes this as positive and desirable clone characteristic. Versini et al. (1990) stated that it is for quality of clone grapes comparison necessary to study specific aroma compounds on which content in grapes the effect of environmental is at minimum, they are monoterpenes and by them were defined differences between clones of 'Traminer Red' and 'Chardonnay' variety. Marais and Rapp (1991) concluded that it is possible to distinguish clones based on terpene content. They proved that clones 457/48, 14Gm D35, 925/643 and FR46/106 of 'Gewiirztraminer' variety appeared to have a greater potential to produce aroma-rich and variety-typical wines than N20 Kieselberg. With respect to 'Weisser Riesling', two clones, namely 37 and 327, could possibly be selected as more flavorful than the others. McCarthy (1992) studied grapes of 10 clones 'Muscat à petite grains blanc' variety and found that there was no difference in the free volatile terpene concentration between clones, but there were significant differences in bounded form of monoterpene concentration. In order to assess the suitability of some genotypes for functional genomics studies on terpenol synthesis in grapevine, Duchene et al. (2009) studied two varieties differing in their aromatic pattern: 'Gewurztraminer' and 'Sauvignon Rose' and two clones of 'Chardonnay' (76 and 809). There are evidences that clonal variation, through somatic mutations, can modify the aromatic profile of fruits. Genovese et al. (2013) reported results that showed different aroma profile (free and bound volatile compounds) in 'Aglianica' and 'Uva di Troia' grapes.

Various authors are using different methods for determination of terpenes in grape and wines. Setkova et al. (2007) developed a rapid headspace solid-phase micro extraction-gas chromatographic–

time-of-flight mass spectrometric method for qualitative profiling volatile fraction of wines. Volatile compounds of grapes are responsible of varietal aroma. In order to obtain an appropriate technique to study grape volatile compounds in pulp and skins of 'Muscat' grapes, Sánchez-Palomo et al., (2005) have developed HS-SPME method coupled with GC-MC. Sixteen volatile compounds have been quantified. Prosen et al. (2007) using synthetic solution developed an extraction procedure for the aroma compounds from musts and wines, using solid-phase micro extraction. The method was suitable for analyzing free aroma compounds in must of different varieties and for monitoring of their release after enzymatic or acidic hydrolysis. Coelho et al. (2007) propose headspace-solid phase micro extraction (HS-SPME) for the variety- and pre-fermentation-related volatile compounds of 'Fernão-Pires' (FP) white grape berries. Two $C_{13}$ norisoprenoids, two aromatic alcohols, two C6 aldehydes, and three C6 alcohols were identified by gas chromatography-quadruple mass spectrometry (GC-qMS). Bordiga et al. (2013) suggest using combination of HS-SPME technique with GCxGC/TOF-MS system for the analysis of wine volatile compounds.

In a last few years, not only on Croatian but also on European wine market, there is a growing interest for wines made of local grapevine varieties with distinguish quality. These wines contribute in raising a regional wine identity and tourism potential. Therefore, efficiency of individual clone selection is very important to provide high quality propagating material and revitalization of forgotten local varieties. 'Škrlet bijeli' is autochthonous variety that grows on very limited areas in continental region of Croatia, in Pokuplje, Vukomeričke gorice and Moslavina. Vineyards planted with 'Škrljet bijeli' represent only 0.3 % of all Croation vineyard area or 66 ha. It is characterized by discrete aroma and freshness, which consumers recognize and prices. Up to now on propagating material market for this particular variety was available only material of the lowest category (CAC; Conformitas Agraria Communitatis). Final phase of individual clonal selection, where is studying the most perspective clone candidates is in progress. The aim of this work was (1) to investigate the presence and stability of specific aroma compounds (monoterpenes, $C_{13}$-norisoprenoids, benzenoids, alcohols $C_6$, alcohols, esters and carbonyl compounds) in must of 10 clone candidates of 'Škrlet bijeli' variety applying GC-MS method, with intention of (2) their mutual distinction and identification of clones by multivariate analysis.

## 2 MATERIALS AND METHODS

### 2.1 Samples

Ten clone candidates of 'Škrlet bijeli' variety produced by propagation of elite vines, selected in process of mass positive clonal selection, and planted in two sites, Popovača and Repušnica, during three vintages were investigated. Both locations are in viticulture region of continental Croatia, sub-region Moslavina. Production viticulture zone is B (Winkler et al., 1974). Both field trails were planted in period from year 2001 to 2004.

Clone candidates coded as ŠK-07, ŠK-11, ŠK-29, ŠK-32, ŠK-33, ŠK-57, ŠK-60, ŠK-69, ŠK-74, ŠK-77 represent progeny of individual elite vine selected from old vineyards. All clone candidates originated from mass clonal selection of 'Škrlet bijeli' which was performed on agricultural traits (yield and sugar accumulation) as well as on good vigour of mother plants. In the field trails, each clone candidate was represented with 3-5 vines planted in same row, that were grafted, at the place, with gem originated from elite vine by method „green on green" on virus-free rootstock: Leaf Roll Virusis.(LR1 and LR3) and Raspberry Ringspot Virus (RRSV). Harvest date was determined based on phenotype evaluation and refractometry tracking of sugar accumulation dynamics and was harvested at the same date for all clone candidates at about 85 ºOe as it is common sugar content at harvest of 'Škrlet bijeli'. The basic agricultural traits (yield and sugar accumulation) are listed in Annexes 1-2.

Sampling for aroma compound analysis carried out according to the plan: first vintage year 2006, the average samples of must for four clone candidates ŠK-29, ŠK-33, ŠK-57 and ŠK-69; second (2007)

and third (2008) vintage year, the average samples of must for ten clone candidates ŠK-29, ŠK-32, ŠK-33, ŠK-60, ŠK-69, ŠK-74, ŠK-77, ŠK-07, ŠK-11, ŠK-57 were prepared from both location. Must samples were frozen at – 28 °C and defrost right before analysis.

### 2.2 Head-space SPME extraction

The SPME extraction conditions were 10 ml of sample that was spiked with internal standard of 3-octanol in concentration of 0,0844 μg $l^{-1}$ (Sigma-Aldrich, St. Louis, MO, USA) in a 20 ml glass headspace vials, with addition of 1,5 g NaCl, extraction time of 45 min and extraction temperature of 50 °C under stirring at 350 r $min^{-1}$. The headspace was sampled using a 50/30 μm divinylbenzene-carboxen-poly(dimethylsiloxane) (DVB-CAR-PDMS) coated fiber in a Supelco fiber holder (Bellefonte, PA, USA). After equilibration, the fiber was removed from the sample and the analytes were thermally desorbed in the injector port of the GC.

### 2.3 GC-MS

A multiPurpose autosampler MPS2 (Gerstel GmbH, Germany) with an agitator and SPME fiber conditioning station was used to extract the volatiles from sample vial headspace. All chromatographic analysis was performed using an Agilent 7890A Series GC system with an Agilent 5975C Mass Selective Detector (Agilent, Palo Alto, USA). The apparatus used was equipped with split/split less injector, J&W DB-Wax column (60 m length x 0.32 i.d. x 0.25 μm film thickness (J&W Scientific, Folsom, CA, USA). The temperature program used was 40 °C for 5 min; 4 °C $min^{-1}$ to 230 °C; 20 min at maximum temperature. Carrier gas (He) flow was 1.2 ml $min^{-1}$. Injections of 1 μl were performed in split less mode while the injector port and the ion source were maintained at 230 °C and 250 °C, respectively. Positive electron impact spectra were recorded at 70 eV in a range *m/z* 30 – 250. Mass spectrometric information of each chromatographic peak was compared to NIST (National Institute for Standards and Technology, USA) mass spectra library. Data is given as relative peak area (RPA) ± standard deviation (SD) presented ratio of area peak of identified compound and peak area of internal standard.

### 2.4 Statistical analysis

Significant differences between clones of 'Šklet bijeli' were determined on the basis of the most abundant aroma compounds: linalool, β-damascenone, terpinolen, nerol and α-terpineol by one-way analysis of variance using software package Statistics (version 8.0, Statsoft Inc., Tulsa, USA), while differences between averages of RPA by Student-Newman-Keul test. Principle component analysis (PCA) and linear discriminant analysis (LDA) were performed to classify the grapevine clone candidates regarding to vineyard site and vintage. A total of 35 major aroma compounds were included in analysis. PCA was performed to provide a data structure study over a reduced dimension, covering the maximum amount of the information present in the basic data. It was conducted using software Statistical Package for the Social Sciences (version 15.0 for Windows; SPSS Inc., Chicago, USA) and statistics software (version 8.0; Statsoft Inc., Tulsa, USA).

**Annex 1**: Yield (kg of grape per stock) of clone candidates in investigated vintages and vineyard sites
**Priloga 1**: Pridelek (kg grozdja po trsu) klonskih kandidatov v proučevanih letnikih in lokacijah

| | 2006 | | 2007 | | 2008 | |
|---|---|---|---|---|---|---|
| Clone candidate | Popovača | Repušnica | Popovača | Repušnica | Popovača | Repušnica |
| ŠK-07 | 3.40 | 2.21 | 5.88 | 2.38 | 3.70 | 3.95 |
| ŠK-11 | 2.87 | 0.76 | 3.82 | 2.91 | 3.76 | 3.39 |
| ŠK-29 | 4.04 | 1.40 | 4.63 | 3.73 | 2.79 | 3.30 |
| ŠK-32 | 3.34 | 1.78 | 2.34 | 1.40 | 3.67 | 1.67 |
| ŠK-33 | 2.20 | 1.55 | 4.04 | 3.62 | 3.00 | 2.10 |
| ŠK-57 | 2.82 | 1.41 | 2.32 | 2.91 | 3.09 | 2.13 |
| ŠK-60 | 3.89 | 1.37 | 2.29 | 2.62 | 3.57 | 3.32 |
| ŠK-69 | 2.44 | 1.62 | 3.80 | 4.95 | 3.27 | 4.09 |
| ŠK-74 | 4.20 | 1.58 | 2.25 | 4.33 | 2.96 | 2.13 |
| ŠK-77 | 4.20 | 1.50 | 2.87 | 4.29 | 3.89 | 3.84 |
| Average | 3.34 | 1.50 | 3.32 | 3.22 | 3.33 | 3.02 |

**Annex 2**: Sugar content (g $l^{-1}$) of clone candidates in investigated vintages and vineyard sites
**Priloga 2**: Vsebnost sladkorja (g $l^{-1}$) klonskih kandidatov v proučevanih letnikih in lokacijah

| | 2006 | | 2007 | | 2008 | |
|---|---|---|---|---|---|---|
| Clone candidate | Popovača | Repušnica | Popovača | Repušnica | Popovača | Repušnica |
| ŠK-07 | 205.0 | 186.8 | 187.4 | 229.7 | 179.4 | 179.8 |
| ŠK-11 | 224.3 | 205.2 | 224.5 | 191.2 | 216.4 | 221.0 |
| ŠK-29 | 217.0 | 188.0 | 206.9 | 224.6 | 223.4 | 168.2 |
| ŠK-32 | 215.3 | 170.7 | 245.1 | 215.5 | 211.5 | 188.7 |
| ŠK-33 | 215.8 | 194.2 | 206.8 | 206.0 | 207.6 | 208.2 |
| ŠK-57 | 217.7 | 185.0 | 215.3 | 200.3 | 189.5 | 205.7 |
| ŠK-60 | 196.3 | 162.8 | 222.3 | 186.4 | 168.3 | 153.5 |
| ŠK-69 | 213.0 | 181.3 | 192.5 | 196.1 | 207.0 | 173.4 |
| ŠK-74 | 196.0 | 190.3 | 221.5 | 201.7 | 209.0 | 182.4 |
| ŠK-77 | 186.7 | 196.3 | 201.9 | 194.5 | 217.7 | 177.4 |
| Average | 208.8 | 186.3 | 213.5 | 204.6 | 204.8 | 188.6 |

## 3 RESULTS AND DISCUSSION

### 3.1 Free terpenes content in grape must

In grape must of ten clone candidates 'Škrlet bijeli', the most abundant aroma compounds were as followed: linalool, β-damascenone, terpinolen, nerol and α-terpineol. However, monoterpene geraniol was not identified.

According to the literature, the most usually analyzed aroma compounds in grape must generally are linalool, geraniol, nerol, α-terpineol and β-damascenone (Sánchez-Palomo et al., 2012; Gómez García-Carpintero et al., 2011).

In order to determine if there are differences among clones on the level of free terpene compounds linalool, β-damascenone, terpinolen, nerol and α-terpineol expressed as mean RPA values for all ten clones, the belonging rank of significant differences was determined by analysis of variance. The results are presented in Table 1.

**Table 1** The influence of clone candidates of 'Škrlet bijeli' on the content of linalool, β-damascenone, terpinolen, nerol and α-terpineol (mean value of RPA ± SD) with belonging rank of significant differences, for both vineyard sites through two (n = 4) or three (n = 6) years, respectively.

**Preglednica 1:** Vpliv klonskih kandidatov 'Škrlet bijeli' na vsebnosti linaloola, β-damascenona, terpinolena, nerola in α-terpineola (povprečna vrednost relativne ploščine vrha ± SD) s pripadajočim rangom značilnih razlik, za obe lokaciji tekom dveh (n = 4) oziroma treh (n = 6) let.

| Clone | n | Linalool | | β-Damascenon | | Terpinolen | | Nerol | | α-Terpineol | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | (RPA / $10^6$) | | | | | | | | | |
| ŠK-07 | 4 | 57440 ± 36976 | a* | 10621 ± 4117 | ab | 1863 ± 557 | a | 1599 ± 2137 | a | 1257 ± 1126 | a |
| ŠK-11 | 4 | 32705 ± 38096 | a | 12198 ± 7591 | ab | 1522 ± 1311 | a | 869 ± 1645 | a | 754 ± 1144 | a |
| ŠK-29 | 6 | 61376 ± 88124 | a | 8479 ± 4461 | a | 2436 ± 2744 | a | 2543 ± 4030 | a | 1523 ± 2311 | a |
| ŠK-32 | 4 | 98760 ± 91769 | a | 14019 ± 7740 | ab | 6518 ± 6917 | a | 3168 ± 3799 | a | 2696 ± 2929 | a |
| ŠK-33 | 6 | 96225 ± 108533 | a | 11924 ± 3845 | ab | 5593 ± 4995 | a | 2627 ± 4793 | a | 2270 ± 3104 | a |
| ŠK-57 | 6 | 94418 ± 90699 | a | 16135 ± 6800 | b | 9573 ± 12949 | a | 2396 ± 4452 | a | 2836 ± 4030 | a |
| ŠK-60 | 4 | 89147 ± 95225 | a | 16826 ± 9992 | b | 7078 ± 7568 | a | 2033 ± 3101 | a | 2983 ± 3279 | a |
| ŠK-69 | 6 | 56035 ± 37655 | a | 9442 ± 2654 | a | 4693 ± 4096 | a | 1152 ± 1597 | a | 1356 ± 1404 | a |
| ŠK-74 | 4 | 61547 ± 54192 | a | 13340 ± 5299 | ab | 4455 ± 4217 | a | 1656 ± 2006 | a | 1779 ± 2001 | a |
| ŠK-77 | 4 | 52773 ± 36890 | a | 10821 ± 3911 | ab | 4373 ± 4546 | a | 1092 ± 1194 | a | 1726 ± 1970 | a |

*Student-Newman-Keul test; values in column marked by the same letter are not significantly different ($p \leq 0.05$)

Neither linalool, with the highest RPA values which ranged from 32705 to 98760, nor terpinolen (RPA ranged from 1522 to 9573), nerol (RPP ranged from 869 to 3168) nor α-terpineol (RPP ranged from 754 to 2983) content were not statisticaly significantly different among clone candidates. RPA values of norisoprenoid compound β-damascenone were statistically different and clone candidats ŠK-29 and ŠK-69 had lower RPA values then clones ŠK-57 and ŠK-60). These results were expected because the study was done on progeny obtained by vegetative propagation of the same plant, and selection of ten clone candidates that were subject of this study based only on phenotype selection of clones from population, selection was not include preliminary aroma compounds analysis.

The impact of vintage and vineyard site on the RPA values of linalool, β-damascenone, terpinolen, nerol and α-terpineol in grape must of clone candidates of 'Škrlet bijeli' with the belonging rank of significant differences was determined by analysis of variance. The results were presented in Table 2 and 3.

**Table 2:** The influence of vintage on the content of linalool, β-damascenone, terpinolen, nerol and α-terpineol (mean value of RPA ± SD) in must with belonging rank of significant differences for 10 clone candidates of 'Škrlet bijeli' for both sites.

**Preglednica 2:** Vpliv letnika trgatve na vsebnosti linaloola, β-damascenona, terpinolena, nerola in α-terpineola (povprečna vrednost relativne ploščine vrha ± SD) v moštu s pripadajočim rangom značilnih razlik za 10 klonskih kandidatov 'Škrlet bijeli' za obe lokaciji.

| | | Linalool | | β-Damascenon | | Terpinolen | | Nerol | | α-Terpineol | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Year | n | (RPA / $10^6$) | | | | | | | | | |
| 2006 | 8 | 58837 ± 31215 | a* | 9362 ± 2961 | a | 4701 ± 2467 | a | 246 ± 134 | a | 938 ± 544 | a |
| 2007 | 20 | 19730 ± 12942 | a | 9765 ± 4492 | a | 1073 ± 622 | a | 141 ± 338 | a | 310 ± 385 | a |
| 2008 | 20 | 127626 ± 77314 | b | 15849 ± 6167 | b | 8898 ± 7756 | b | 4460 ± 3445 | b | 3950 ± 2631 | b |
| F exp | | 24.23 | | 8.29 | | 13.67 | | 23.41 | | 2711 | |
| Pr > F | | < 0.0001 | | 0.0009 | | < 0.0001 | | < 0.0001 | | < 0.0001 | |

*Student-Newman-Keul test; values in column marked by the same letter are not significantly different ($p \leq 0.05$); p-value is the significance; Pr > F - This is the p-value associated with the F-statistic. It is used in testing the null hypothesis that all of the model coefficients are 0; F - This is the F-statistic is the mean square model divided by the mean square error; F exp – this is the variance between treatments devided by the variance within treatments

**Table 3:** The influence of vineyard site on the content of linalool, β-damascenone, terpinolen, nerol and α-terpineol (mean value of RPA ± SD) with belonging rank of significant differences for 10 clone candidates of 'Škrlet bijeli', for both vineyard sites through three years.

**Preglednica 3:** Vpliv lokacije vinograda na vsebnosti linaloola, β-damascenona, terpinolena, nerola in α-terpineola (povprečna vrednost relativne ploščine vrha ± SD) s pripadajočim rangom značilnih razlik za 10 klonskih kandidatov 'Škrlet bijeli' za obe lokaciji.

| | | Linalool | | β-Damascenon | | Terpinolen | | Nerol | | α-Terpineol | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Location | n | (RPA / $10^6$) | | | | | | | | | |
| Popovača | 24 | 78129 ± 90991 | a* | 11826 ± 6206 | a | 6288 ± 8190 | a | 2539 ± 3921 | a | 2469 ± 3082 | a |
| Repušnica | 24 | 59333 ± 42874 | a | 12639 ± 5606 | a | 3587 ± 2764 | a | 1377 ± 1778 | a | 1393 ± 1414 | a |
| F exp | | 1.45 | | 0.11 | | 1.74 | | 1.85 | | 2.97 | |
| Pr > F | | 0.235 | | 0.739 | | 0.194 | | 0.182 | | 0.092 | |

*Student-Newman-Keul test; values in column marked by the same letter are not significantly different ($p \leq 0.05$); p-value - this is the significance; Pr > F - This is the p-value associated with the F-statistic. It is used in testing the null hypothesis that all of the model coefficients are 0; F - This is the F-statistic is the Mean Square Model divided by the Mean Square Error; F exp – this is the variance between treatments/variance within treatments

Results of vintage year and vineyard site influence on RPA values of five the most abundant terpene compounds linalool, β-damascenone, terpinolen, nerol and α-terpineol in must (Table 2 and 3), show during year 2008, significantly higher RPA values for all five dominant aroma compounds which indicates favourable climatological conditions for their individual synthesis. At the same time, RPA values for all five the most abundant terpene compounds were not significantly influenced by vineyard site.

It is not possible to make a real judgment on must aroma based on processing data of individual aroma compounds because different combination of compound concentration at the end bring different wine olfactory experience (Robinson, 2011; Botelho, 2008). To determine if there is stable correlation between clones, vintage years and location and all 35 detected aroma compounds, PCA method was used.

Results of PCA analysis imply that the loading values of the variables associated with the first five principal components were as followed: *cis*-ocimene, *trans*-ocimene, myrcene, limonene, geranic oxide and hotrienol, were the dominant variables in the first principal component, which accounted for 32 % of the total variance. The 2-methyl-1-butanol, 3-methyl-1-butanol, ethyl acetate, hexyl acetate, isoamyl acetate, acetaldehyde, 2-hexenal and 2-hexene-1-ol (E) dominated the second principal component that explained up to 26 % of the total variance. The first five principal components thus accounted for 83 % (PC3 14 %, PC4 8 % and PC5 5 %) of the variation among the samples analyzed. Out of these 35 parameters, 12 were recognized by PCA as being less important. Therefore, the remaining 22 parameters were included in the LDA test (Table 4).

**Table 4:** Content of aroma compounds present in grape musts of 10 clone candidates of 'Škrlet bijeli'
**Preglednica 4:** Vsebnosti aromatičnih spojin, prisotnih v moštu 10 klonskih kandidatov 'Škrlet bijeli'

| | **Aromatic compound*** | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample | A** | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S | T | U | V |
| P200629*** | 432 | 9285 | 177 | n.d. | 810 | 44 | | 6 | 76 | 1878 | 2722 | 5 | 19 | 34 | 300 | 60 | 142 | 21 | 49090 | 591 | 24 | 156 |
| P200633 | 355 | 2946 | 134 | 96 | 83 | 474 | 24 | 40 | 697 | 424 | 727 | 59 | 141 | 368 | 2693 | 67 | 83 | 29 | 38395 | 1352 | 105 | 697 |
| P200657 | 373 | 7243 | 235 | 105 | 122 | 442 | 21 | 36 | 685 | 506 | 837 | 60 | 148 | 328 | 2361 | 45 | 119 | 25 | 48568 | 1396 | 115 | 728 |
| P200669 | 491 | 11215 | 178 | 110 | 217 | 536 | 29 | 57 | 815 | 979 | 2180 | 76 | 133 | 421 | 3232 | 57 | 136 | 21 | 46481 | 621 | 138 | 918 |
| P200707 | 328 | 2002 | 3732 | 81 | 86 | 414 | 47 | 52 | 571 | 298 | 551 | 115 | 1299 | 159 | 1182 | 6 | 80 | 42 | 61337 | 12181 | n.d. | 474 |
| P200711 | 237 | 2242 | 2004 | 3 | 56 | 60 | n.d. | n.d. | 72 | 65 | 145 | 9 | 1049 | 23 | 117 | 5 | 46 | 26 | 52399 | 17743 | n.d. | 217 |
| P200729 | 413 | 25950 | 2319 | n.d. | 1364 | 16 | n.d. | n.d. | 31 | 539 | 1120 | n.d. | 725 | 4 | 22 | 3 | 420 | 26 | 77309 | 8422 | n.d. | 80 |
| P200732 | 393 | 8537 | 4659 | 6 | 103 | 112 | 4 | 7 | 93 | 291 | 590 | 18 | 1475 | 38 | 246 | 4 | 185 | 32 | 80501 | 7636 | n.d. | 341 |
| P200733 | 465 | 38886 | 1751 | 35 | 2854 | 175 | 9 | 16 | 235 | 1154 | 1810 | 32 | 339 | 79 | 484 | 4 | 648 | 24 | 65414 | 1070 | 45 | 544 |
| P200757 | 450 | 3459 | 2668 | 53 | 155 | 185 | 10 | 16 | 242 | 486 | 826 | 35 | 741 | 83 | 503 | 3 | 155 | 32 | 67941 | 2637 | 53 | 526 |
| P200760 | 392 | 12400 | 1733 | 17 | 1221 | 152 | 7 | 10 | 193 | 1001 | 2152 | 24 | 368 | 68 | 387 | 7 | 245 | 34 | 58110 | 440 | n.d. | 322 |
| P200769 | 245 | 1328 | 980 | 19 | 118 | 90 | 2 | 6 | 142 | 279 | 574 | 14 | 426 | 43 | 223 | 6 | 80 | 16 | 47649 | 2976 | n.d. | 263 |
| P200774 | 402 | 9047 | 2701 | 7 | 159 | 96 | 3 | 7 | 115 | 366 | 694 | 15 | 915 | 42 | 243 | 6 | 194 | 28 | 62514 | 3801 | n.d. | 227 |
| P200777 | 226 | 2191 | 1786 | 37 | 67 | 304 | 19 | 26 | 326 | 196 | 384 | 51 | 912 | 132 | 862 | 4 | 80 | 32 | 54642 | 11820 | 46 | 590 |
| P200807 | 975 | 72796 | 59 | 12 | 4385 | 223 | 14 | 22 | 190 | 671 | 1483 | 33 | 156 | 93 | 756 | 73 | 2050 | 15 | 56091 | 3964 | 26 | 344 |
| P200811 | 142 | 2249 | 272 | 14 | 45 | 219 | 94 | 66 | 204 | 22 | 64 | 161 | 1588 | 89 | 650 | 10 | 85 | 9 | 36842 | 19595 | 26 | 418 |
| P200829 | 588 | 9445 | 553 | 93 | 675 | 2067 | 170 | 179 | 1386 | 914 | 1471 | 344 | 439 | 571 | 5238 | 30 | 379 | 34 | 123024 | 3368 | 31 | 276 |
| P200832 | 428 | 6993 | 44 | 162 | 143 | 1305 | 128 | 185 | 1905 | 219 | 509 | 263 | 351 | 692 | 6338 | 19 | 196 | 12 | 59320 | 10002 | 42 | 612 |
| P200833 | 884 | 92124 | 120 | 176 | 1602 | 2569 | 251 | 304 | 2390 | 766 | 1389 | 541 | 190 | 885 | 8033 | 338 | 929 | 53 | 84514 | 3376 | 74 | 919 |
| P200857 | 295 | 10949 | 423 | 578 | 133 | 3320 | 263 | 432 | 4905 | 83 | 463 | 534 | 1157 | 1613 | 12311 | 34 | 132 | 70 | 42657 | 17773 | 67 | 672 |
| P200860 | 203 | 1654 | 707 | 349 | 46 | 2318 | 178 | 243 | 2522 | 35 | 235 | 362 | 1174 | 935 | 7460 | 13 | 83 | 42 | 34643 | 18186 | 42 | 407 |
| P200869 | 136 | 1746 | 716 | 136 | 21 | 817 | 82 | 133 | 1380 | 26 | 144 | 146 | 1686 | 452 | 4067 | 10 | 63 | 8 | 29348 | 13378 | 41 | 518 |
| P200874 | 193 | 2127 | 448 | 164 | 36 | 915 | 95 | 130 | 1184 | 72 | 220 | 198 | 1189 | 384 | 3581 | 26 | 162 | 18 | 61264 | 21516 | 52 | 760 |
| P200877 | 151 | 2039 | 813 | 203 | 28 | 928 | 88 | 128 | 1559 | 32 | 160 | 179 | 1938 | 499 | 4139 | 8 | 64 | 7 | 26104 | 13920 | 58 | 870 |
| R200629 | 360 | 7802 | 411 | 9 | 542 | 108 | 91 | 62 | 140 | 1065 | 2007 | 132 | 156 | 78 | 669 | 64 | 234 | 11 | 59173 | 2626 | n.d. | 330 |
| R200633 | 538 | 78267 | 145 | 51 | 4485 | 543 | 36 | 69 | 687 | 1731 | 2985 | 82 | 25 | 412 | 3791 | 67 | 828 | 8 | 41660 | 526 | 91 | 853 |
| R200657 | 833 | 69384 | 154 | 85 | 4046 | 783 | 59 | 82 | 918 | 1767 | 2771 | 134 | 28 | 533 | 4702 | 113 | 1054 | 11 | 48843 | 1000 | 119 | 1007 |
| R200669 | 518 | 90488 | 260 | 36 | 7057 | 499 | 28 | 56 | 538 | 1987 | 3779 | 66 | 22 | 327 | 3078 | 146 | 1624 | 23 | 32974 | 882 | 110 | 900 |
| R200707 | 415 | 62791 | 914 | 19 | 4099 | 180 | 210 | 189 | 157 | 1153 | 1900 | 294 | 487 | 86 | 1095 | 36 | 1513 | 24 | 64440 | 3738 | 50 | 639 |
| R200711 | 224 | 13746 | 909 | 8 | 1861 | 57 | 21 | 22 | 73 | 873 | 1195 | 33 | 475 | 35 | 378 | 12 | 1376 | 53 | 63692 | 3819 | 22 | 203 |
| R200729 | 195 | 26649 | 1334 | n.d. | 722 | 39 | 1515 | 782 | 40 | 418 | 725 | 1775 | 862 | 17 | 271 | 19 | 472 | 22 | 65137 | 10472 | n.d. | 221 |
| R200732 | 468 | 97154 | 1834 | 6 | 7985 | 146 | 1952 | 1025 | 120 | 1326 | 2109 | 2433 | 666 | 73 | 841 | 103 | 3489 | 31 | 74030 | 1305 | 33 | 380 |
| R200733 | 400 | 38595 | 1285 | 4 | 2517 | 101 | 40 | 33 | 97 | 663 | 1323 | 54 | 433 | 56 | 518 | 21 | 2002 | 24 | 59930 | 3558 | 21 | 297 |
| R200757 | 386 | 60035 | 840 | 7 | 4709 | 146 | 108 | 79 | 129 | 1570 | 2030 | 129 | 246 | 78 | 748 | 17 | 2036 | 25 | 80490 | 1710 | 27 | 351 |
| R200760 | 297 | 29803 | 922 | 17 | 1588 | 140 | 39 | 34 | 123 | 468 | 904 | 60 | 525 | 73 | 611 | 12 | 1860 | 25 | 66148 | 11042 | 30 | 460 |
| R200769 | 196 | 2093 | 837 | 4 | 394 | 69 | 3 | 9 | 68 | 876 | 1200 | 10 | 365 | 35 | 331 | 9 | 431 | 21 | 92184 | 4165 | 21 | 180 |
| R200774 | 295 | 11357 | 1045 | 18 | 1087 | 139 | 9 | 20 | 156 | 492 | 787 | 23 | 972 | 80 | 699 | 10 | 764 | 19 | 65886 | 12623 | 38 | 313 |
| R200777 | 300 | 21365 | 2484 | 4 | 1422 | 75 | 4 | 7 | 79 | 1026 | 1350 | 16 | 957 | 32 | 227 | 13 | 516 | 26 | 74599 | 10441 | 18 | 202 |
| R200807 | 1522 | 103793 | 173 | 28 | 6620 | 635 | 127 | 92 | 398 | 1936 | 2760 | 239 | 361 | 161 | 1595 | 81 | 2693 | 24 | 79533 | 2899 | 40 | 418 |
| R200811 | 1166 | 49889 | 109 | 66 | 2100 | 568 | 139 | 112 | 549 | 1231 | 1993 | 251 | 78 | 174 | 1734 | 30 | 1103 | 23 | 83628 | 2525 | 35 | 425 |
| R200829 | 715 | 136871 | 75 | 38 | 22703 | 811 | 1194 | 813 | 656 | 2814 | 4301 | 1725 | 145 | 228 | 2398 | 59 | 2050 | 18 | 53826 | 1804 | 58 | 326 |
| R200832 | 1935 | 164594 | 73 | 103 | 7920 | 1184 | 188 | 205 | 1026 | 1910 | 3172 | 354 | 51 | 431 | 4276 | 40 | 3388 | 45 | 86157 | 1857 | 97 | 1430 |
| R200833 | 730 | 24316 | 75 | 85 | 976 | 676 | 480 | 370 | 811 | 532 | 1062 | 805 | 231 | 263 | 2605 | 35 | 866 | 27 | 73882 | 9542 | 57 | 612 |
| R200857 | 350 | 4684 | 996 | 106 | 269 | 705 | 110 | 135 | 975 | 863 | 1103 | 211 | 752 | 315 | 2938 | 117 | 278 | 23 | 83945 | 7493 | 69 | 850 |
| R200860 | 1403 | 68263 | 162 | 396 | 3655 | 757 | 70 | 100 | 1830 | 1050 | 1745 | 164 | 81 | 424 | 3486 | 19 | 826 | 34 | 66365 | 4360 | 107 | 1121 |
| R200869 | 188 | 2782 | 988 | 42 | 198 | 534 | 61 | 68 | 637 | 298 | 487 | 121 | 1059 | 228 | 1985 | 7 | 126 | 14 | 41303 | 15781 | 35 | 259 |
| R200874 | 427 | 13648 | 99 | 87 | 835 | 721 | 172 | 164 | 933 | 761 | 1204 | 306 | 220 | 336 | 2803 | 18 | 426 | 17 | 65548 | 9222 | 64 | 703 |
| R200877 | 305 | 22093 | 63 | 56 | 1805 | 492 | 54 | 68 | 695 | 948 | 1281 | 110 | 23 | 236 | 2020 | 11 | 410 | 10 | 40714 | 505 | 42 | 370 |

*mean values of aroma compounds expressed in relative peak area (x$10^6$) calculated for three replicates; ** aroma compounds: A=acetaldehyde, B=ethyl acetate, C=hexanal, D=geranic oxide, E=isoamyl acetate, F=myrcene, G=α-felandren, H=α-terpinene, I=limonene, J=2-methyl-1-butanol, K=3-methyl-1-butanol, L=β-phellandrene, M=2-hexenal, N=*trans*-ocimene, O=*cis*-ocimene, P=cinnamen, Q=hexyl acetate, R=6-methyl-5-hepten-2-one, S=1- hexanol, T=2-hexene-1-ol (E), U=Z-linalool oxide, V= hotrienol; ***sample code: location (P-Popovača, R-Repušnica), vintage year (2006-2008), code of clones; n.d.=indication that aroma compound is at level lower to detection limit

### 3.2 Influence of vineyard site and clone candidates on aroma compounds content

Using LDA method, six parameters were selected as the most discriminating variables: *trans*-ocimene, 2-methyl-1-butanol, myrcene, α-phelandrene, *cis*-ocimene and 3-methyl-1-butanol. The other five parameters isoamyl acetate, acetaldehyde, Z-linalool oxide, ethyl acetate and limonene also contribute significantly to better separation among the samples. When the LDA was applied to the data (48 samples, 22 variables), three discriminate functions explained 80 % of the total variance. Function 1 explains 43.8 % of the total variance, function 2 explains 23.4 %. The scores of the samples and parameters for these first two functions are plotted on Figure 1. As it can be seen, the samples are well separated depending on vineyard site and clone candidates. The accuracy of the placement of each sample into 20 groups was 100 %.

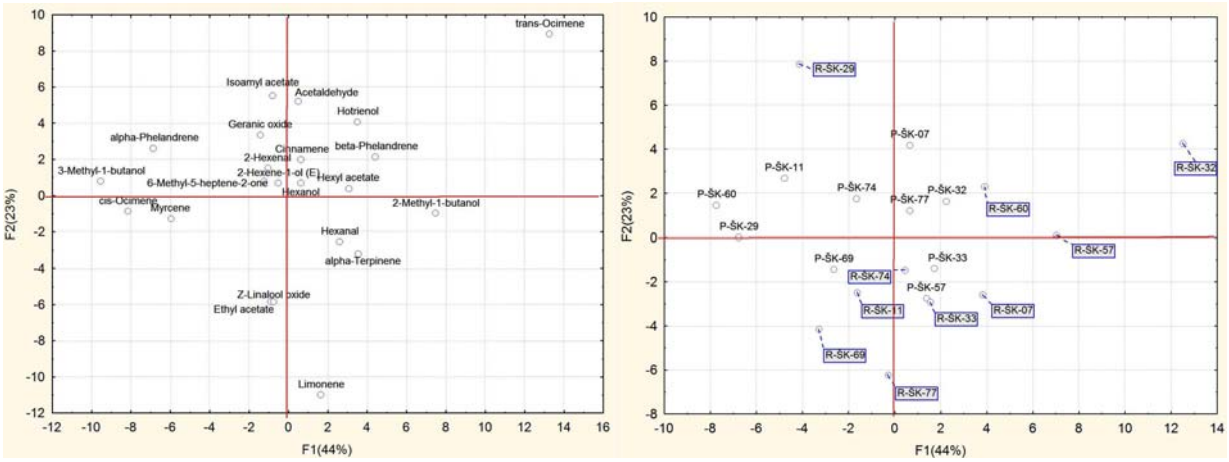


**Figure 1:** Projection of the scores of the samples (right) and parameters (left) for 48 must samples depending on vineyard site and clone candidates in the plane defined by the two standardized canonical discriminant function coefficients.

**Slika 1:** Projekcija rezultatov vzorcev (desno) in parametrov (levo) za 48 vzorcev mošta v odvisnosti od lokacije vinograda in klonskih kandidatov v ravnini, ki ju določata standardizirani funkciji diskriminantnih koeficientov.

From Figure 1 it can be seen, that only two clone candidates (ŠK-69 and ŠK-33) on two vineyard sites (Popovača and Repušnica) do not show differences in analyzed parameters.

### 3.3 Influence of vintage year on aroma compounds in vineyard site Popovača

Using LDA method, four parameters were selected for vintage year of grape production and clone candidate as the most discriminating variables: myrcene and α-phelandrene, as well as trans-ocimene and cis-ocimene. The other ten parameters: (2-hexene-1-ol (E), α-terpinene, acetaldehyde, 2-hexenal, 3-methyl-1-butanol, 2-methyl-1-butanol, isoamyl acetate, hexanal, ethyl acetate and geranic oxide also contribute significantly to better separation among the samples. When the LDA was applied to the data (24 samples, 22 variables), three discriminate functions explained 84.6 % of the total variance. Function 1 explains 48.0 % of the total variance, function 2 explains 26.1 % and function 3 10.5 %. The scores of the samples and parameters for these first two functions are plotted on Figure 2. As it can be seen, the samples are well separated depending on grape production year. The accuracy of the placement of each sample into 10 groups was 1007%. Clone candidates' ŠK-60, ŠK-69, ŠK-77, ŠK-29, ŠK-11 and ŠK-07 during three or two vintage years did not show differences in analyzed parameters.

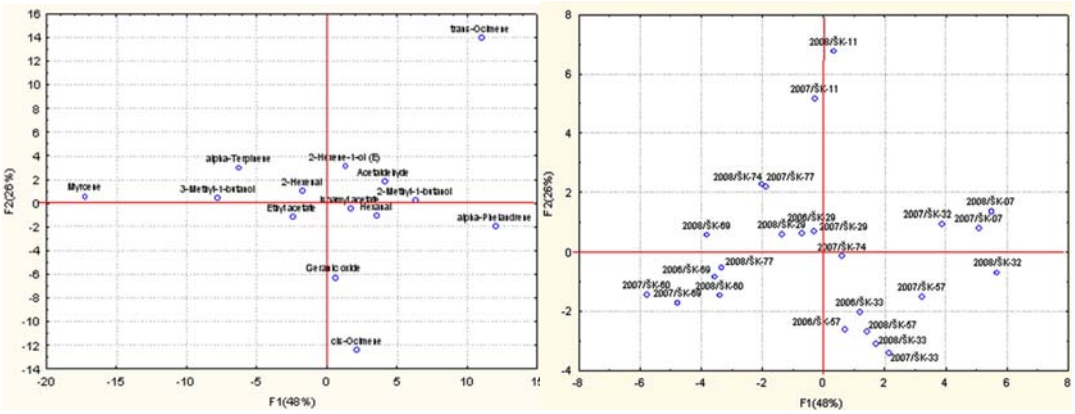


**Figure 2:** Projection of the scores of the samples (right) and parameters (left) for 24 must samples from vineyard site Popovača depending on vintage year and clone candidates used in the plane defined by the two standardized canonical discriminant function coefficients.

**Slika 2:** Projekcija rezultatov vzorcev (desno) in parametrov (levo) za 24 vzorcev mošta iz lokacije vinograda Popovača v odvisnosti od letnika trgatve in klonskih kandidatov v ravnini, ki ju določata standardizirani funkciji diskriminantnih koeficientov.

### 3.4 Influence of vintage year on aroma compounds in vineyard site Repušnica

Using LDA method, six parameters were selected for vintage year of grape production and clone candidate as the most discriminating variables: 3-methyl-1-butanol, ethyl acetate and trans-ocimene, as well as 2-methyl-1-butanol, α-phelandrene and α-terpinene. The other eight parameters: 2-methyl-1-butanol, acetaldehyde, hexanal, geranic oxid, isomyl acetate, myrcene, 2-hexanal and hexyl acetate also contribute significantly to better separation among the samples. When the LDA was applied to the data (24 samples, 22 variables), two discriminant functions explained 82.7 % of the total variance. Function 1 explains 60.8 % of the total variance, function 2 explains 21.9 %. The scores of the samples and parameters for these first two functions are plotted on Figure 3. As it can be seen, the samples from site Repušnica are well separated depending on vintage year. The accuracy of the placement of each sample into 10 groups was 100 %. Clones ŠK-32, ŠK-57, ŠK-77 and ŠK-29 during three or two production years did not show differences in analyzed parameters.

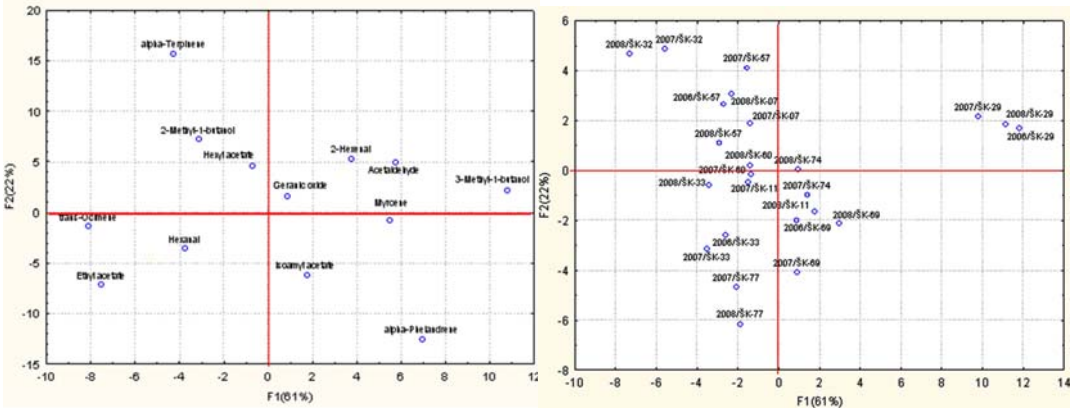


**Figure 3:** Projection of the scores of the samples (right) and parameters (left) for 24 must samples from vineyard site Repušnica depending on vintage and clone candidates used in the plane defined by the two standardized canonical discriminant function coefficients.

**Slika 3:** Projekcija rezultatov vzorcev (desno) in parametrov (levo) za 24 vzorcev mošta iz lokacije vinograda Repušnica v odvisnosti od letnika trgatve in klonskih kandidatov v ravnini, ki ju določata standardizirani funkciji diskriminantnih koeficientov.

If compared both vineyard sites it can be concluded that only two clones ŠK-77 and ŠK-29 during investigated vintage years did not show differences in analyzed parameters.

## 4 CONCLUSION

Analyzing free volatile terpene compounds responsible for primary aroma compounds responsible for flavour of 'Škrlet bijeli' such as linalool, terpinolen, nerol, α-terpineol and β-damascenone were detected, while contrary to expectation, monoterpene geraniol was not detected. Differences of RPA values for the first four compounds were not significant among clone candidates. However, remarkable differences of RPA values among clone candidates were established for some less represented compounds, as eg. norisoprenoid compound β-damascenone. It is noteworthy that significantly higher RPA values for all five dominant aroma compounds were established in the 2008 vintage, which indicates favourable climatological conditions for their synthesis and they can be used as quantitate indicator for prediction of wine aroma intensity. At the same time, RPA values for all five the most abundant aroma compounds were not significantly influenced by vineyard site and therefore could not effectively discriminated among them.

Meanwhile, other aroma compounds were identified (*trans*-ocimene, 2-methyl-1-butanol, myrcene, α-phelandrene, *cis*-ocimene and 3-methyl-1-butanol) that noticeably less participate in total flavour description, but they still enable notable clone candidates discrimination using LDA method according to the individual compounds (only within individual vineyard site). Results of these aroma compounds showed that influence of vineyard site (soil, climate, fertilization and other) was dominant over clone genetic potential when it is placed in other environment (or ecological) conditions, that their RPA values for individual clones and their order (rank) were not consistent on different location. However, within individual site, clones were through three very different vintages retained their mutual relations regarding to aroma compounds synthesis and it was possible to differentiate them.

Analysis of must aroma compounds enabled positive discrimination of two clones ŠK-32 and ŠK-57 in comparison to others. It is necessary to initiate additional comparative study on must and further the wines of these clones in order to find answer what is must aroma profile analysis can help in clonal selection procedure.

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DOI: 10.14720/aas.2016.107.2.20
**Agrovoc descriptors:** databases, information systems, data processing, information processing, information storage, agriculture, pesticides, analytical methods, new technology

**Agris category code**: U30, C30

# Presence of nanotechnology in agriculture: bibliometric approach

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**ABSTRACT**

Increasing number of scientific publications points to quick developments in the field of nanoscience and nanotechnology. Nanotechnology offers potentials of unimaginable proportions. Innovative possibilities present themselves in many areas of human activity, including agriculture, for example in precision farming, reduction of pollution and increasing crop yields. We bibliometrically assessed interactions between nanotechnology and agriculture. With co-word analysis in particular, we examined aspects of agro-nano applications related to plant protection. In order to analyze and map the structure of knowledge, we employed selected terms from a general citation database Web of Science (WOS) as well as specialized bibliographic database CAB Abstracts which covers life sciences with a special emphasis on agriculture. Our thematic maps (visualization) present some principal themes and relations among them. Pesticides, biosensors and detection are the main keywords in the network of words from article titles and network of the KeyWords+. Analysis of controlled terms (descriptors, classification codes) from CAB Abstracts in connection with pesticides shows two important directions of research: pollution and environmental topics, and topics related to human health, experimental animals and related.

**Key words:** bibliometrics, scientometrics, co-word analysis, nanotechnology, agriculture, pesticides, databases

**IZVLEČEK**

**UPORABA NANOTEHNOLOGIJE V KMETIJSTVU: BIBLIOMETRIČNI PRISTOP**

Naraščajoče število znanstvenih objav kaže na hiter razvoj področja nanoznanosti in nanotehnologije. Nanotehnologiji pripisujejo danes potencial neslutenih razsežnosti. Inovativne možnosti reševanja aktualnih problemov se kažejo na vseh področjih človekovega delovanja, s ciljem preciznega kmetovanja, zmanjšanja onesnaževanja in povečanja pridelka, tudi na področju kmetijstva. V našem delu smo prepletanje nanotehnologije s področjem kmetijstva bibliometrično ovrednotili. S sobesedno analizo smo proučili predvsem tisti vidik agro-nano aplikacij, ki se nanaša na fitofarmacevtska sredstva. Pri analizi in kartiranju strukture znanja smo uporabili različne ključne besede iz splošne zbirke WOS in specializirane zbirke CAB Abstracts, ki zajema širše področje kmetijstva. S tematskimi kartami smo prikazali najbolj poudarjena vsebinska področja in relacije med njimi. Osrednje ključne besede v omrežju besed iz naslovov člankov in v omrežju KeyWords+ se nanašajo na fitofarmacevtska sredstva, biosenzorje in detekcijo. Analiza deskriptorjev iz zbirke CAB Abstracts izpostavi predvsem dve pomembni smeri raziskav; onesnaževanje oziroma okoljske študije ter vsebine, ki se nanašajo na zdravje človeka, poskusne živali in s tem povezane modele proučevanja.

**Ključne besede:** bibliometrija, scientometrija, sobesedna analiza, kmetijstvo, fitofarmacevtska sredstva, nanotehnologija, bibliografske podatkovne zbirke

## 1 INTRODUCTION

Our era is marked by the transition towards the knowledge society. New technologies, such as biotechnology, nanotechnology and information technologies are at the forefront, promoting development in other areas (Roco and Bainbridge, 2002). Nanotechnology in particular is hailed as the new technological revolution, which has potentials to influence the society more than the industrial revolution. As indicated by Wood et al. (2003) "it is so profound that it will touch all

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aspects of economy and society". Substantial financial resources are allocated to research and development in this field (National …, 2011). The concept of "nano" represents something very small. Nanoparticles, whose specific properties nanotechnology exploits, are those small pieces of material, in which at least one dimension encompasses less than 100 nm (Commission ..., 2011). The very small size of these particles gives reason for very specific physical, chemical and biological properties. In comparison with the particles in larger dimensions, the nanoparticles are more chemically active, which can be favourable, if some chemical reaction is desirable, or negative if it is not. Although nanomaterials (natural or intentionally produced) are already present in many aspects of our life, the understanding its potential toxicity is still in its infancy (Tarver, 2006; The potential ..., 2009). Regulations governing the risks to human health and environment are very inadequate (Cush et al., 2012).

Employing innovative approaches nanotechnology has opened up new possibilities for the tackling of problems related to food safety and human health. In conventional agriculture intensive use of agrochemicals pollutes soil, groundwater as well as crop products. The use of encapsulated nanomaterials can reduce potentially toxic agrochemicals by employing controlled delivery (e.g. quick-release of agrochemicals, moisture-release etc.). Nanotechnology also has the capacity to decompose harmful components (Manjunatha et al., 2016). The reason is in specific characteristics of nanoparticles whereby, due to the small size of particle and consequently larger surface area, different systemic activity, solubility and mobility is reached. It has been predicted that nanotechnology will completely transform food industry in the future, including processing, packaging, and therefore also consumption (Tiju in Morrison, 2006; Rai in Ingle, 2012). The applications of nanotechnology in the field of agronomy have been summarized by various authors (Nanoscale …, 2003; Opara, 2004; Scrinis and Lyons, 2007; Ruffini Castiglione and Cremonini, 2009; Ghormade et al., 2011; Misra et al.., 2013; Huang et al., 2015: Manjunatha et al., 2016) as follows:

- controlled release of nano-agrochemicals used in plant nutrition and protection (also growth hormones or herbicides) and subsequent reduction of toxicity and environmental pollution (less loss through leaching or leaking),
- controlled delivery of genetic material to plant cells due to the small size of nanoparticles (molecular treatment for development of plant resistance to stress, pests or diseases),
- detection of different needs of plants connected with nutrients, diseases or pesticide residues by sensitive sensors,
- monitoring soil/environmental conditions and plant growth with sensors,
- detoxification of pesticide (herbicide) residues (e.g. atrazine, triazine); to avoid limitations in the selection of crops in rotation.

Science has created a huge body of scientific and technological data, information and knowledge in connection with nano technologies. As is evident in Figure 1, the number of records with general "nano" content has been constantly growing, especially in recent years. Similar increase of this topic, in relation to plant protection and fertilizers, has also been noticed by Gogos et al. (2012).

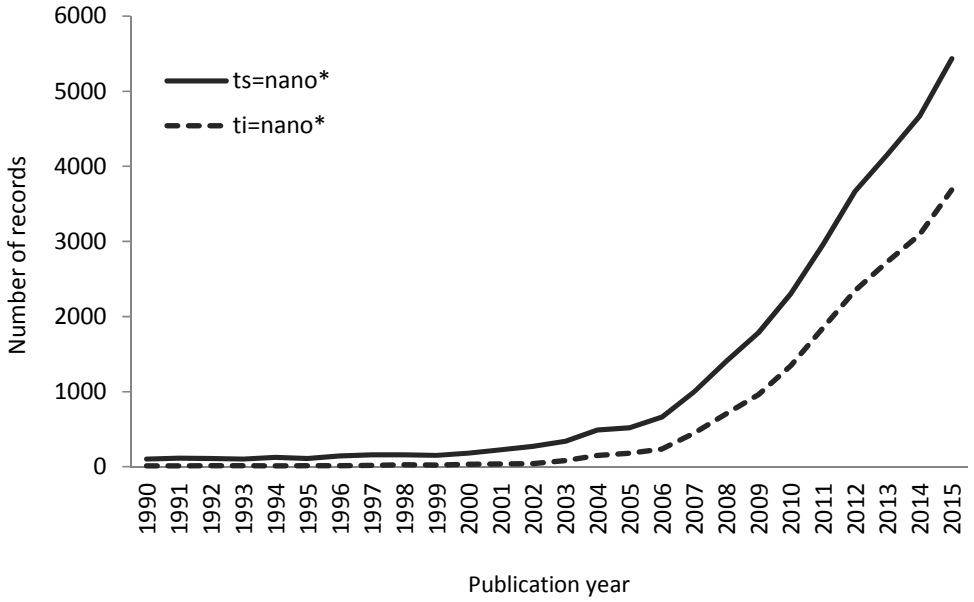



**Figure 1:** Number of records found in WOS regarding the search word-root nano* in an article title (ti), and records found by nano* in topic field (ts)

Fast development and promising scientific discoveries in the field of nanoscience and nanotechnology has also attracted attention of information science, especially bibliometrics. Thus, this field has become a model subject in many studies of this kind. Huang et al. (2011) identified more than 120 such studies. Much information is now available, however, tracking, monitoring and mapping thereof in an ever-changing system of scientific discoveries has become a very complex task. Using bibliometrics we can thus evaluate development of science by studying different models based on topics, authors, institutions, countries, etc. in a comprehensible way through quantitative studies, statistical analysis and graphical vizualisation.

Co-word analysis is a bibliometric technique which can identify topics and relations among the topics. This method uses patterns of co-occurrence of words or phrases. This helps to understand associations between various concepts within a subject area. Namely, appearance of two keywords or phrases within the same document shows the relationship between topics to which they relate (Cambrosio et al., 1993).

In this work we intend to assess relations between nanotechnology and agriculture. Since the most important part of agro-nano applications is related to pesticides, as reported in literature (e.g. Khot et al., 2012), we analyzed more specifically this particular area. We aim to examine the knowledge structure with co-word analysis where we study the patterns of co-occurence of words or phrases (concepts) in specific sets of database records, in order to identify research activities within a scientific area. Research activities in this field as demonstrated by co-word structure, and relations between different research topics, will be visualized through thematic maps. We wish to identify the principal themes and relations among them.

## 2 MATERIALS AND METHODS

We used the data retrieved from the three Web of Science Citation Indexes – SCI (Science Citation Index Expanded), SSCI (Social Science Citation Index), and A&HCI (Arts & Humanities Citation Index) accessed via WOS Core Collection (Thomson Reuters), as well as the data from CAB Abstracts database (CAB International) which is a principal international database for applied life sciences in relation to agriculture, environment, veterinary sciences, food science and nutrition.

In order to follow research topics we monitored different subject concepts, such as key words (descriptors), title words and classification codes. For the purposes of configuration and assessment of experimental data we prepared several operational databases in order to create and visualize different networks. In WOS we employed words from article titles, as well KeyWords+. These keywords are "index terms created by Thomson Reuters from significant, frequently occurring words in the titles of an article's cited references". Based on WOS classification, we additionally analyzed the scatter of content across different subject areas (WOS categories). In CAB database, we used subject headings (descriptors) and CABIcodes. Both codes and descriptors are based on proprietary subject indexing of records in this database. CABIcodes are classification codes which define the broader subject scope of articles. Every record has at least one CABI code. Usually, there are more.

To delineate the scope of the field of nanoscience and nanotechnology we used the union of complex queries (Warris, 2004; Porter et al., 2008; Maghrebi et al., 2011) which defined the selection of relevant database records. These search methodologies were described in detail in our previous work (Stopar et al., 2016). Since important agro-nano applications are related to pesticides, our analysis mainly focused on this aspect ("nano-pesticide"). In addition, we combined the above search query (i.e. delineation of the field nanoscience) with concepts which apply to pesticides in a general sense, such as pesticid*, insecticid*, herbicid*, fungicid* and acaricid*. In case of CAB Abstracts, the share of those nano-related records which also relate to pesticides amounts to some 14 %. In case of WOS, this share is only 0.4 %. Data was retrieved at the end of 2014.

Evaluation of data was conducted with the use of a bibliometric tool Bibexcel (Persson, 2010). Integral part of this tool is an algorithm which performs the stemming of words. In our experiment, such words were used to identify co-occurrence of article-title terms. With the process of stemming, inflected or derived words were reduced to their word stem or root form (e.g. nanoparticles/nonoparticl; determination/determin; detection/detect; pesticides/pesticid; application/ applic).

Co-word analysis was used as a technique for the identification of relations and connections between various topics through the frequency of terms. It is presumed that such co-words analysis displays research activities within a scientific field. Visualization was prepared with the program Pajek, which is a program package for analysis and visualization of large networks (Mrvar and Batagelj, 2016). This software provides a possibility to display (visualize) networks and groups. It can elucidate important "knots" and individual points (vectors). It also reveals links between the points. The position on a picture and the strength of links show relations among the items. Images were prepared in vector EPS format (Encapsulated PostScript). GSview was used for this purpose.

## 3 RESULTS AND DISCUSSION

### 3.1 WOS Core Collection (SCI, SSCI, AHCI) - KeyWords+ and title words

First part of the experiment presents the data based on WOS. The methodology described in the Methods section retrieved 3994 records.

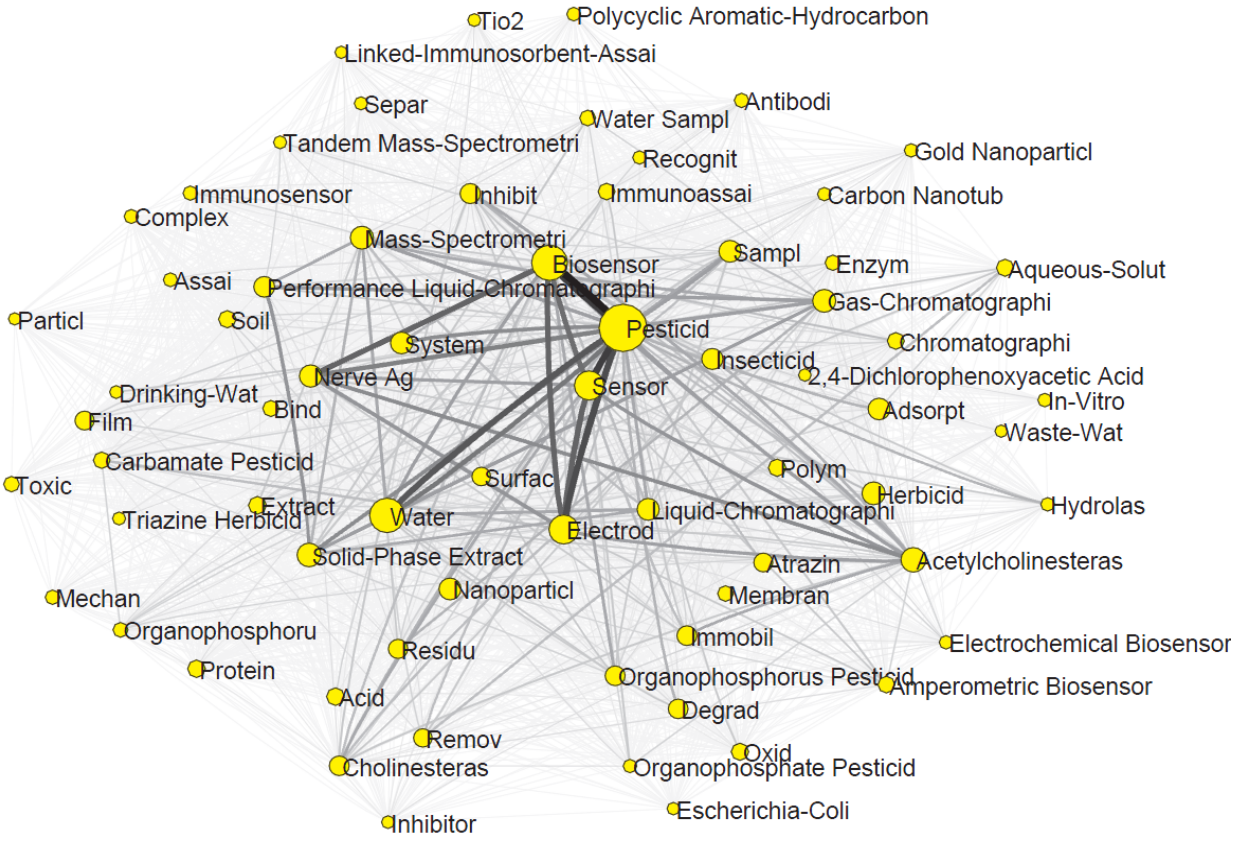
Analysis of the scatter of content across different WOS categories shows that the records have been indexed with 207 different WOS categories. Some categories occur with negligible frequency. In terms of content the categories cover areas of analytical chemistry, electrochemistry, biochemistry, biophysics, biotechnology, as well as areas relating to environmental science, food technology etc. About 36 % (1441) of records are mapped to WOS category *Chemistry, Analytical.* The category *Nanoscience & Nanotechnology* has been assigned to less than 8 % of records (305).

The highest number of records was found in the journal *Biosensors & Bioelectronics,* followed by chemistry-related journals such as *Analytica Chimica Acta*, *Talanta*, *Analytical Chemistry*,

*Sensors and Actuators B-Chemical*, *Journal of Agricultural and Food Chemistry*, *Journal of Chromatography A* etc.

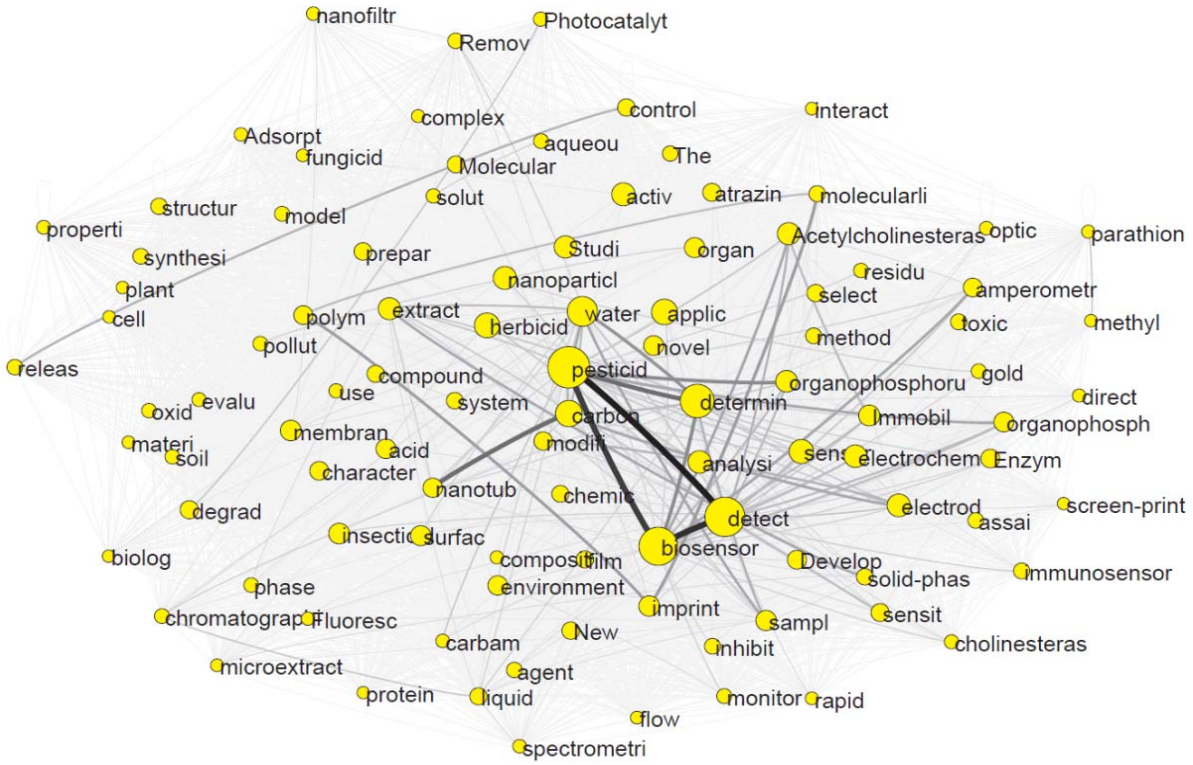
This sample of data was further assessed with co-word analysis. We used word-roots from article titles and KeyWords+. Figure 2 presents network of 70 KeyWords+ which occur with the highest frequency. In Figure 3, network of 100 most frequent title words is shown. As explained in Method section, word-roots have been used in both cases: we mapped related concepts to the same root in order to reduce the number of seemingly different concepts which, however, denote the same meaning.

The words related to the detection of pesticides, along with detection of biosensors, occur most frequently as is evident from circle size and ties between the words.




Legend:
circles: KeyWords+ / circle size: number of records / labels: the names of the most frequent KeyWords+ / lines: ties between pairs of KeyWords+

**Figure 2:** The network of the 70 KeyWords+ (word-roots) with the highest occurrence (WOS; sample: nano_pesticides)




Legend:

circles: words from article titles / circle size: number of records / labels: the most frequent words / lines: ties between words

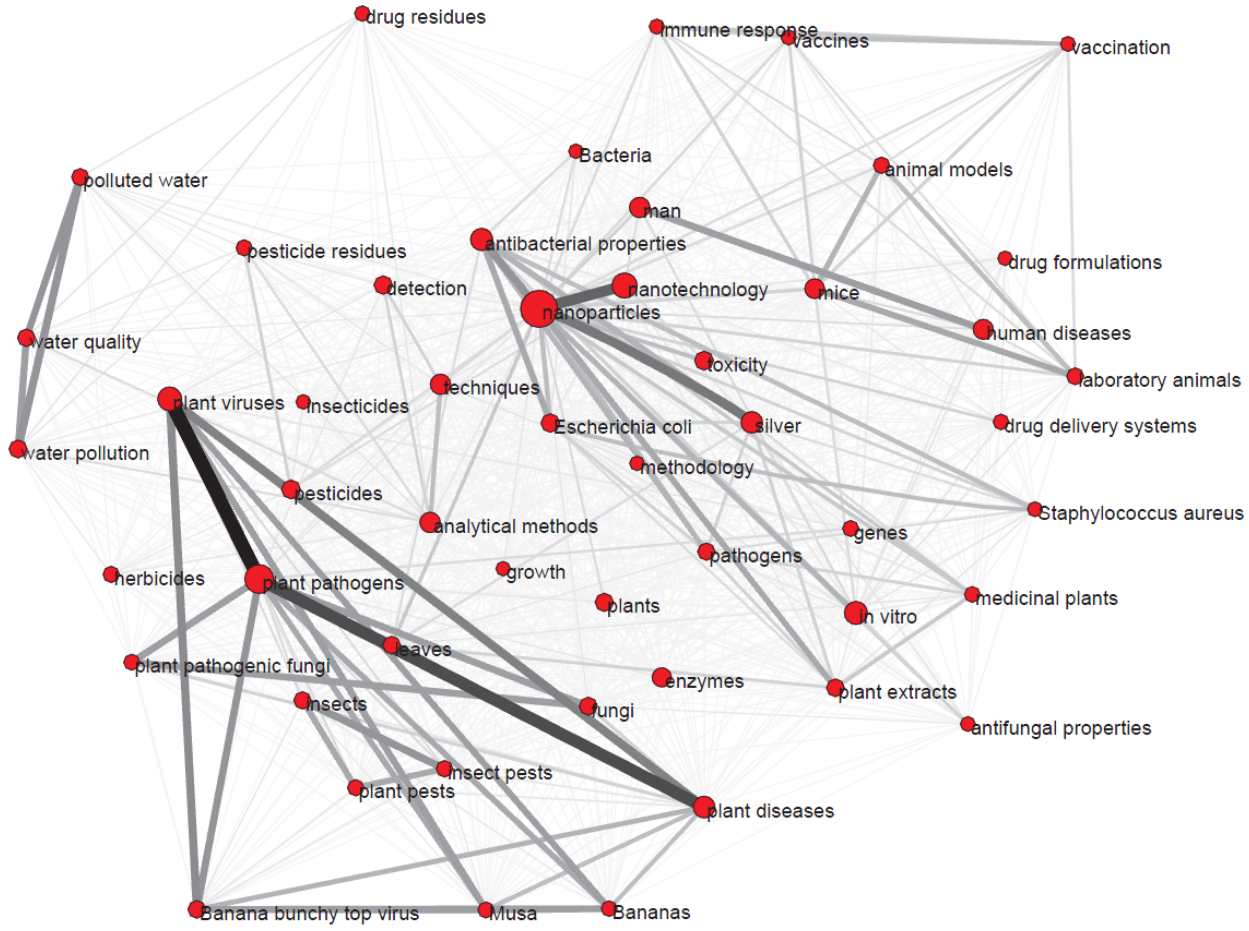
**Figure 3:** Network of the 100 words (word-roots) from article titles with the highest occurence (WOS; sample: nano_pesticides)

Figures 2 and 3 also indicate several other directions of research, for example, controlled release of pesticides, monitoring, degradation, detection of residues (e.g. herbicide, atrazine, triazine), nanoimprinting techniques (e.g. polym - imprint), studies on a molecular level, etc.

In the above case we employed the terms from the titles which are characterized by natural language. The terms in such context have a meaning only in relation to one another. At this point we wish to emphasize the importance of the specialists in the identification of relevant terms and detection of the topic in question. Thus, any similar results will strongly depend on terminology employed.

### 3.2 CAB Abstracts - descriptors and CABI codes

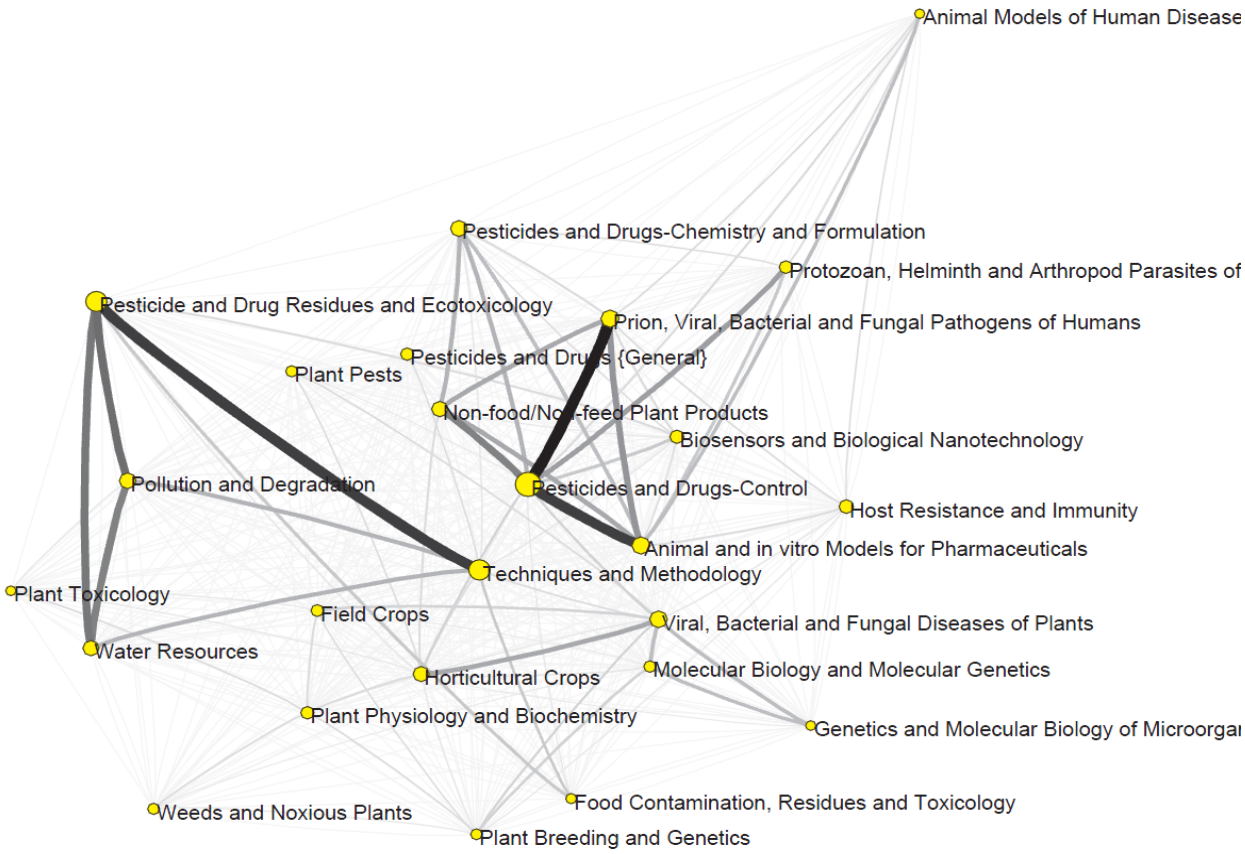
The second part of the experiment was based on CAB Abstracts. Data from this database has already been used for various bibliometric assessments (e.g. Bartol in Hočevar, 2011).




Legend:
circles: CAB descriptors / circle size: number of records / labels: names of the most frequent CAB descriptors / lines: ties between pairs of CAB descriptors

**Figure 4:** CAB descriptors (subject headings) with the highest occurrence (CAB Abstracts; sample: nano_pesticides)

The analysis of records related to the use of pesticides (sample: nano_pesticides; CABI codes and descriptors; Figures 4 and 5) indicates several important directions in the network. These directions are especially expressed in the topics of pollution and environmental studies, as well as topics related to human health and experimental animals (in relation to the models of animal examination). In Figure 5 the strongest links are detected between CABI codes *Pesticides and Drugs-Control* and *Prion, Viral, Bacterial and Fungal Pathogens of Humans*, as well as *Animal and in vitro Models for Pharmaceuticals.* There also exists a specific "nano CABI code" *Biosensors and Biological Nanotechnology* which shows connections with pesticides.



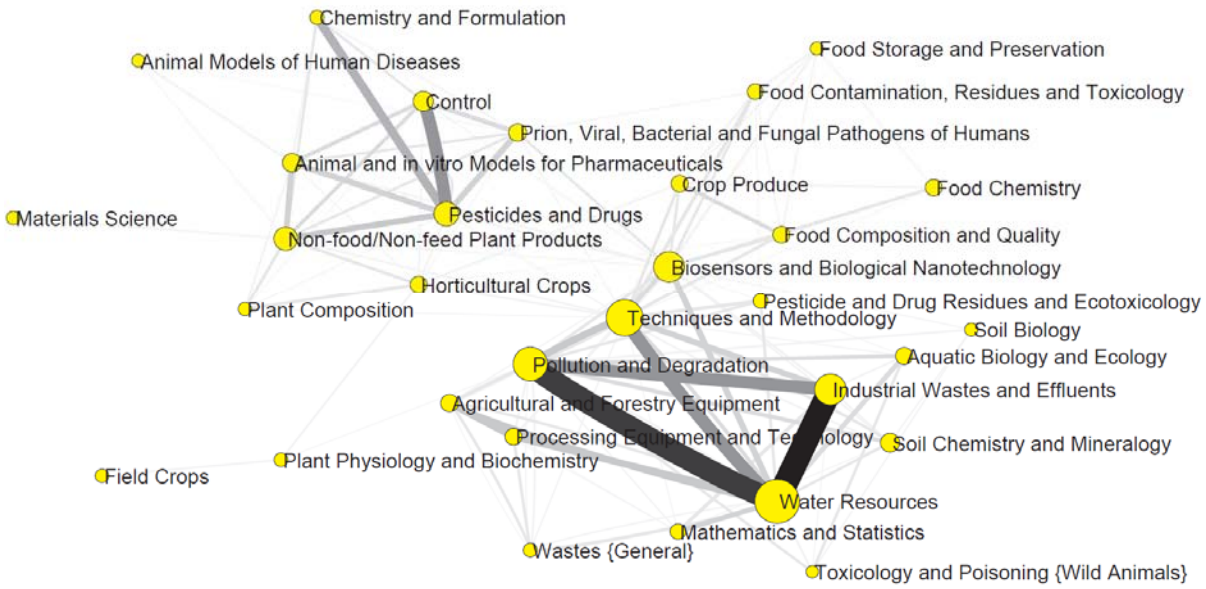

Legend:

circles: CABI codes / circle size: number of records / labels: the scope of the most frequent CABI codes / lines: ties between pairs of CABI codes

**Figure 5:** CABIcodes with the highest frequencies (CAB Abstracts; sample: nano_pesticides)

At the end, we present an additional analysis which is not directly related to the two above pesticide-based CAB models. CAB Abstracts database is dedicated to applied life sciences in relation to agriculture, environment, veterinary, food and nutrition so we also wished to examine the presence of nanoscience in a broader context - not just as applicable to only the more narrow aspect of pesticides. In this sense, we present results of co-word analysis of CABI codes which investigates the context of nanoscience more generally (Figure 6).

The results are based on the occurrence of word root nano* in article titles (publication year 2012). Figure shows CABI codes with the highest occurrence.




Legend:

circles: CABI codes / circle size: number of records / labels: the scope of the most frequent CABI codes / lines: ties between pairs of CABI codes

**Figure 6:** Network of the CABI codes (CAB Abstracts; search query: nano*.ti and 2012.yr)

We can observe that environmental issues are represented the most frequently. This is evident from the circle size. The strongest links can be found between the codes *Water Resources* and *Pollution Degradation* and *Industrial Wastes and Effluents*. More strongly expressed are also the ties between codes *Pesticides and Drugs* and *Control* or between codes *Techniques and Methodology* and *Water Resources* or *Pollution and Degradation* and *Industrial Wastes and Effluents*. The central place in the network is occupied by nanosensors. This is represented by CABI code *Biosensors and Biological Nanotechnology* which is connected with pesticides, crop production, water resources, quality of food and pathogens. A couple of major groups are revealed in the network, such as:

- water resources, wastes, pollution;
- nutrition (quality, chemistry, toxicology, contamination, storage and preservation);
- pesticides, diseases, protection, models.

## 4 CONCLUSIONS

Identification of current as well as emerging topics is important. It serves to better understand the developments in scientific research. Co-word analysis presents a significant tool to this end. The topics and connections can be ascertained by an analysis of the co-occurrence of various keywords or phrases. The aim of our study was to analyze the knowledge structure in nanoscience. Analyzing specific terms (keywords, descriptors) in databases CAB and WOS we thus evaluated the connections between nanoscience and agriculture (nano-agro concepts).

Examination based on CAB database, which is predominantly an agricultural database, shows strong emphasis on environmental studies where an important part of research also focuses on pesticides. In the networks of CABI codes the prominent place is occupied by sensors. Research topics linking human health and experimental animals are also strongly present. WOS-based examination also detected strong presence of topics related to biosensors and pesticides. The importance of sensors has also been emphasized by some other authors (for example, Cozzens et al.,

2013). Some other directions of research such as controlled release, degradation, monitoring, detection of residues, studies at the molecular level, are also noticeable.

Bibliographic databases offer a large quantity of "organized" information, however, from the "raw" bibliographic data alone it is difficult to draw final conclusions on the connections and the flow of knowledge. Bibliometric mapping of science and subsequent visualization can still show important relations of selected elements and present the data in a more comprehensible way which makes it easier to understand and interpret what is possibly important.

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DOI: 10.14720/aas.2016.107.2.21
**Agrovoc descriptors:** plants, DNA, transposons, adaptation, evolution stress, genotype environment interaction, plant defence reactions

**Agris category code**: F30

# Vloga transpozonskih elementov v evoluciji in prilagoditvah kmetijskih rastlin

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**IZVLEČEK**

Transpozonski elementi (TE) so odseki DNK, ki predvsem pri rastlinah predstavljajo največji delež genoma. Prav zaradi njihove številčnosti in sposobnosti prehajanja znotraj genoma, lahko ključno vplivajo na fenotipske spremembe in evolucijo rastlinskih vrst, predvsem pa tudi omogočajo prilagoditev rastlin na stresne dejavnike. Preko genetskih in epigenetskih mehanizmov delovanja spreminjajo zgradbo genov, vplivajo na njihovo izražanje in ustvarjajo nova regulatorna omrežja. Delež genoma, ki ga predstavljajo in vpliv, ki ga imajo na letega, se med posameznimi vrstami močno razlikuje, bili pa so odkriti v vseh do sedaj raziskanih rastlinskih genomih. S svojim delovanjem med drugim pogosto povzročijo škodljive mutacije, zato je njihovo izražanje s strani gostitelja navadno dobro nadzorovano. Poznavanje mehanizmov delovanja transpozonskih elementov in njihovo nadaljnje raziskovanje bodo še v večji meri omogočali njihovo uporabo, na primer za namen izboljšanja agronomsko pomembnih lastnosti poljščin, za odpornost na bolezni in škodljivce in za zatiranje invazivnih vrst.

**Ključne besede:** transpozonski elementi, mobilna DNK, evolucija rastlin, prilagajanje na stres

**ABSTRACT**

**EVOLUTIONARY AND ADAPTIVE ROLE OF TRANSPOSABLE ELEMENTS IN AGRICULTURAL PLANTS**

Transposable elements (TE) are stretches of DNA that represent the greatest fraction of genomes, especially in plants. Because of their high copy numbers and ability to mobilize through genome, they are able to influence the phenotypic traits and evolution of plants and also plant adaptation to environmental stress. By genetic and epigenetic mechanisms, they change the gene structure, influence gene expression and create new regulatory networks. The fraction of genome that they represent and the influence they have is variable among species; however they were detected in practically every plant genome researched up to date. Deleterious mutations may be caused by their activity which is also another reason why their expression is tightly regulated by the host organism. Gaining knowledge of TE's mechanisms and research development in the future will allow us to use them, for example for crop improvement purposes, resistance development against diseases and pathogens and suppression of invasive species.

**Key words:** transposable elements, mobile DNA, plant evolution, stress adaptation

## 1 UVOD

Vir genetskih sprememb in posledično gonilo evolucije so predvsem mutacije in spremembe v uravnavanju izražanja genov, katerih povzročiteljev je več vrst. Med drugim so to tudi transpozonski ali mobilni elementi, ki ustvarjajo večji del genomske variabilnosti in lahko organizmom omogočajo hitro adaptacijo na stresne razmere. Slednja je še posebej pomembna pri višjih rastlinah, ki kot odgovor na stresne dejavnike, ne morejo zamenjati svojega življenjskega okolja (Capy in sod. 2000).

Transpozonski elementi (TE) so odseki DNK, ki so se sposobni vključiti na nova mesta v genomu, lahko povečajo število svojih kopij in se pri tem poslužujejo ene ali več encimskih funkcij

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avtonomnega elementa (Lisch, 2013). Transpozicijsko aktivnih je zgolj majhen delež TE v genomu. Večina namreč ostaja v neaktivnem, dormantnem stanju, vseeno pa predstavljajo prav ti elementi največji delež večine genomov, predvsem rastlinskih (Kejnovsky in sod., 2012).

Zaradi robustnega načina podvojevanja (amplifikacije), ki izkorišča gostiteljeve celične mehanizme, so bili v preteklosti označeni kot t.i. sebična, parazitska in "odpadna" DNK (Capy in sod. 2000). Poimenovanje transpozonski elementi so privzeli šele desetletja potem, ko se je Barbara McClintock v 1940-ih letih kot prva zavedla njihovega pomena in kontrolne funkcije ter jih glede na njihovo vlogo ustrezno poimenovala kontrolni elementi in obrazložila, da je pravi pomen njihovega obstoja nadzor, ne pa transpozicija (Comfort, 1999). Pozneje je predpostavila tudi, da so prav okoljske spremembe tiste, ki slednjo izzovejo, ta pa ustvari genetsko raznolikost, ki omogoči preživetje gostitelja v stresnih razmerah (McClintock, 1984). Za večino ostalih raziskovalcev tistega časa je bila njena hipoteza preveč optimistična. Sklepali so, da je aktivacija TE možna zgolj takrat kadar zatajijo gostiteljevi obrambni mehanizmi, ki sicer v normalnih razmerah transpozicijo zavirajo (Casacuberta in González, 2013).

## 2 TRANSPOZONSKI ELEMENTI

Transpozonski elementi so prisotni v praktično vseh organizmih, do sedaj odkrite izjeme so le redke (npr. *Plasmodium falciparum* (William H. Welch, 1897)). Velik pomen ima njihova prisotnost predvsem za rastline, pri katerih TE ustvarjajo izjemne razlike v velikosti genomov. Ugotovljeno je bilo, da je število genov v praktično vseh rastlinskih genomih primerljivo, in sicer med 5.000 in 50.000, kar pomeni, da so razlike med vrstami desetkratne, gledano z vidika velikosti celotnih genomov, pa je razlika tudi do 200.000-kratna (za primerjavo – navadni repnjakovec - *Arabidopsis thaliana* (L.) Heynh. ima 26.200 genov, celoten genom je velik 120 Mbp, koruza - *Zea mays* L. pa ima 30.000 genov v 2.061 Mbp velikem genomu). Nekodirajoči del genomov je v glavnini sestavljen iz ponavljajoče se DNK, kamor uvrščamo tudi TE (Wicker, 2012).

### 2.1 Značilnosti

Izvor TE še vedno ni popolnoma jasen, njihova prisotnost v organizmih pa nakazuje, da so se pojavili že zelo zgodaj v evolucijskem razvoju evkariontov ali celo v času zadnjega skupnega prednika, še pred pojavom epigenetskih mehanizmov, ki jih sicer uravnavajo (Smith, 2015).

V grobem TE delimo v dva glavna razreda – razred I in II, glede na prisotnost oz. odsotnost RNK kot vmesnika transpozicije.

Elementi razreda I ali retrotranspozoni so najpogostejši elementi v rastlinskih genomih in jih nadalje ločimo na LTR (long terminal repeats) in ne-LTR retrotranspozone. Proces premeščanja LTR retrotranspozonov (slika 1, razdelek a), ki so na obeh koncih zamejeni z dolgimi terminalnimi ponovitvami (LTR-long terminal repeat) in kodirajo Gag proteine, reverzno transkriptazo, proteazo in integrazo, se prične v 5' LTR. Ta vsebuje promotor katerega prepozna gostiteljeva RNK polimeraza II in prepiše TE v mRNK (pomeni prisotnost RNK intermediata TE). V prvem koraku se Gag proteini organizirajo v virusom podobne delce, ki vsebujejo mRNK, reverzno transkriptazo in integrazo. Reverzna transkriptaza prepiše mRNK v dsDNK (double stranded DNA). V drugem koraku nato integraza to cDNK (complementary DNA) vstavi na novo pozicijo v genomu. TE, ki je bil matrica za prepis pa ostane na svoji izvorni lokaciji (Levin in Moran, 2011).

Ne-LTR retrotranspozoni, elementi SINE (short interspersed nuclear elements) in LINE (long interspersed nuclear elements) ne vsebujejo LTR zaporedij in kodirajo enega ali dva odprta bralna okvirja (ORF-open reading frame) (slika 1, razdelek b). Prav tako kot pri LTR retrotranspozonih, tudi v tem primeru pride do tvorbe mRNK, se pa ti elementi premikajo preko TPRT mehanizma (target-site-primed reverse transcription), pri katerem v elementu zakodirana endonukleaza prelomi eno verigo DNK in

izpostavi 3'-OH konec, ki je uporabljen kot začetno mesto za reverzno transkripcijo RNK. Nastala cDNK se nato enako integrira na novo mesto v genomu (Levin in Moran, 2011). Z vsakim premikom se v primeru vseh predstavnikov razreda I ustvari nova kopija elementa, zato govorimo o replikativnem ali t.i. »kopiraj in prilepi« mehanizmu transpozicije (Kejnovsky in sod., 2012).

Transpozicija elementov razreda II ali DNK transpozonov (slika 1, razdelek c) pa poteka preko t.i. »izreži in prilepi« mehanizma, ki predstavlja konzervativen način replikacije, pri katerem ne nastajajo nove kopije elementov (Kejnovsky in sod., 2012). TE se namreč iz prvotnega mesta fizično izreže in vključi na novo lokacijo s pomočjo encima transpozaza, ki je kodiran znotraj elementa in prepozna TIR (Toll/interleukin-1 receptor (TIR) homology domain**)** domene, ki TE razreda II omejujejo. Dvojni zlom, ki po transpoziciji ostane na prvotni lokaciji, popravijo popravljalni mehanizmi gostiteljskega organizma (Lisch, 2013). Število kopij teh elementov se poveča zgolj izjemoma, v primeru, da izrez in reintegracija nastaneta med DNK podvojevanjem in se TE premakne z že podvojenega dela DNK na še neprepisanega (Grandbastien, 2015).

V rastlinah se najpogosteje pojavljajo TE, ki so predstavniki *hAT* (*hobo*, *Activator*, *Tam3*), *CACTA* in *MULE* (*Mutator* tipa) superdružin elementov razreda II (Lisch, 2013). Pri vseh razredih ločimo med TE še tiste, ki so avtonomni, kar pomeni, da kodirajo vse potrebne encime za transpozicijo in ne-avtonomne, ki se za potrebe premikanja zanašajo na mehanizacijo avtonomnih TE (Wei in Cao, 2016). Najbolj zastopani neavtonomni TE so MITE (Miniature Inverted-repeat Transposable Elements), ki so večinoma delecijski derivati avtonomnih elementov ali sekvenc, ki so avtonomnim TE podobne na njihovem terminalnem delu, za transpozicijo pa izkoriščajo prisotnost avtonomnih elementov in encimov, ki jih le-ti kodirajo (Lisch, 2013). Drug podrazred elementov razreda II (Kejnovsky in sod., 2012; Wicker, 2012) oz. dodaten razred glede na alternativen način klasifikacije (Lisch, 2013), predstavljajo *helitroni*. Elemente v razred II združuje odsotnost RNK intermediata, ne pa nujno tudi skupen izvor (Wicker, 2012).

*Helitroni* se premikajo preko podvojevanja na način kotalečega se kroga (»rolling-circle«) mehanizma transpozicije, ki se začne z zlomom verige DNK na terminalnem delu *helitrona* in v tarčni regiji integracije. Sledijo premestitev sekvence in njena integracija s formacijo heterodupleksa, DNK sinteza na mestu izreza in podvojitev DNK vstavljene sekvence (Lisch, 2013).

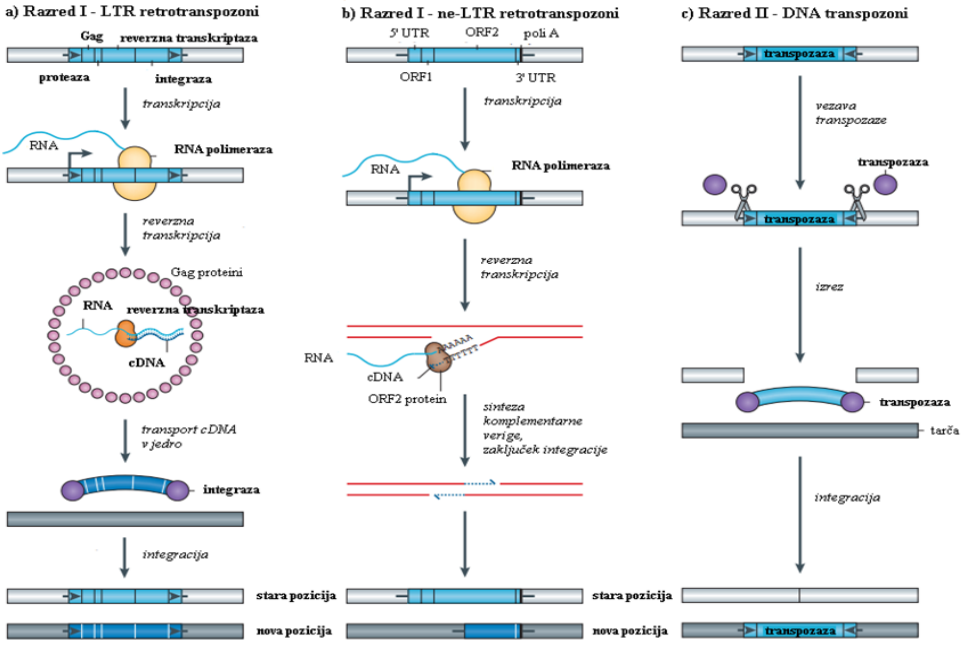



**Figure 1:** Zgradba in mehanizmi transpozicije različnih TE (Levin in Moran, 2011: 617)

## 2.2 Vloga in delovanje TE v rastlinskem genomu

TE so bili v preteklosti večinoma obravnavani kot negativni elementi genoma. Zaradi pozitivne selekcije na ravni DNK, ki je bila posledica sposobnosti hitrejše replikacije TE sekvenc kot gostiteljevih, so bili pogosto okarakterizirani kot sebična DNK. Po drugi strani pa je bila na ravni gostiteljskega organizma prisotna negativna selekcija zaradi TE insercij, ki so vodile v za gostitelja škodljive mutacije, osnova za hipotezo o »odpadni« DNK (Kidwell in Lisch, 2001). Zanemarjena in predvsem nerazumljena je bila njihova vloga v uravnavanju izražanja genov in spreminjanja fenotipov (slika 2) (Wei in Cao, 2016).

Dinamičnost mehanizmov TE in njihovo delovanje na različne načine prispevata k izražanju genov ter s tem variabilnosti fenotipov in evoluciji rastlin. Lahko pa TE tudi vplivajo na nova ali preusmerijo delovanje obstoječih regulatornih omrežij preko genetskih in epigenetskih mehanizmov, kar vodi v fenotipsko plastičnost ter dalje v adaptacijo in s tem naravno selekcijo (Wei in Cao, 2016).

Veliko število raziskanih in v literaturi opredeljenih primerov opisuje posledice delovanja TE in njihov vpliv na spreminjanje fenotipskih lastnosti rastlin. Mehanizme, preko katerih TE vplivajo na evolucijo rastlin in njihovo prilagoditev na stresne dejavnike, Wei in Cao (2016) delita na genetske in epigenetske.

### 2.2.1 Genetski mehanizmi

#### 2.2.1.1 Insercijska inaktivacija

Delovanje TE se najbolj očitno kaže v inaktivacijah genov (slika 2, razdelek a) zaradi insercij elementov v protein kodirajoča območja, največkrat zaradi predčasnega pojava terminacijskega kodona ali prekinitve bralnega okvirja (Smith, 2015).

Insercija TE je tako pri žlahtni vinski trti (*Vitis vinifera* L.) povzročila spremembo barve plodu. Vstavitev *Gret1* LTR retrotranspozona, ki je pri temno rdeči sorti 'Cabernet' odsotna, je pri beli sorti 'Chardonnay' vodila v izgubo funkcije *Vvmby1A* gena in s tem posledično tudi do izgube rdeče barve (Lisch, 2013).

#### 2.2.1.2 Domestikacija TE in vpliv na regulacijo ekspresije gostiteljevih genov

Določeni TE so lahko udomačeni (slika 2, razdelek b) zaradi prispevka njihovih kodirajočih sekvenc in regulatornih elementov. To še posebej velja za LTR retrotranspozone, katerih LTR deli lahko delujejo kot promotorji in viri regulatornih sekvenc (*cis*-regulatorni elementi itd.) (Bui in Grandbastien, 2012).

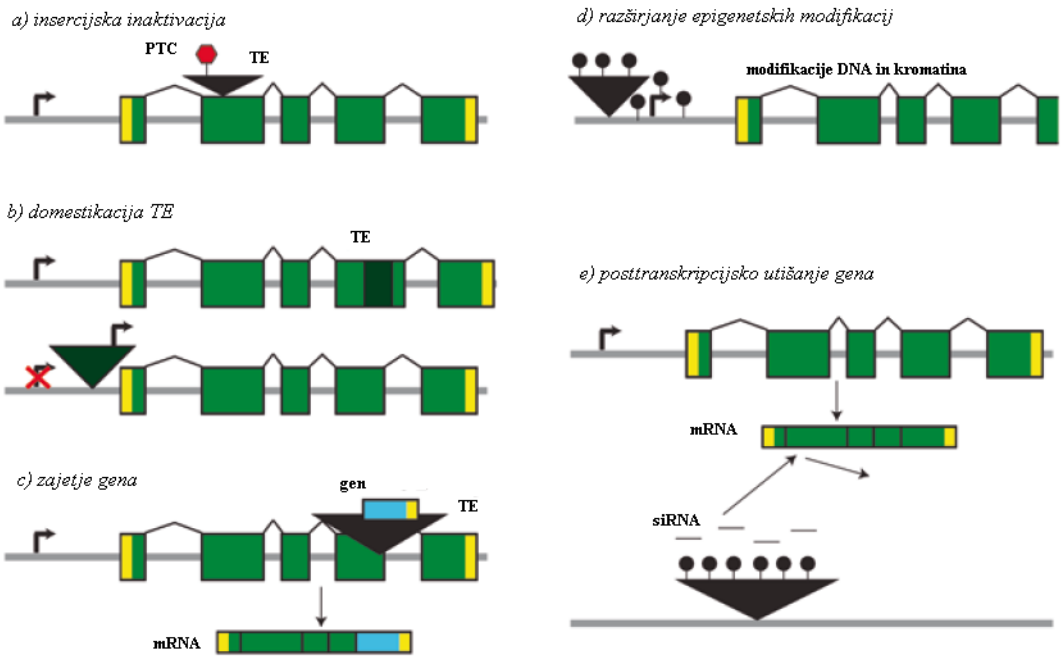
Primer domestikacije *Copia* tipa elementa, ki je vstavljen v *RPP7* gen za odpornost na oomiceto *Hyaloperonospora parasitica* (Pers.) Constant., je poznan pri navadnem repnjakovcu (Tsuchiya in Eulgem, 2013), podoben pojav pa je bil raziskan tudi pri vrsti tobaka *Nicotiana glutinosa* L., kjer MITE domestikacija v *N* genu povzroči alternativno izrezan ekson in tako omogoča odpornost na mozaični virus tobaka (Smith, 2015).

Drug primer opisuje toleranco na aluminij (Al) pri različnih vrstah trav in tudi žitih, ki je povezana z insercijo LTR retrotranspozona. Ta nadzoruje povečanje in relokalizacijo ekspresije citratnih transporterskih genov v koreninski vršiček. Ti geni kodirajo proteine, ki Al odstranjujejo iz rastline in jim omogočajo rast tudi v onesnaženih tleh (Grandbastien, 2015).

#### 2.2.1.3 Zajetje gena

TE lahko zajamejo, podvojijo in mobilizirajo tudi gene in genske fragmente gostitelja (slika 2, razdelek c), ki jih retrotranspozoni podvojijo preko reverzne transkripcije njihove mRNK in tako nastane retrogen, DNK gen kopiran nazaj iz RNK z reverzno transkripcijo. Fragmenti retropoziranih genov so lahko združeni z gostiteljevimi in tako povzročijo nastanek himernih proteinov (Contreras in sod., 2015).

TE za katere je omenjena aktivnost najbolj značilna so *helitroni*, ki s svojim mehanizmom pomnoževanja pogosto zajamejo gostiteljske gene. Tako več kot ena tretjina *helitronov* koruze vsebuje vsaj en genski fragment gostitelja in s premeščanjem genskih sekvenc potencialno generirajo nove genske strukture z različnimi funkcijami (Contreras in sod., 2015).




**Slika 2:** Vpliv delovanja TE (Smith, 2015: 147)

### 2.2.2 Epigenetski mehanizmi

Navkljub mnogim primerom potencialnih prednosti insercije TE, je najbolj pogost učinek njihove vstavitve v bližino promotorja gostiteljevega gena, njegova inaktivacija s strani epigenetskih mehanizmov (Contreras in sod., 2015). Utišanje vstavljenih TE gostitelji največkrat dosežejo z DNK metilacijo, histonskimi modifikacijami in RNK interferenco, ki pa lahko hkrati vplivajo tudi na bližnje gene, najpogosteje z zmanjšanjem njihove ekspresije ali utišanjem (slika 2, razdelek d) (Wei in Cao, 2016).

Koruza in soja sta dve kratkodnevni rastlini, katerih določeni kultivarji so se kot posledica insercije TE uspeli prilagoditi na rast tudi v drugih, manj optimalnih geografskih območjih, v katerih vladajo razmere za rast dolgodnevnih rastlin. Gen *Gmphy*A2 za fitokrom A v soji je zaradi vstavitve *Copia* tipa retrotranspozona hipermetiliran in se posledično veliko manj prepisuje. V koruzi se podobno insercija *CACTA* tipa elementa v *ZmCCT* promotor odraža v epigenetskem uravnavanju občutljivosti na fotoperiodo. Zaradi manjše občutljivosti na dolžino dneva, so specifični kultivarji teh dveh poljščin sposobni uspevati v neoptimalnih svetlobnih razmerah (Wei in Cao, 2016).

Bolj zapletene prerazporeditve TE lahko vodijo v produkcijo miRNK (microRNA), ki izvirajo iz TE in lahko uravnavajo protein-kodirajoče gene preko post-transkripcijskih genskih utišanj. Vpliv na izražanje genov pa lahko TE dosežejo tudi preko utišanja genov, ki niso v neposredni bližini (slika 2, razdelek e).

## 3 VLOGA TE V GENOMU RASTLIN

Posledice prisotnosti TE v genomih so raznolike in precej zapletene (primeri v preglednici 1), saj so neposredno odvisne od mesta insercije elementa, odziva gostiteljskega organizma in njegovih obrambnih mehanizmov na vključitev, interakcij med njima, pomembno pa je tudi trenutno stanje gostitelja in morebitni okoljski vplivi. Delovanje TE je kompleksno in zelo specifično, organizmu pa lahko omogočijo selekcijsko prednost ali pa v njem povzročijo celo uničujoče mutacije (Tsuchiya in Eulgem, 2013).

Kot primer koristne prisotnosti TE je v nadaljevanju opisan primer rdeče pomaranče, ki so

ga preučevali Butelli s sod. (2012), pri katerem gre za insercijo *Copia* tipa retrotranspozona v bližino gena *Ruby*, ki je transkripcijski aktivator tvorbe antocianinov.

**Preglednica 1:** Primeri vpliva TE na variabilnost pri rastlinah (povzeto po Wei in Cao, 2016: 27-28)

| Regulatorni mehanizem | TE klasifikacija | Reguliran gen | Rastlinski fenotip |
|---|---|---|---|
| Insercijska mutageneza | Razred II, Ac/Ds | SBEI | nagubana semena graha – odziv na osmotski stres |
| | Razred I, *Gret1* | *Vvmby1A* | spremembe v barvi grozdja |
| | Razred II, Ac/Ds | *C* | različno obarvanje koruznih semen |
| Regulatorni elementi | Razred I, LTR, *Renovator* | *Pit* | odpornost na glivo *M. oryzae* (T.T. Hebert) M.E. Barr. pri rižu |
| | Razred II, MITE | *AltSB* | toleranca na aluminij pri sirku |
| | Razred II, MITE, *mPing* | Os01g0299700, Os02g0135500, Os02g0582900 | odziv na stres pri rižu |
| | Razred I, *Copia*-like, *COPIA-R7* | *RPP7* | odziv na prisotnost oomicete *H. parasitica* pri n. repnjakovcu |
| Epigenetska regulacija | Razred II, MITE | *MAIF1* | ABA signaliziranje in odziv na abiotski stres pri rižu |
| | Razred II, MITE | *ZmNAC111* | vpliv na toleranco na sušne razmere pri koruzi |
| | Razred I, *Copia*-like, *SORE-1* | *GmphyA2* | neobčutljivost na fotoperiodo pri soji |

### 3.1 Vloga TE pri sintezi antocianinov

Rdeče pomaranče so sadje za katero so dokazali, da njihovo uživanje pripomore k izboljšanju zdravja, pozitivno vpliva na stanje kardiovaskularnega sistema, predvsem zaradi velike vsebnosti vitamina C, karotenoidov in antocianinov, ki omogočajo antioksidativno delovanje. Kljub velikem povpraševanju, tudi zaradi vizualne pestrosti, pa je oskrbovanje trga z njimi omejeno zaradi nezanesljive proizvodnje, pri kateri je polna tvorba barve pomaranč odvisna od nizkih temperatur (Butelli in sod., 2012).

Večina modernih sort rdečih pomaranč se je razvila iz starih italijanskih, taka je npr. 'Doppio Sanguigno', med novejši pa spadata 'Tarocco' in 'Moro' (Butelli in sod., 2012).

Antocianini so naravni pigmenti, ki jih lahko zasledimo v rdečem, vijoličnem in modro obarvanem sadju, cvetovih in tudi vegetativnih organih rastlin. Njihova sinteza je v večji meri regulirana na ravni transkripcije. Regulatorni kompleks, ki ga sestavljajo predvsem Myb proteini in družina transkripcijskih faktorjev z WD ponovitvami, uravnava izražanje strukturnih genov, ki so potrebni za sintezo antocianinov, njihovo modifikacijo in transport. V rdečih pomarančah je ekspresija določenih antocianin biosinteznih genov močno povečana v primerjavi z navadnimi pomarančami, ki imajo oranžno obarvana tkiva plodu. Raznolikost v intenzivnosti pigmenta in tkivni specifičnosti je odvisna pretežno od aktivnosti R2R3 Myb transkripcijskih faktorjev v omenjenem kompleksu. Delni cDNK fragment, ki kodira ohranjeno Myb DNK (MYB-myeloblastosis) vezavno domeno tipično za R2R3 Myb transkripcijski faktor, so izolirali iz pomaranč

sorte 'Moro' s pomočjo degenerativnega PCR in ga poimenovali *Ruby* (Butelli in sod., 2012).

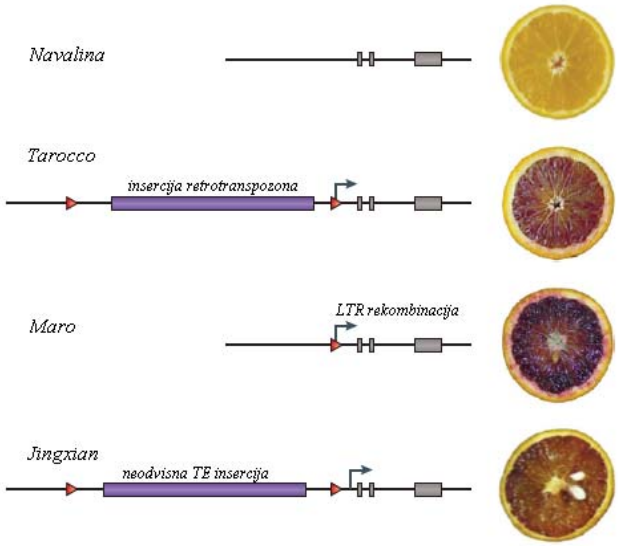
Gen *Ruby* so nato klonirali iz treh sort rdečih pomaranč ('Sanguinelli', 'Maltaise Sanguine' in 'Moro') ter iz treh sort navadnih ('Navalina', 'Salustiana' in 'Cadenera'). Primerjava sekvenc je pokazala popolno ujemanje nukelotidnega zaporedja v treh eksonih in dveh intronih, ki sestavljajo gen, zato so sklepali, da se razlika v izražanju gena pojavi zaradi različnega uravnavanja na ravni *Ruby* transkripcije. Pri navadnih pomarančah se ta gen namreč ne izraža. Z izolacijo in raziskavo regulatornih regij gena so ugotovili, da se pri sortah rdečih pomaranč pojavlja insercija TE, ki pripada družini *Copia* tipa TE in ga opredelili kot Tcs1 (Butelli in sod., 2012).

Tcs1 je 5413 nukleotidov dolg TE z vsemi tipičnimi značilnostmi elementov, ki spadajo v družino *Copia* – prisotni so odprt bralni okvir, ki kodira Gag in Pol proteina za reverzno transkripcijo in integracijo ter dva enaka LTR. Glede na prisotnost potrebnih proteinov in identičnost obeh LTR so sklepali, da gre za insercijo do katere je prišlo šele nedavno (Butelli in sod., 2012).

Med nadaljnjim raziskovanjem rdeče pomaranče sorte 'Moro' so našli zaporedje TATA 32 baznih parov (bp) nad začetnim mestom transkripcije, in sicer znotraj LTR elementa. LTR prav tako vsebuje izrezovalno mesto za prvi intron *Ruby* transkripta. Dognali so, da insercija Tcs1 na mestu *Ruby* deluje kot promotor, ki nadzoruje povečano izražanje gena in omogoča, da se transkripcija prične prav v LTR (Butelli in sod., 2012).

Različne študije so potrdile, da so TE lahko zelo občutljivi na stresne dejavnike, kot je npr. velika slanost, nizke ali visoke temperature, poškodbe in bakterijske ter virusne infekcije. Ta odzivnost na stres se je z insercijami različnih TE v regije na 5' strani gostiteljevih genov v mnogih primerih prenesla tudi na te gene. TE aktivnost lahko torej poveča celotno število genov, ki jih inducira stres, poleg tega pa nagnjenost k reprogramiranju izražanja genov razumemo tudi kot selekcijsko prednost. TE bi namreč lahko predstavljali mehanizem preko katerega se genom z reorganizacijo samega sebe odzove na stres (Lisch, 2013).

Transkripcija aktivnih TE je navadno s strani gostitelja zatirana, a se lahko kot odgovor na različne stresne razmere ponovno aktivira, kar je McClintockova (1984) v svoji teoriji razložila kot strategijo organizma, da v neoptimalnih razmerah z ojačano transkripcijo poveča svoje možnosti za preživetje. Tako je tudi s TE Tcs1, ki je aktiviran, kadar so sadeži rdeče pomaranče izpostavljeni mrazu in s svojim delovanjem povzroči od nizkih temperatur odvisno akumulacijo antocianinov (Butelli in sod., 2012).




**Slika 3:** Shematski prikaz območja gena pri različnih sortah pomaranč (Lisch, 2013: 53-54)

Pri sorti 'Navalina' (slika 3) je torej prisotna funkcionalna, nativna oblika *Ruby* gena, ki se v plodu izraža omejeno, kar se odraža v oranžni barvi plodu. Pri sorti 'Tarocco' se ta zaradi insercije TE izraža bolj, pri 'Maro' pa je rekombinacija med LTR zaporedjema povzročila še povečano izražanje, ki se odraža kot še intenzivnejše rdeče obarvanje plodu (Lisch, 2013).

Večina sort rdečih pomaranč izvira iz omenjenih sicilijanskih genotipov, na Kitajskem pa so pri sorti 'Jingxian' potrdili, da je prišlo do neodvisnega dogodka, pri katerem je soroden, podobno velik LTR retrotranspozon, ki so ga poimenovali Tsc2, prav tako povzročil odziv na nizke temperature in tkivno specifično obarvanje s spodbujanjem ekspresije *Ruby* gena preko aktivatorske sekvence v regiji na 5' strani gena (slika 3) (Butelli in sod., 2012).

V svoji študiji so Butelli s sod. (2012) dokazali razvoj pomaranč iz pomela in manadarin, pri katerih je tudi prisoten *Ruby* alel, ter izvor rdečih pomaranč iz starih mediteranskih oz. omenjene kitajske sorte zaradi insercije TE. Prav tako so razložili vlogo TE kot promotorja in regulatorja genske ekspresije ter podal priložnost za nadaljnjo evolucijo rdečih pomaranč s pomočjo genskega inženiringa, ki bi lahko omogočil pridelavo tudi na podnebno manj primernih območjih.

## 4 3HH H4 POTENCIALNO IZKORIŠČANJE TE

### 4.1 Vpliv na izboljšanje poljščin

Predvsem poljščine, na čelu s koruzo, so rastline, katerih genomi so v veliki meri sestavljeni iz TE.

Za proizvodnjo koruze so podnebni dejavniki, predvsem razpoložljivost vode, izjemno pomembni. Suša bistveno vpliva na pridelavo po svetu, razumevanje genetske osnove za naravno variabilnost tolerance na sušo pri tej poljščini, pa bi lahko močno pripomoglo k izboljšanju njenega gojenja. Odziv rastlin na sušo je kompleksen in odvisen od časovne periode ter jakosti stresa s katerim se rastlina srečuje. Mao in sod. (2015) so v svoji študiji ugotovili, da MITE insercija v promotor *NAC* gena negativno vpliva na toleranco koruze na sušne razmere.

Glede na predvideno možno vlogo NAC-tipa genov v odzivu na sušne razmere, so sekvencirali gen *ZmNAC111* v 262 inbridiranih koruznih linijah. Detektiranih je bilo 157 mutacij tipa SNP ter 119 tipa InDel (insercij in delecij). Še posebej obetavna je bila novo identificirana mutacija InDel velika 82 bp (InDel-572), locirana 572-bp nad start kodonom *ZmNAC111* gena.

InDel-572 je MITE insercija v *ZmNAC111* promotorju. Analiza sekvence InDel-572 v promotorski regiji *ZmNAC111* je pokazala, da je insert sestavljen iz dolgih terminalnih invertiranih ponovitev (vsaka velikosti 38 bp), 4 bp velikih zank in dveh dodatnih »TA« nukleotidov na koncu in enega neposredno pred insercijo. Opis predstavlja tipično strukturo MITE insercije v genomu, ki je navadno kratka, velikosti približno nekaj sto bp in je sestavljena iz TIR zaporedij, tarčnih mest direktnih ponovitev in se preferenčno vstavi pri TA oz. TAA nukleotidnem zaporedju. BLAST primerjava podatkovne baze koruznih TE je pokazala, da gre za *Tc1/Mariner* superdružino MITE elementov.

Z RT-qPCR analizo so ugotovili, da je ekspresija *ZmNAC111* veliko večja v primerkih, ki MITE insercije nimajo, torej vstavitev TE v promotor zavre ekspresijo gena in s tem poveča občutljivost koruze na pomanjkanje vode.

Genomski fragment, ki vsebuje *ZmNAC111* so prenesli tudi v repnjakovec (*Arabidopsis*) in tako dokazali, da je molekulski mehanizem tolerance na sušo ohranjen med vrstami. Dodatne transgene študije pri tej vrsti in koruzi so dokazale, da lahko prekomerna ekspresija *ZmNAC111* gena izboljša toleranco na sušo v transgenih rastlinah.

Ob dodatnih raziskavah bi se *ZmNAC111* gen lahko izkazal za potencialnega kandidata v genskem inženiringu, alel, ki TE ne vsebuje, pa bi lahko postal selekcijska tarča za gensko izboljšanje rastlin za odpornost na sušo (Mao in sod., 2015).
Kljub temu, da v omenjenem primeru vstavitev TE ne pomeni prednosti, ampak nasprotno, bi lahko enak pristop ubrali v primerih, ko ima vstavitev TE

potencialno izboljšavo pri prilagajanju na stresne dejavnike.

### 4.2 Zatiranje invazivnih vrst

Kritičen dejavnik uspeha invazivnih vrst je njihova sposobnost hitre prilagoditve na novo okolje, ki navadno zanje pomeni nenaden in intenziven stres. Količina in narava genetske variabilnosti, ki je na voljo za selekcijo med invazijo v veliki meri določa prilagoditveni potencial (Stapley, 2015).

Presenetljivo sposobnost invazivnih vrst, da se prilagodijo in naselijo nove habitate kljub ozkemu grlu, ki ga predstavlja pomanjkanje alelnih oblik genov, so opredelili kot t.i. genetski paradoks invazivnih vrst. Za ta pojav obstaja več možnih razlag, ena izmed njih pa vključuje TE, ki naj bi generirali genetsko variabilnost kot odgovor na okoljske in genetske spremembe in s tem pospešili prilagoditev. Pogostost pojava potencialno koristnih alelov je kot rezultat delovanja TE veliko večja od možnosti pojava naključnih mutacij, kar pomeni, da je zaradi TE aktivnosti hitra prilagoditev veliko bolj verjetna. V svoji študiji je Stapley (2015) tako predpostavila, da s TE posredovana prilagoditev omogoči preživetje in uspeh invazivnih vrst v novem okolju. V tem primeru gre predvsem za tiste TE, ki se odzivajo na okoljski stres, so še vedno ali pa so bili do nedavnega transkripcijsko aktivni, se nahajajo v evkromatinskih regijah ter se vstavljajo v regije v bližini genov, kot so regulatorne regije in introni. Navaja tudi več primerov TE, ki so podvrženi selekciji in so prilagojeni biotskemu, abiotskemu ali genomskemu stresu, npr. divji ječmen (*Hordeum vulgare* L.) pri katerem število kopij TE variira glede na nadmorsko višino in navadni repnjakovec, pri katerem so TE pod vplivom pozitivne selekcije, če se nahajajo v bližini genov.

Z napredkom v poznavanju TE in regulaciji njihovega delovanja bi lahko z zaviranjem s TE posredovane prilagoditve našli pristop, ki bi omogočal nadzor in omejitev invazivnih rastlin v okolju v katerem povzročajo škodo.

## 5 ZAKLJUČEK

TE predstavljajo pomemben vir genetske in epigenetske variabilnosti in imajo vpliv na gensko regulacijo ter posledično tudi fenotipsko variabilnost.

V svojih študijah je Barbara McClintock prva predpostavila, da genomske spremembe, ki jih v stresnih razmerah povzroči delovanje TE pripomorejo k prilagoditvi in celo speciaciji. S prepoznavanjem specifičnih stresnih dejavnikov, ki vodijo v nastanek novih regulatornih omrežij, ki se odzivajo na določen stres, bi morda lahko izkoriščali možnost aktivacije nativnih TE in s tem pridobivanje novih varietet poljščin, ki bi bile bolj odporne na okoljske spremembe (Smith, 2015).

Napredek v fenomiki, genomiki in mapiranju lastnosti s pomočjo raziskovanja celotnega genoma čedalje bolj omogoča določanje števila mutacij, ki so bile pomembne za domestikacijo in naknadno modifikacijo lastnosti ter so bile povzročene zaradi aktivnosti TE. Določena orodja, ki so bila uporabljena za kartiranje lastnosti v udomačenih rastlinah pa bodo verjetno pripomogla tudi pri raziskovanju naravnih rastlinskih populacij, npr. divjih sorodnikov kulturnih rastlin, kar nam bo dalo boljši vpogled tudi v naravno selekcijo (Lisch, 2013).

Velik izziv, ki ga ponujajo TE je med drugim v njihovi ponovljivi naravi in vsesplošnem prepričanju, da je njihova prisotnost v genomih organizmov brez večjega pomena. Prav zaradi tega v sklopu različnih analiz največkrat sploh niso obravnavani, njihova vloga pa je zato še težje določljiva. Transpozonske elemente bi tako morali natančno anotirati in jih vključevati v raziskave kot potencialen vir funkcionalne variabilnosti. Še posebej je to pomembno pri TE za katere je znano, da prenašajo regulatorne informacije (Lisch, 2013).

V prihodnosti bo verjetno potrebnih še veliko raziskav na področju transpozonskih elementov, da bomo zares lahko razumeli njihovo vlogo in namen. Do sedaj se je že izkazalo, da njihov obstoj ni samo sebične narave in da ti elementi vendarle niso zgolj odpadni deli genoma, kljub temu, da bistvo njihove obsežne prisotnosti še ni popolnoma jasno.

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**Agris category code**: F60, h20

# Fiziološki odziv žlahtne vinske trte *Vitis vinifera* L. na okužbo z zvijanjem listov vinske trte povezanih virusov (GLRaV-1 in GLRaV-1 + GLRaV-3)

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**IZVLEČEK**

Bolezen zvijanja listov vinske trte je ena najpomembnejših in najbolj razširjenih virusnih bolezni vinske trte. Povzročitelji te bolezni so z zvijanjem listov vinske trte povezani virusi (Grapevine leafroll associated viruses, GLRaVs). S poskusom v rastlinjaku smo od junija do septembra, v rastni sezoni 2014, spremljali in primerjali fiziološke procese na trtah okuženih z enim (GLRaV-1), oziroma z dvema (GLRaV-1 in GLRaV-3) virusoma iz prej omenjenega kompleksa virusov. V sredini rastne sezone (meseca julija) se je negativni učinek na spremljane fiziološke procese v listih trte močneje izrazil pri mešani okužbi. Neto-fotosinteza (Pn) listov, okuženih z GLRaV-1 in GLRaV-3, je bila v primerjavi s Pn listov okuženih le z GLRaV-1 enkrat manjša. Podobno zmanjšanje smo opazili tudi pri prevodnosti listnih rež in transpiraciji ter v parametrih, povezanih s fotokemično učinkovitostjo fotosintetskega aparata (hitrost transporta elektronov po tilakoidi).

**Ključne besede:** *Vitis vinifera* L.; bolezen zvijanja listov vinske trte; GLRaV; fotosinteza

**ABSTRACT**

**PHYSIOLOGICAL RESPONSE OF GRAPEVINE *Vitis vinifera* L. TO GRAPEVINE LEAFROLL ASSOCIATED VIRUSES (GLRaV-1 and GLRaV-1 + GLRaV-3)**

Grapevine leafroll disease is one of the most severe viral diseases of grapevine caused by Grapevine leafroll-associated viruses (GLRaVs). Physiological processes were monitored on grapevines with single (GLRaV-1) and mixed (GLRaV-1 and GLRaV -3) viral infection under greenhouse conditions from June to September, in vegetation period 2014. In the mid of the season (July) negative effects of the virus infections on physiological processes were more severe in mixed than in single infection. The net-photosynthesis (Pn) of the leaves infected with GLRaV-1 and GLRaV-3 reached only a half of the Pn in GLRaV-1 infected grapevines. Similar reduction was found for stomatal conductance, transpiration and parameters related to photochemical efficiency (electron transport rate).

**Key words:** *Vitis vinifera* L.; grapevine leafroll disease; GLRaV; photosynthesis

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## 1 UVOD

Bolezen zvijanja listov vinske trte je ena najpomembnejših in najbolj razširjenih virusnih bolezni vinske trte na svetu (Martelli in Boudon-Padieu, 2006; Martelli, 2014). Prizadene lahko vse sorte in podlage, vendar bolezenska znamenja niso nujno značilna oziroma prepoznavna pri vseh sortah vinske trte (Pearson in Goheen, 1998). Omenjena bolezen skrajšuje življenjsko dobo trt, zmanjšuje pridelek (v povprečju od 15 do 20 %, v nekaterih primerih tudi do 40 %) in posledično povzroča gospodarsko škodo (Endeshaw in sod., 2014; Martelli, 2014).

Bolezen zvijanja listov vinske trte povzročajo z zvijanjem listov vinske trte povezani virusi (grapevine leafroll-associated viruses; GLRaV) iz družine *Closteroviridae* (Martelli, 2014). Do danes je opisanih pet z zvijanjem listov vinske trte povezanih virusov: GLRaV-1, -2, -3, -4 in -7 (Martelli, 2014). Izmed virusov, ki so povezani z boleznijo zvijanja listov vinske trte, sta v Evropi najbolj razširjena GLRaV-1 in GLRaV-3 (Martelli in sod., 2002; Sforza in sod., 2003), ki se pogosto pojavljata tudi v mešanih okužbah z drugimi virusi (Prosser in sod., 2007; Jooste in sod., 2015). Največjo škodo pri pridelavi grozdja povzročajo okužbe vinske trte z GLRaV-3 (Cabaleiro in Segura, 2006; Douglas in Krüger 2008), ki je, kar se tiče vpliva na pridelavo, tudi najbolj proučevan.

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Bolezen negativno vpliva na vinsko trto, pri kateri izzove morfološke in fiziološke spremembe. Omenjene spremembe so povezane z motnjami asimilacije $CO_2$, zmanjšajo se prevodnost listnih rež, transpiracija in število kloroplastov (Almási in sod., 1996). Te motnje se lahko pojavijo še pred pojavom vidnih bolezenskih znamenj. Bolezenska znamenja se kažejo kot predčasno medžilno rumenenje pri belih in rdečenje pri rdečih sortah vinske trte. Pozno poleti in jeseni je značilno tudi zvijanje listov (Korošec-Koruza, 1992; Martelli in Boudon-Padieu, 2006; Martelli, 2014). Jagode na grozdih okuženih trt običajno dozorevajo počasneje in neenakomerno, posledica česar so spremenjene vsebnosti sladkorjev, kislin in fenolnih snovi ter obarvanosti jagod. S tem je zmanjšana kakovost grozdja, posledično pa vina in sadnih sokov (Cabaleiro in sod., 1999; Sampol in sod., 2003; Bertamini in sod., 2004; Endeshaw in sod., 2014; Martelli, 2014).

Razumevanje vpliva GLRaV na vinsko trto otežuje kompleksnost virusov in zelo raznolik fiziološki odziv rastlin na virusne okužbe (Balachandran in sod., 1997). Na fiziološke parametre in količino pridelka vinske trte vplivajo tako virus kot tudi različek ali izolat virusa (Rowhani in sod., 2015). Cabaleiro in sod. (1999) poročajo, da GLRaV-3 ali GLRaV-1 v mešani okužbi z virusom A vinske trte (*Grapevine virus A*; GVA) zmanjšujejo kakovost grozdja. Nasprotno so Tomažič in sod. (2005) ugotovili, da je bila kakovost grozdja pri trtah okuženih z GLRaV-1 boljša kot pri trtah brez tega virusa, verjetno tudi zaradi manjšega pridelka in posledično hitrejšega dozorevanja jagod okužene trte (Tomažič in sod., 2005).

Po nam znanih podatkih do zdaj še ni bilo raziskave, ki bi primerjala vpliv mešane okužbe z GLRaV-1 in GLRaV-3 (v nadaljevanju: mešana okužba) in okužbe le z GLRaV-1 na metabolizem vinske trte. V predstavljeni raziskavi smo ugotavljali, kakšen vpliv ima okužba z GLRaV-1 v primerjavi z mešano okužbo na izbrane fiziološke procese in parametre (neto fotosintezo, dejansko in potencialno fotokemično učinkovitost PSII, prevodnost listnih rež, transpiracijo, hitrost transporta elektronov po tilakoidi in vsebnost klorofila) žlahtne vinske trte.

## 2 MATERIALI IN METODE DELA

### 2.1 Zasnova poskusa

V novembru 2013 smo po predhodnem testiranju s DAS-ELISA testom na GLRaV-1 in GLRaV-3 narezali rozge žlahtne vinske trte ('Blaufränkisch'('Modra Frankinja'), 'Rheinriesling' ('Renski rizling') in 'Pinot Gris' ('Sivi Pinot') okužene z GLRaV-1 ter z GLRaV-1 in GLRaV-3. Rozge, dolge okrog 40 cm, smo ukoreninili in posadili v petlitrske lonce z mešanico zemlje za pikiranje potaknjencev in vermikulita v razmerju 2 : 1. Tako pripravljene rastline smo vzdrževali v rastlinjaku v naslednjih rastnih razmerah: naravna dnevna fotoperioda, temperatura od 25 do 30 °C (dnevna) in 15 °C (nočna), zračna vlaga od 40 do 60 %. Trte smo zalivali vsak četrti dan oziroma po potrebi in tudi 24 ur pred vsakimi fiziološkimi meritvami. V času poskusa smo trte tretirali s sistemičnima pripravkoma z aktivno snovjo heksitiazoks (akaricid) in tebukonazol (fungicid) ter pognojili z raztopino gnojila NPK 6-3-6 z mikrohranili in dodatkom morskih alg (Unichem). Zaradi bujne rasti smo nove mladike ustrezno prikrajševali.

Meritve izmenjave plinov ($CO_2$ in $H_2O$), s katerimi smo izmerili neto fotosintezo (Pn), prevodnost listnih rež ($g_s$) ter transpiracijo (E), ter meritve fluorescence (ocena fotokemične učinkovitosti), vsebnosti klorofila (Chl) in hitrost transporta elektronov po tilakoidi (ETR) smo opravljali od začetka junija do sredine septembra na tretjem polno razvitem listu. Meritve so bile opravljene med 9. in 12. uro v rastlinjaku na Kmetijskem inštitutu Slovenije leta 2014. Po vsakem merjenju (3. junij, 17. julij, 11. in 25. avgust, 8. september), smo odvzeli del lista za preverjanje prisotnosti virusov s PCR. Odvzem vzorca smo opredelili kot sistematično napako (enaka poškodba na merjenih listih pri vseh trtah v poskusu), ki je imela zanemarljiv vpliv na meritve, saj poteka odziv na ranitveni stres navadno le nekaj ur (León in sod., 2001). Opazovali smo tudi pojavljanje bolezenskih znamenj na preučevanih trtah.

### 2.2 Preverjanje prisotnosti GLRaV-1 in GLRaV-3

*Serološka detekcija*

Prisotnost GLRaV-1 in GLRaV-3 v rastlinskem materialu pred rezanjem rozg in po njihovi saditvi v lonce smo preverili s testom DAS-ELISA. Pri tem smo uporabili protitelesa in pufre skladno z navodili proizvajalca (BIOREBA AG, Švica). Vse vzorce smo testirali v dveh tehničnih ponovitvah. Na podlagi rezultatov testa ELISA smo pripravili dve obravnavanji s po osmimi trtami: obravnavanje A - okužene z GLRaV-1 in obravnavanje B - okužene z GLRaV-1 in GLRaV-3 (mešana okužba).

*Molekularna identifikacija*

Skupno RNA iz listov obravnavanih trt smo izolirali s kompletom MagMAX™-96. Total RNA Isolation Kit (Ambion, Thermo Fisher Scientific, ZDA) po priporočilih proizvajalca. Za izolacijo smo uporabili napravo MagMAX Express (Ambion, Thermo Fisher Scientific, ZDA). Za izolacijo smo uporabili 4–5 mg (dva krogca premera 5 mm) rastlinskega tkiva. Komplementarno DNA (cDNA) smo iz 2 µl izolirane RNA sintetizirali z uporabo kompleta High-Capacity cDNA Archive Kit (Applied Biosystems, Thermo Fisher Scientific, ZDA) po priporočilih proizvajalca. Izbrani del virusnega genoma smo pomnožili v cikličnem termostatu Veriti 96-Well Thermal Cycler (Applied Biosystems). Za pomnoževanje GLRaV-1 smo uporabili začetna oligonukleotida LR1hsp70-417F/LR1hsp70-737R (Osman in sod., 2007), za GLRaV-3 pa LR3_18345F/LR3-18488R (Bester in sod., 2014). PCR je potekala v naslednjih razmerah: denaturacija pri 94 °C (5 min); 35 ciklov pomnoževanja: denaturacija pri 94 °C (30 s), naleganje začetnih oligonukleotidov pri 58 °C za GLRaV-1 in pri 54 °C za GLRaV-3 (30 s) in podaljševanje pri 72 °C (50 s); ter zaključno podaljševanje pri 72 °C (7 min). Prisotnost specifičnih pomnoženih produktov PCR smo preverili na 1 % agaroznem gelu.

### 2.3 Meritve fizioloških parametrov in procesov

Pn, $F_v'/F_m'$, $g_s$, E in ETR smo merili s prenosnim merilnikom fotosinteze LI-6400 (Licor, ZDA). V merilno kiveto smo vstavili izbrani list vinske trte tako, da je bila z listom pokrita celotna površina okenca merilne kivete. Koncentracija $CO_2$ je bila uravnana na 380 µmol $CO_2$ $mol^{-1}$, temperatura lista na 25 °C in svetloba na 1000 µmol $m^{-2}$ $s^{-1}$, kar je bilo v svetlobnem saturacijskem območju fotosinteze. Posamezno meritev smo končali, ko je

bila dosežena stabilna razlika med koncentracijo $CO_2$ v zraku, ki je bil v kiveto dovajan, in zrakom, ki je iz kivete izstopal.

Potencialno fotokemično učinkovitost (Fv/Fm) smo merili s fluorometrom Mini PAM (Heinz Walz GmbH, Effeltrich, Germany). Pred meritvami smo na liste za 10 minut namestili ščipalko za prilagoditev listov na temotne razmere. S tem smo zagotovili, da so bili vsi fotosistemi II 'odprti' oziroma, da lahko sprejmejo fotone za biokemijsko delo (Stanje 1). Izračunali smo jo kot $F_v/F_m = (F_m - F_0)/F_m$, tako da smo najprej izmerili minimalno fluorescenco ($F_0$) z uporabo merilne svetlobe majhne jakosti (0,15 µmol $m^{-2}s^{-1}$ fotosintetsko aktivnega sevanja). Nato smo uporabili satuacijski pulz svetlobe (7000 µmol $m^{-2}$ $s^{-1}$ fotosintetsko aktivnega sevanja, 0,8 s), ki je »zaprl« vse fotosisteme II (Stanje 2), da smo izmerili maksimalno fluorescenco ($F_m$) (Lichtenthalter in sod., 2005).

Koncentracijo klorofila v listih smo izmerili z napravo SPAD-502 klorofilmeter (Minolta, Osaka, Japonska). Na vsakem listu smo opravili tri meritve vsebnosti klorofila in izračunali povprečno vrednost.

### 2.4 Statistična analiza

Za ugotavljanje statistično značilnih sprememb parametrov v času smo obdelali podatke s programom R (R Development Core Team, 2008) s paketom *Rcmdr* (v. 2.2-3) in programom GraphPad Prism 5.00 (GraphPad Software, Inc., La Jolla, CA, USA). Obravnavanji A (GLRaV-1) in B (mešana okužba) smo primerjali z dvosmerno analizo variance (ANOVA, $p \leq 0,05$). Proučevana dejavnika sta bila vrsta okužbe (enojna, mešana) in termin meritve. Statistično značilne razlike med obravnavanji po posameznih terminih meritev smo računali z Bonferonnijevim posttestom. Trte obravnavanja A, ki so bile okužene še z GLRaV-3, niso bile vključene v analizo (preglednica 1). Vse meritve na slikah in preglednici 2 so predstavljene kot povprečje s standardno napako (povprečje ± s.n.).

## 3 REZULTATI

### 3.1 Prisotnost GLRaV-1 in GLRaV-3 v vzorcih vinske trte

V nekaterih trtah skupine A, ki so bile glede na rezultate testa DAS-ELISA in prvega RT-PCR okužene samo z GLRaV-1, smo pozneje v sezoni potrdili tudi prisotnost GLRaV-3. Te trte (A3, A5 in A6) so bile popolnoma izključene iz fizioloških analiz (preglednica 1).

**Preglednica 1:** Prisotnost GLRaV-1 in GLRaV-3 glede na vzorčenje med junijem in septembrom v rastlinjaku na Kmetijskem inštitutu Slovenije leta 2014

**Table 1:** The presence of GLRaV-1 and GLRaV-3 according to the sampling between June and September in greenhouse of Agricultural institute of Slovenia in 2014

| Rastlina / Plant | Meritev / Measurement | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| A1 | + | + | + | + | + |
| A2 | + | + | + | + | + |
| A3* | + | ++ | + | + | + |
| A4 | + | + | + | + | + |
| A5* | + | + | ++ | ++ | + |
| A6* | + | + | ++ | + | + |
| A7 | + | + | + | + | + |
| A8 | + | + | + | + | + |
| B1-B8 | ++ | ++ | ++ | ++ | ++ |

A, B – razporeditev trt v obravnavanja glede na rezultate testa ELISA in zaporedna številka trte; Rastlina: A – okužene z GLRaV-1; B – okužene z GLRaV-1 in GLRaV-3; Meritev: 1, 2, 3, 4, 5 – zaporedna številka meritve (1 – 3. 6. 2014, 2 – 14. 7. 2014, 3 – 11. 8. 2014, 4 – 25. 8. 2014, 5 – 8. 9. 2014); + – zaznana prisotnost GLRaV-1 s PCR; ++ – zaznana prisotnost GLRaV-1 in GLRaV-3 s PCR; * - trte, ki niso bile vključene v statistično analizo

A, B – distribution of grapevines in treatments based on the results of the ELISA test and the number of the grapevine; Plant: A – GLRaV-1-infected; B – GLRaV-1 and GLRaV-3-infected; Measurement: 1, 2, 3, 4, 5 – measurement dates (1 – 3 June 2014, 2 – 14 July 2014, 3 – 11 August 2014, 4 – 25 August 2014, 5 – 8 September 2014); + – GLRaV-1 confirmed by PCR; ++ – GLRaV-1 and GLRaV-3 confirmed by PCR; * - grapevines which were not included in the statistical analysis

### 3.2 Vpliv okužbe z GLRaV-1 in mešane okužbe z GLRaV-1 in GLRaV-3 na vinsko trto

Dvosmerna analiza variance je pokazala, da je vrsta okužbe (enojna, mešana) značilno vplivala na vse merjene parametre, razen na $F_v'/F_m'$ in Chl. Termin merjenja je imel značilen vpliv na vse merjene parametre. Pri Pn, $F_v'/F_m'$ in ETR je bila značilna interakcija obeh dejavnikov (termin merjenja in vrsta okužbe), kar pomeni, da se je pri teh parametrih vrsta okužbe različno odražala glede na termin merjenja.

Bonferonnijev posttest je pokazal značilno razliko med trtami z enojno okužbo in tistimi z mešano okužbo pri Pn, ETR, E in $g_s$ v sredini julija. Listi trt, okuženih z GLRaV-1, so imeli dvakrat večjo Pn (9,19 ± 1,43 μmol $CO_2$ $m^{-2}$ $s^{-1}$) od listov z mešano okužbo (4,16 ± 0,60 μmol $CO_2$ $m^{-2}$ $s^{-1}$). Značilno manjši vpliv okužbe z GLRaV-1 v primerjavi z mešano okužbo se je pokazal tudi pri $g_s$, E in ETR Podrobne povprečne vrednosti parametrov in procesov so navedene v preglednici 2.

Na listih trt v našem poskusu se bolezenska znamenja značilna za bolezen zvijanja listov vinske trte niso razvila. Koncentracija klorofila se med obravnavanjema ni bistveno razlikovala. V sredini julija in začetku avgusta je bila pri mešani okužbi koncentracija klorofila sicer nekoliko manjša kot pri okužbi z GLRaV-1, vendar ne značilno.

**Preglednica 2:** Povprečne vrednosti fizioloških parametrov in standardna napaka znotraj obravnavanj A (GLRaV-1) in B (GLRaV-1 + GLRaV-3) med junijem in septembrom 2014 v rastlinjaku na Kmetijskem inštitutu Slovenije

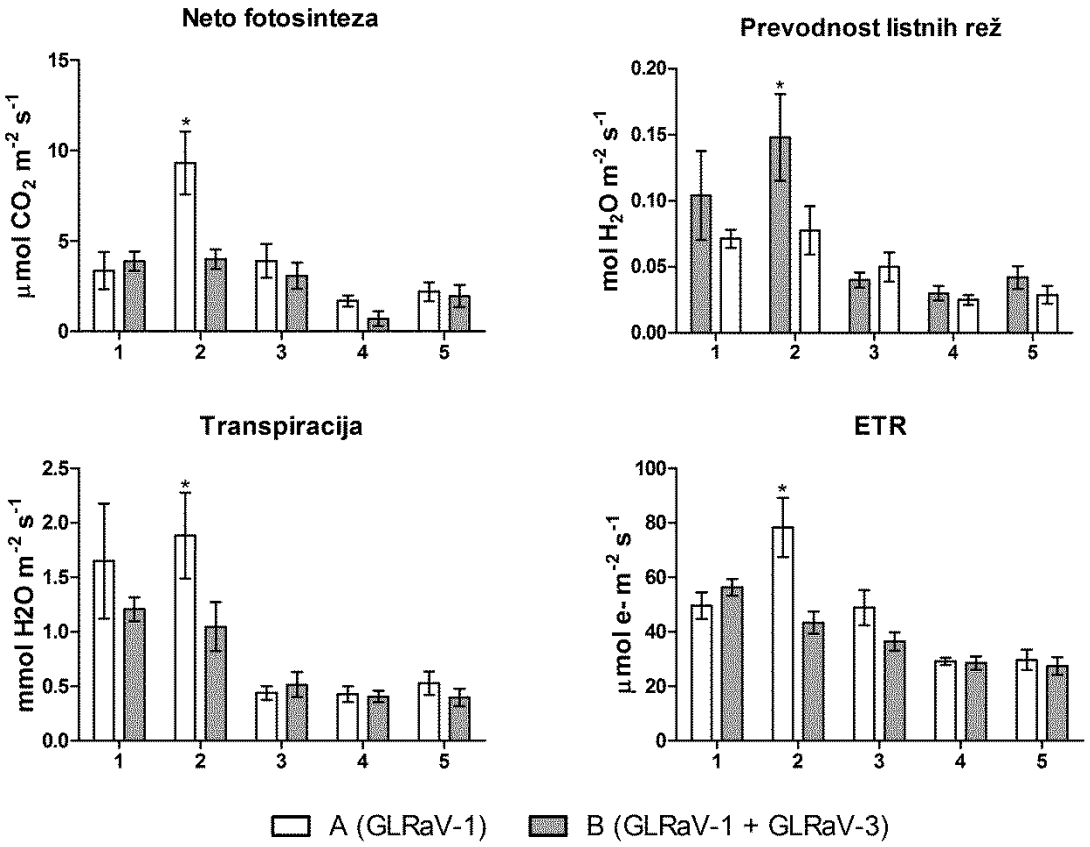
**Table 2:** Mean values and standard error of physiological parameters within the treatments A (GLRaV-1) and B (GLRaV-1 + GLRaV-3) between June and September 2014 in greenhouse of Agricultural institute of Slovenia

| | t | A | B | |
|---|---|---|---|---|
| Pn | 1 | 3,84 ± 0,97 | 3,88 ± 0,62 | |
| | 2 | 9,19 ± 1,43 | 4,16 ± 0,60 | * |
| | 3 | 3,79 ± 0,77 | 3,08 ± 0,72 | |
| | 4 | 1,33 ± 0,44 | 0,89 ± 0,43 | |
| | 5 | 1,88 ± 0,53 | 2,02 ± 0,69 | |
| $F_v'/F_m'$ | 1 | 0,48 ± 0,05 | 0,47 ± 0,02 | |
| | 2 | 0,52 ± 0,02 | 0,46 ± 0,01 | |
| | 3 | 0,39 ± 0,01 | 0,44 ± 0,01 | |
| | 4 | 0,48 ± 0,02 | 0,44 ± 0,01 | |
| | 5 | 0,48 ± 0,02 | 0,52 ± 0,02 | |
| $F_v/F_m$ | 1 | 0,76 ± 0,02 | 0,78 ± 0,01 | |
| | 2 | 0,78 ± 0,01 | 0,77 ± 0,01 | |
| | 3 | 0,76 ± 0,01 | 0,78 ± 0,01 | |
| | 4 | 0,71 ± 0,02 | 0,74 ± 0,01 | |
| | 5 | 0,72 ± 0,02 | 0,73 ± 0,01 | |
| $g_s$ | 1 | 0,11 ± 0,03 | 0,07 ± 0,01 | |
| | 2 | 0,16 ± 0,03 | 0,08 ± 0,02 | * |
| | 3 | 0,03 ± 0,01 | 0,05 ± 0,01 | |
| | 4 | 0,03 ± 0,01 | 0,02 ± 0,00 | |
| | 5 | 0,04 ± 0,01 | 0,03 ± 0,01 | |
| E | 1 | 1,80 ± 0,49 | 1,21 ± 0,12 | |
| | 2 | 2,05 ± 0,28 | 1,10 ± 0,25 | * |
| | 3 | 0,37 ± 0,08 | 0,51 ± 0,12 | |
| | 4 | 0,37 ± 0,08 | 0,40 ± 0,06 | |
| | 5 | 0,47 ± 0,13 | 0,38 ± 0,09 | |
| ETR | 1 | 51,78 ± 4,53 | 56,28 ± 3,06 | |
| | 2 | 76,07 ± 9,15 | 43,33 ± 4,01 | * |
| | 3 | 48,78 ± 5,30 | 36,36 ± 3,33 | |
| | 4 | 27,79 ± 1,72 | 28,46 ± 2,44 | |
| | 5 | 28,21 ± 3,36 | 27,38 ± 3,28 | |
| Chl | 1 | 30,57 ± 1,01 | 30,38 ± 0,78 | |
| | 2 | 34,18 ± 3,30 | 31,05 ± 1,64 | |
| | 3 | 35,85 ± 2,61 | 30,24 ± 1,06 | |
| | 4 | 33,77 ± 2,20 | 31,18 ± 1,46 | |
| | 5 | 29,90 ± 2,37 | 28,44 ± 1,52 | |

A, B – razporeditev trt v obravnavanja glede na rezultate molekularnih analiz (A – okužene z GLRaV-1, B – okužene z GLRaV-1 in GLRaV-3); Pn – neto fotosinteza; $g_s$ – prevodnost listnih rež; E – transpiracija; $F_v'/F_m'$ – dejanska fotokemična učinkovitost PSII; $F_v/F_m$ – potencialna fotokemična učinkovitost PSII; ETR – hitrost transporta elektronov po tilakoidi; Chl – klorofil (SPAD meritev); t – termin meritev; 1, 2, 3, 4, 5 – zaporedna številka meritve (1 – 3. 6. 2014, 2 – 14. 7. 2014, 3 – 11. 8. 2014, 4 – 25. 8. 2014, 5 – 8. 9. 2014); * – statistično značilna razlika med obravnanvanjem A in B ($p < 0,05$)

A, B – distribution of grapevines in treatments based on the molecular analyses (A – GLRaV-1 infected, B – GLRaV-1 and GLRaV-3 infected); Pn – neto photosynthesis; $g_s$ – stomatal conductance; E – transpiration; $F_v'/F_m'$ – effective quantum yield of PSII; $F_v/F_m$ – maximum quantum efficiency of PSII; ETR – electron transport rate; Chl –

chlorophyll (SPAD measurement); t – measurement date; 1, 2, 3, 4, 5 – serial number of the measurements (1 – 3 June 2014, 2 – 14 July 2014, 3 – 11 August 2014, 4 – 25 August 2014, 5 – 8 September 2014); * – statistically significant difference between treatments A and B ($p < 0,05$)




**Slika 1:** Neto fotosinteza, prevodnost listnih rež, transpiracija in hitrost transporta elektronov po tilakoidi (ETR) pri obravnavanjih A (okužba z GLRaV-1) in B (okužba z GLRaV-1in GLRaV-3) v petih terminih meritev na žlahtni vinski trti (*Vitis vinifera* L.) leta 2014. Prikazana so povprečja s standardno napako. 1, 2, 3, 4, 5 – zaporedna številka meritve (1 – 3. 6. 2014, 2 – 14. 7. 2014, 3 – 11. 8. 2014, 4 – 25. 8. 2014, 5 – 8. 9. 2014); * – statistično značilna razlika med obravnavanjema A in B ($p < 0,05$)

**Figure 1:** Net photosynthesis, stomatal conductance, transpiration and thylakoid electron transport rate (ETR) in treatment A (infection with GLRaV-1) and B (infection with GLRaV-1 and GLRaV-3) measured on grapevine (*Vitis vinifera* L.) in 2014. Average values with standard error are presented. The measurements were performed five times (1 – 3. 6. 2014, 2 – 14. 7. 2014, 3 – 11. 8. 2014, 4 – 25. 8. 2014, 5 – 8. 9. 2014); * –significant difference between treatments A and B at $p < 0.05$

## 4 DISKUSIJA

Po nam znanih podatkih predstavlja študija prvo primerjavo učinka okužbe z GLRaV-1 in mešane okužbe (GLRaV-1 in GLRaV-3) na žlahtno vinsko trto. V primerjavi z okužbo le z GLRaV-1 smo pri mešani okužbi pri drugem merjenju, v sredini julija, izmerili manjšo neto fotosintezo, počasnejši transport elektronov po tilakoidi, manjšo transpiracijo in prevodnost listnih rež. Sampol in sod. (2003) prav tako poročajo o manjši neto fotosintezi (tudi do 45 %, odvisno od starosti lista), prevodnosti listnih rež ter vsebnosti karotenoidov in fluorescenci klorofila kot posledici okužbe vinske trte z GLRaV ter virusom pahljačavosti listov vinske trte (*Grapevine fanleaf virus*; GFLV) v lončnih poskusih. Podobne rezultate merjenj aktivnosti fizioloških procesov v z GLRaV

okuženimi rastlinami so ugotovili tudi pri drugih sortah (Bertamini in sod., 2004; Moutinho-Pereira in sod., 2012).

Tudi drugi virusi, predstavniki drugih rodov in družin, negativno vplivajo na fiziološke parametre v vinski trti. Reynard in Gugerli (2015) sta ugotovila, da z rdečo packavostjo listov vinske trte povezani virus (*Grapevine red blotch-associated virus*; GRBaV), ki povzroča podobna bolezenska znamenja kot GLRaV, negativno vpliva na neto fotosintezo, transpiracijo in prevodnost listnih rež. Tako kot v našem poskusu z GLRaV, je bil tudi pri GRBaV vpliv viden že sredi julija, še pred pojavom bolezenskih znamenj. Neto fotosinteza, transpiracija in prevodnost listnih rež so bili za okoli 30 % manjši v primerjavi s kontrolno skupino. Gambino in sod., (2012) so ob koncu rastne dobe izmerili manjšo vsebnost klorofila in zmanjšano neto fotosintezo na sorti 'Bosco' okuženi z razbrazdanjem debla skalne in vinske trte povezanim virusom (*Grapevine Rupestris stem pitting-associated virus*; GRSPaV). Podobno so Basso in sod. (2010) zabeležili značilno zmanjšanje vsebnosti klorofila in fotosintetskega potenciala pri trtah z bolezenskimi znamenji sort 'Cabernet franc' in 'Cabernet sauvignon', ki so bile prav tako okužene z GRSPaV.

Agrotehnični in ampelotehnični ukrepi in razmere okolja vinske trte vplivajo na pojavnost bolezenskih znamenj. O vplivu razmer gojenja na pojavnost znamenj bolezni zvijanja listov vinske trte so poročali Barba in sod. (1989), Cabaleiro in sod. (1997, 1999) ter Christov in sod. (2007). Pri gojenju rastlin okuženih z GLRaV v *in vitro* razmerah Barba in sod. (1989) niso opazili bolezenskih znamenj na listih, čeprav je bila koncentracija virusa po čiščenju pri trtah gojenih v *in vitro* razmerah celo 30-krat večja kot pri trtah v vinogradu, ki so imele bolezenska znamenja. Tudi Christov in sod. (2007) niso opazili bolezenskih znamenj na listih *in vitro* gojenih rastlin vzgojenih iz zimskih brstov trt, ki so na polju kazale znamenja okužbe. Podobno tudi v našem poskusu na listih trt v rastlinjaku nismo opazili bolezenskih znamenj značilnih za bolezen zvijanja listov vinske trte.

Pri razlagi rezultatov moramo torej upoštevati tudi rastne razmere, saj se vrednotenja v razmerah *in vitro*, v lončnih poskusih (v rastlinjaku) in na prostem lahko razlikujejo. Cabaleiro in sod. (1997, 1999) v lončnih poskusih niso izmerili značilnih sprememb neto fotosinteze, medtem ko so razlike ugotovili pri poskusu v vinogradu. Neto fotosinteza pri trtah okuženih z GLRaV-3 je bila za od 53 do 65 % manjša kot pri neokuženih trtah. Okužba z enim ali obema virusoma v našem poskusu ni značilno vplivala na maksimalno potencialno učinkovitost PSII ($F_v/F_m$), ki je pri vitalnih rastlinah v dobrem fiziološkem stanju približno 0,83. $F_v/F_m$ se zmanjša, kadar je rastlina izpostavljena močnemu ali dolgotrajnemu stresu, ki povzroči nepovratne posledice fotosinteznega aparata (Vodnik, 2001). Na zmanjšanje razmerja $F_v/F_m$ lahko vplivajo tudi drugi dejavniki, npr. povišanje okoljske temperature. Konstantnost razmerja $F_v/F_m$ med merjenji (preglednica 2) nakazuje, da so bile razmere v rastlinjaku primerne za izvajanje poskusa z vinsko trto in da okužba z GLRaV ni povzročila nepovratnih poškodb fotosinteznega aparata. V našem primeru to pomeni, da na podlagi meritev $F_v/F_m$ ne moremo ločiti vpliva okužbe z enim ali obema virusoma pri vinski trti v rastlinjaku. Pri dejanski učinkovitosti PSII ($F_v'/F_m'$) je opaziti majhne, neznačilne razlike med trtami okuženimi z GLRaV-1 ali z GLRaV-1 in GLRaV-3 (preglednica 2).

Največ raziskav vpliva GLRaV na vinsko trto je bilo narejenih pri trtah okuženih z GLRaV-3, mnogo manj pri trtah okuženih z GLRaV-1 in le nekaj pri trtah z mešanimi okužbami. Raziskovalci so se osredotočali predvsem na kakovost in količino pridelka ter vsebnost snovi v jagodah (Martelli, 2014). Spring in sod. (2012) v svoji raziskavi niso ugotovili razlik pri vplivu na pridelek med trtami okuženimi z GLRaV-1 in GFkV ter tistimi okuženimi samo z GLRaV-1. Pri primerjavi vpliva na pridelek med okuženimi in zdravimi trtami so Tomažič in sod. (2003) ugotovili, da okužba trte z GLRaV-1 zmanjša pridelek. Podobno so slabšo rodnost trt opazili Endeshaw in sod. (2014) ob okužbi z GLRaV-3 in Moutinho-Pereira in sod. (2012) ob mešani okužbi z GLRaV-1 in GLRaV-3. Santini in sod. (2011) so ugotovili zmanjšanje pridelka pri mešani okužbi z GLRaV-1 in GVA ne pa tudi pri mešani okužbi z GLRaV-3 in GVA.

Raziskovalci so neodvisno od bolezenskih znamenj in njihovih povzročiteljev dokazali, da je odziv trt na fiziološke procese in pridelek med sortami

različen (Zufferey in sod., 2000). Zato je pomembno, da študije opravljamo na domačih sortah trt v lokalnih razmerah. Le na tak način lahko dobimo realne podatke o delovanju procesov v trti.

## 5 ZAKLJUČEK

V okviru raziskave smo merili fiziološke parametre povezane s fotosintezo na trtah okuženih samo z GLRaV-1 ali okuženih z GLRaV-1 in GLRaV-3 v rastlinjaku. Ugotovili smo, da se v začetku junija vrednosti merjenih parametrov med različnimi obravnavanji niso značilno razlikovale. Značilne razlike med okužbo z enim virusom ter mešano okužbo so se pojavile le pri neto fotosintezi, potencialni fotokemični učinkovitosti PSII, prevodnosti listnih rež, transpiraciji, hitrosti transporta elektronov po tilakoidi in vsebnosti klorofila pri merjenju sredi julija. Na podlagi rezultatov merjenj v rastlinjaku težko posplošimo, kakšen je vpliv GLRaV na vinsko trto, še posebno pred pojavom bolezenskih znamenj, ali kakšen je njihov vpliv na trte na prostem.

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**Agrovoc descriptors:** Slugs, noxious molluscs, mollusc control, pest control, control methods, ecological control, cultural pest control

**Agris category code**: H01, h10

# Možnosti okoljsko sprejemljivega zatiranja gospodarsko škodljivih polžev s poudarkom na rezultatih domačih raziskav zatiranja lazarjev (Arionidae)

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**IZVLEČEK**

Polže uvrščamo med gospodarsko pomembne škodljivce gojenih in samoniklih rastlin. Škodljivi so predvsem polži brez hišic, ki jih uvrščamo v družini lazarjev (Arionidae) in slinarjev (Limacidae). Polži so vsejede živali. Občutljivi so na izsušitev, zato so aktivni ponoči in v oblačnem, deževnem vremenu. Gospodarsko škodo povzročajo na vrtninah, poljščinah, sadnem drevju, grmovnicah, travi, zeliščih in okrasnih rastlinah. Prvi zapisi o zatiranju polžev segajo v začetek 20. stoletja. Sledilo je pomembno odkritje v letu 1934, ko so v Južni Afriki odkrili metaldehid, ki je prinesel pomemben napredek v varstvu rastlin pred polži. Naslednji pomemben korak v razvoju limacidov je bil dosežen z odkritjem karbamatov. Zaradi dokazanega neciljnega delovanja metaldehida in ostalih snovi z limacidnim delovanjem raziskovalci iščejo alternativne rešitve v varstvu rastlin pred polži. V prispevku predstavljamo različne okoljsko sprejemljive načine zatiranja polžev, kot so ustrezna obdelava tal, nastavljanje pasti s pivom, postavljanje prehodnih ovir in uporaba t.i. elektroograj, s poudarkom na rezultatih domačih raziskav zatiranja lazarjev.

**Ključne besede:** polži, zatiranje, okoljsko sprejemljivi načini

**ABSTRACT**

**POSSIBILITIES OF ENVIRONMENTALLY ACCEPTABLE CONTROL METHODS AGAINST ECONOMICALLY IMPORTANT SLUGS WITH EMPHASIS ON THE RESULTS OF DOMESTIC RESEARCHES OF CONTROLLING ARIONIDAE SLUGS**

Slugs from the Arionidae and Limacidae families are classified as an important economic agricultural pests. They are omnivorous animals. Slugs are sensitive to drying out, so they are active at night and in cloudy, rainy weather. They cause economic damage to the vegetables, crops, fruit trees, shrubs, grasses, herbs and ornamental plants. First reports of slugs control are dating from the early 20$^{th}$ century. This was followed by an important discovery in the year 1934 when in South Africa metaldehyde was discovered. This discovery has brought significant progress in the protection of plants against slugs. The next major step in the development of molluscicides was achieved with the discovery of carbamates. Due to the proven non-target effect of metaldehyde and other substances with molluscicidal activity researchers are looking for alternative solutions in the protection of plants against slugs. In this paper we present a variety of environmentally acceptable methods of slugs control, such as proper soil cultivation, beer trapping, barriers and the use of so-called electrical fences against slugs.

**Key words:** slugs, control, environmentally acceptable methods

## 1 UVOD

Številčnost lazarjev (Arionidae) se je v zadnjih letih močno povečala. Čeprav so ti polži gospodarsko zelo škodljivi (Frank, 1998; Hammond in sod., 1999; Ahmadi, 2004), pa imajo v naravi pomembno vlogo pri ohranjanju biološkega ravnovesja, saj se hranijo z različnimi

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rastlinskimi in živalskimi ostanki (Burnie, 2001; Ortan, 2014). Polže uvrščamo med gospodarsko pomembne škodljivce, še posebno v letih in razmerah, ki so za njih ugodna (mile zime, leta z veliko dežja). Gospodarsko škodo v večji meri povzročajo le polži brez hišic (Godin, 1983; Laznik in sod., 2011). To so predstavniki iz družin lazarjev (Arionidae) in slinarjev (Limacidae) (Milevoj, 2007).

Polži so občutljivi na izsušitev, zato so aktivni ponoči in v oblačnem, deževnem vremenu. Večina se prehranjuje s svežimi in odmrlimi rastlinskimi deli. Zelo radi se hranijo z mladimi rastlinami, npr. sadikami. Škodo povzročajo na vrtninah, poljščinah, sadnem drevju, grmovnicah, travi, zeliščih in okrasnih rastlinah (Godin, 1983). Ob množičnem pojavu lahko polži povzročijo velik izpad pridelka. S hranjenjem na rastlinah, kar je vidno v obliki manjših ali večjih luknjic, povzročajo količinski in kakovostni izpad pridelka (Milevoj, 2007).

## 2 DEJAVNIKI, KI VPLIVAJO NA POJAV POLŽEV

Pojavljanje lazarjev vzpodbuja gojenje rastlin v monokulturah in uporaba fitofarmacevtskih sredstev, ki omejujeta številčnost naravnih sovražnikov na takšnih območjih (Milevoj, 2007). Podobno velja za izsuševanje vlažnih biotopov, kar prizadane naravne sovražnike polžev (Dmitrieva, 1978). Populacije polžev se večajo tudi zaradi opustitve gojitve perutnine na prostem, ki se hrani z jajčeci polžev. Pojav polžev je večji ob milih zimah in vlažnih poletjih. Zelo dobra skrivališča zanje so neobdelana zemljišča, zeleni pasovi ob prometnicah, zarasla, zapleveljena ter mulčena zemljišča. Njihovo število se poveča ob pretirani uporabi dušika na zemljiščih (Milevoj, 2007).

Pojav lazarjev zaznamo že zgodaj spomladi, ko se temperature dvignejo nad 5 °C. Mladi lazarji se iz jajčec izležejo marca ali aprila. Na začetku ostanejo blizu svojih skrivališč in se z rastlinskimi ostanki hranijo le v neposredni bližini skrivališč. Število lazarjev se začne hitreje večati v sredini aprila, ko povprečna dnevna temperatura doseže 10 °C (Young in Port, 1989). Od začetka do sredine maja populacija doseže prvi vrh. Lazarji se nato selijo in intenzivno iščejo hrano, kar jim omogoča hitro rast. Drugi vrh populacije dosežejo nekje med začetkom in sredino avgusta. Večja populacija lazarjev se pogosto pojavi tudi konec septembra, včasih pa šele v sredini oktobra (Kozlowski in Sionek, 2000).

## 3 ŠKODLJIVOST

Gospodarsko škodljivi so predvsem polži brez hišice, in sicer predstavniki dveh družin: lazarji (Arionidae) in slinarji (Limacidae). Že nekaj polžev na kvadratni meter lahko povsem uniči pridelek (Milevoj, 2007; Laznik in Trdan, 2009). Lazarji spadajo med pljučarje in polifage, ki se hranijo s široko paleto živil. Pasejo se na razpadajočih zelenih delih rastline in na rastočih organizmih, kot so glive, alge, mah, šotišče in lišaji (Kozlowski in Kozlowska, 1998; Kozlowski, 2000). Njihova osnovna hrana so gojene in divje rastoče rastline. Najraje se hranijo z novo rastočimi rastlinami (s kaljenimi semeni, sadikami, listi, vejicami, koreninami, sadjem) in pogosto povzročajo poškodbe na posevkih, zlasti na mladih komaj posajenih rastlinah (Esenko, 2008). Opravljen je bil poskus, v katerem so opazovali, s katerimi rastlinami se lazarji najraje prehranjujejo. Največ poškodb je bilo zabeleženih na vrtni solati, zelju, korenju, rdeči pesi, redkvici, peteršilju in fižolu, medtem ko je bilo na sončnicah, detelji in krompirju poškodb najmanj. Poškodbe so bile zabeležene tudi na okrasnih rastlinah, zeliščih in jagodnjaku(Kozlowski in Kozlowska, 1998; Kozlowski, 2000).

## 4 ZATIRANJE

Za uspešno zatiranje polžev moramo najprej ugotoviti, kakšno škodo povzročajo ter kolikšen je njen obseg (Laznik in Trdan, 2009). Pri odločanju o izbiri načina zatiranja polžev je poleg stroškov zatiranja pomembna tudi njegova okoljska neoporečnost. Včasih se lahko pred polži uspešno obvarujemo že z ustrezno obdelavo tal, kolobarjenjem, pravočasno in kakovostno setvijo ipd. (Vakselj, 1992). Lahko si pomagamo z različnimi biotičnimi varstvenimi ukrepi, če pa z navedenimi ukrepi nismo učinkoviti, lahko uporabimo še kemične načine zatiranja. Zaradi jesenskega odlaganja jajčec je zelo priporočljivo zatirati polže tudi jeseni, ker tako zmanjšamo njihovo spomladansko populacijo (Ortan, 2014). Žal pa v jesenskem času ljudje na polže pogosto pozabijo, saj se ti zaradi nižjih temperatur poskrijejo v svoja skrivališča in so zato slabše opazni.

Uporaba prvih vab sega v leto 1899, ko so začeli proti polžem nastavljati vabe z bakrovim acetoarzenatom ter ostalimi bakrovimi in arzenovimi spojinami. Od leta 1939 so bile v uporabi vabe z raznimi anorganskimi solmi, na primer z barijevim, kalcijevim in natrijevim fluorsilikatom. Leta 1934 so v Južni Afriki odkrili metaldehid, ki je prinesel pomemben napredek pri zatiranju polžev (Henderson in Triebskorn, 2002). Naslednji velik korak v razvoju je bil dosežen s karbamati: izolanom, sevinom, metiokarbom, kloetokarbom, tiodikarbom in bensultapom. Izvedene so bile tudi raziskave s kofeinom, ki je kot čisti koncentrat sicer zelo strupen, celo bolj kot metaldehid (Hata in sod., 1997). V devetdesetih letih prejšnjega stoletja so ponovno začeli raziskovati učinkovitost vab na osnovi različnih kovin (železo, aluminij) in organskih nosilcev. V poljskih poskusih so se za najbolj učinkovite pripravke izkazale snovi z metaldehidom in karbamati (Henderson in Triebskorn, 2002).

Pri zatiranju polžev na vrtovih, kjer se velikokrat prerazmnožijo, je pomembno, da tla obdelujemo in za vrt redno skrbimo (Vakselj, 1992). Pazimo na razpoke, ki nastanejo v tleh, saj te polžem omogočajo idealno zatočišče in skrivališče za odlaganje jajčec. Koristno je, da jih čim prej zasujemo. V primeru težkih tal je priporočljivo jeseni počakati na prvo zmrzal, nato pa tla preorati. Spomladi pa tla čim prej zrahljamo in uničimo skrivališča, s čimer spravimo jajčeca iz skrivališč na površje, kjer se hitro posušijo (Dmitrieva, 1978). Koristno je tudi, da v območju okrog vrta pokosimo travo, ki prav tako predstavlja ustrezno zatočišče za polže. Izogibamo se prepogostemu zastiranju s pokošeno travo, saj travna zastirka prav tako predstavlja idealno zatočišče za polže (Dmitrieva, 1978). Spomladi, ko začnemo s setvijo in sajenjem rastlin, je pomembno, da to storimo pravočasno, in sicer takrat, ko imajo rastline dobre razmere za rast. S tem rastlinam omogočimo, da hitro (z)rastejo in so manj časa v občutljivi fazi (Purvis, 1996).

Ročno pobiranje polžev se izvaja v zgodnjih jutranjih urah ali pa zvečer in ob deževnem vremenu, ko so polži aktivni skozi ves dan. Ta metoda je bolj primerna za majhna zemljišča, na večjih zemljiščih je pobiranje prenaporno (Esenko, 2008). Precej učinkovito metodo za zmanjševanje škodljivosti polžev na manjših površinah predstavljajo tudi vabe s pivom (Ortan, 2014). V tla vkopljemo jogurtov lonček, napolnjen s pivom. Lončka ne napolnimo do vrha, da polži zlezejo v lonček in se v pivu utopijo. Za zmanjšanje škode, ki jo napravijo polži, si lahko pomagamo tudi s postavitvijo ovir (Schüder in sod., 2003). Namen ovir je, da polžem prepreči hranjenje in povzročitev poškodb na gojenih rastlinah. Namestimo jih med mestom, kjer se polži skrivajo in mestom, kjer se hranijo. V ta namen uporabljamo pripravke, ki polže izsušujejo in povzročajo močno izločanje sluzi (Prior, 1985). Ker se z izločanjem sluzi polži v kritičnih razmerah zaščitijo le enkrat, mora biti takšna ovira dovolj široka. Po tleh lahko potrosimo lesni pepel ali apno, in sicer v debelini od 1 do 3 cm (Resnik, 2015). Oba pripravka sta higroskopična, povzročata izsušitev in izločanje sluzi (Schüder in sod., 2003). Učinek je viden le ob suhem vremenu. Priporočljiva širina varovalnega pasu je od 10 do 20 cm. Poraba žganega apna je od 20 do 30 kg/ar (Vakselj, 1992; Resnik, 2015).

Žgano apno ali apneni dušik lahko uporabimo tudi drugače, in sicer tako, da ga potrosimo po talnem površju (Vakselj, 1992). Zemljišče najprej pokosimo in odstranimo pokošeno gmoto. Pozno zvečer ali zgodaj zjutraj ob suhem ali rahlo

vlažnem vremenu apno oziroma apneni dušik potrosimo v dveh odmerkih, od 3 do 4 kg/ar žganega apna oziroma od 1,5 do 2 kg/ar apnenega dušika. Med nanosoma počakamo vsaj od 15 do 30 min. Snovi morata priti v neposreden stik s polži (Resnik, 2015).

Apneni dušik je iz naravnih sestavin: apnenca, premoga in zračnega dušika. Dokazano je bilo, da je mogoče z redno uporabo tega gnojila uspešno zmanjšati pojav nekaterih talnih škodljivcev (strun [*Agriotes* spp.]) in bolezni (golšavost kapusnic [*Plasmodiophora brassicae* Woronin], bela gniloba [*Sclerotinia sclerotiorum* (Lib.) de Bary]), prav tako pa lahko vplivamo tudi na trajno zmanjšanje populacije polžev ter manjšo zapleveljenost. Posebnost gnojila je počasno sproščanje dušika, ki se ne izpira ob močnih padavinah. Rastlina poleg dušika sprejme tudi kalcij, ki rastlinam omogoča večjo odpornost na bolezni in škodljivce. Z uporabo apnenega dušika ne spreminjamo bistveno pH tal, lahko pa celo zmanjšamo njihovo kislost (Šušek, 2015). Da čim bolj učinkovito zatremo polže, je priporočljivo apneni dušik posuti od 8 do 10 dni pred sajenjem. Takrat imajo polži brez hišice nezavarovano mehko telo, ki je zelo občutljivo za poškodbe in jedke snovi. Dokazano je, da so mladi polži in jajčeca zelo občutljivi za gnojenje z apnenim dušikom (Ryder in Bowen, 1977). Pri uporabi 30 g apnenega dušika/$m^2$ je bilo dva dni po nanosu živih polžev samo 24 %, pri količini 50 g/$m^2$ pa je bilo preživelih polžev le še 11 %. Pozorni moramo biti samo pri razgradnji gnojila, kjer nastajajo plini, ki so fitotoksični in lahko do neke mere delujejo fungicidno in herbicidno (Šušek, 2015).

f Med naravne sovražnike polžev uvrščamo race. Za najučinkovitejše so se izkazale pekinška, indijska ali domača raca (Ortan, 2014). Poskrbeti moramo, da imajo na voljo dovolj vode, saj imajo polži slinasto telo in bi zaradi tega lahko škodljivo vplivali na prebavni trakt živali. Pomagajo nam lahko tudi domače kokoši, ki se hranijo z jajčeci polžev, ki jih polži odložijo v tla (Vakselj, 1992).

Za enega od uspešnih načinov biotičnega zatiranja polžev se je v tujini že izkazala tudi uporaba parazitske ogorčice *Phasmarhabditis hermaphrodita* (Schneider) (Wilson in sod., 1993). Pripravek je patentiran pod uradnim imenom Nemaslug®. Parazitske ogorčice polžev trenutno prodajajo v 14-ih evropskih državah. V Sloveniji parazitskih ogorčic polžev za te namene še nismo uporabljali in preučevali, saj je omejena vrsta še vedno na seznamu t.i. tujerodnih organizmov (Laznik in sod., 2009; Resnik, 2015).

## 5 REZULTATI DOMAČIH RAZISKAV

V laboratorijskem poskusu smo na Biotehniški fakulteti v Ljubljani preučevali učinkovitost delovanja izbranih okoljsko sprejemljivih snovi pri zatiranju lazarjev in raziskali potencialne možnosti uporabe teh snovi za omejevanje njihovega škodljivega delovanja na gojenih rastlinah. V poskus smo vključili naslednje snovi: lesni pepel, hidrirano apno, diatomejsko zemljo in žagovino. Njihov vpliv smo preučevali posamično ali v kombinaciji. Izvedli smo dva poskusa, in sicer kontaktni učinek posamezne snovi ali kombinacije snovi pri valjanju in učinek postavitve prehodne ovire. Ob stiku s snovjo so se lazarji v trenutku skrčili in začeli močno izločati sluz. Tretiranega polža smo položili v petrijevko, ki je vsebovala list solate in vlažen tampon. Največjo smrtnost preučevanih polžev smo ugotovili pri uporabi hidriranega apna, kjer so lazarji v trenutku poginili. Tudi v drugem poskusu, v katerem smo na sredino posode položili svež list solate in vlažen tampon, okrog pa posuli različne preučevane snovi, se je za najučinkovitejšo oviro lazarjem izkazalo hidrirano apno oziroma kombinacija hidriranega apna z drugimi snovmi (Resnik, 2015).

V letih 2008 in 2009 smo v laboratorijskih razmerah preizkušali limacidno delovanje 26 snovi v 89 različnih obravnavanjih (Mihičinac, 2010). Poskusi, v katere smo vključili lazarje (*Arion* spp.), so potekali v dveh serijah, in sicer z injiciranjem aktivne snovi v prebavilo polžev in z uporabo pelet. V naši raziskavi smo pri injiciranju 100 % smrtnost polžev ugotovili v obravnavanju z bakterijo *Bacillus thuringiensis* Berliner 1915 var. *kurstaki* (0,25 ml v 10 % koncentraciji/osebek), kofeinom (0,25 ml v 10 % koncentraciji/osebek), natrijevim dodecil sulfatom (0,25 ml v 10 % koncentraciji/osebek; 0,125 ml v 10 %

koncentraciji/osebek; 0,125 ml v 5 % koncentraciji/osebek; 0,0625 ml v 10 % koncentraciji/osebek) in pirimikarbom (0,25 ml v 10 % koncentraciji/osebek; 0,125 ml v 10 % koncentraciji/osebek; 0,125 ml v 5 % koncentraciji/osebek; 0,0625 ml v 10 % koncentraciji/osebek), medtem ko smo največjo (100 %) smrtnost polžev pri uporabi pelet dosegli z natrijevim dodecil sulfatom v 0,5 % koncentraciji z dodatkom kumine (Mihičinac, 2010).

V laboratorijskem poskusu smo z metodo vbrizgavanja v prebavilo lazarjev (*Arion* spp.) preizkušali limacidno učinkovitost štirih različnih snovi: pripravkov Delfin WG in Pirimor 50 WG ter kofeina in natrijevega dodecil sulfata (Verlič, 2012). Učinkovitost smo preizkušali pri štirih različnih koncentracijah (1,25; 2,5; 5; 10 %) in v treh različnih odmerkih (0,062; 0,125; 0,25 ml). Največji limacidni potencial je imel natrijev dodecil sulfat, ki je pokazal zadovoljivo delovanje že pri manjših koncentracijah. Pri vbrizgavanju smo 100 % smrtnost lazarjev ugotovili v obravnavanjih s pripravkom Delfin WG (0,25 ml v 10 % koncentraciji, 0,25 ml v 5 % koncentraciji in 0,25 ml v 2,5 % koncentraciji), z natrijevim dodecil sulfatom (0,25 ml v 10 % koncentraciji, 0,125 ml v 10 % koncentraciji, 0,062 ml v 10 % koncentraciji, 0,25 ml v 5 % koncentraciji, 0,125 ml v 5 % koncentraciji, 0,062 ml v 5 % koncentraciji in 0,25 ml v 2,5 % koncentraciji), kofeinom (0,25 ml v 10 % koncentraciji, 0,125 ml v 10 % koncentraciji in 0,25 ml v 5 % koncentraciji) in pripravkom Pirimor 50 WG (0,25 ml v 10 % koncentraciji, 0,125 ml v 10 % koncentraciji, 0,062 ml v 10 % koncentracij, 0,25 ml v 5 % koncentraciji in 0,125 ml v 5 % koncentraciji). Najboljše limacidno delovanje na lazarje smo dosegli z natrijevim dodecil sulfatom v 2,5 % koncentraciji (Verlič, 2012).

V laboratorijskem poskusu smo preučevali vpliv jakosti in napetosti električnega toka pri električni oviri, ki smo jo postavili z namenom preprečevanja prehoda polža (*Arion* spp.) k viru hrane (Laznik in sod., 2011). Preučevali smo napetost (2 V, 4 V, 6 V, 8 V in 10 V) v kombinaciji z jakostjo (0,1 mA, 0.01 mA in 0.001 mA). Rezultati naše raziskave so pokazali, da na uspešnost ovire vplivata oba dejavnika, tako jakost kot napetost el. toka. Pri najnižji napetosti (2 V) je oviro prestopilo 41 % preučevanih polžev, pri najvišji napetosti (10 V) pa je oviro prešlo le 1 % polžev. Do podobnih ugotovitev smo prišli tudi pri jakosti električnega toka. Najboljšo kombinacijo je predstavljalo razmerje 10 V in 0.01 mA, kjer nismo zabeležili prehoda polžev čez oviro (Laznik in sod., 2011).

## 6 ZAKLJUČEK

Zatiranje gospodarsko pomembnih škodljivcev v kmetijstvu poteka predvsem s kemičnimi sredstvi za varstvo rastlin, ki pa imajo lahko na okolje tudi negativne vplive (Hata in sod., 1997; Henderson in Triebskorn, 2002). Za številne aktivne snovi so raziskovalci ugotovili, da izkazujejo neciljno delovanje na organizme (Martin, 1993; Purvis, 1996). Zaradi teh dejstev in vedno večje okoljske osveščenosti in skrbi za okolje, raziskovalci širom sveta iščejo načine v varstvu rastlin, ki so okoljsko sprejemljivi in nimajo negativnih vplivov na okolje in ljudi (Laznik in sod., 2011). Z ustrezno obdelavo tal lahko pripomoremo k zmanjšanju številčnosti polžev, z drugimi okoljsko sprejemljivimi ukrepi njihovega zatiranja, nekateri med njimi so predstavljeni v prispevku, pa lahko vplivamo na manjšo uporabo limacidov ali pa slednjih celo ni potrebno uporabiti.

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# CONTENT ANALYSIS OF THE PAPERS IN THE ACTA AGRICULTURAE SLOVENICA

# VSEBINSKA OBDELAVA PRISPEVKOV V ACTA AGRICULTURAE SLOVENICA let. 107 št. 2

Karmen STOPAR[a], Tomaž BARTOL[b]

## SUBJECT INDEX BY AGROVOC DESCRIPTORS
## PREDMETNO KAZALO PO DESKRIPTORJIH AGROVOC



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## VSEBINSKO KAZALO PO SKUPINAH ZNANJA (PREDMETNIH KATEGORIJAH)

## NAVODILA AVTORJEM

(letniki z liho številko - rastlinska proizvodnja)

### Prispevki

Sprejemamo izvirne znanstvene članke s področja agronomije, hortikulture, rastlinske biotehnologije, raziskave živil rastlinskega izvora, agrarne ekonomike in informatike ter s sorodnih področij - **letniki z liho številko** (npr. 97, 99) - v slovenskem in angleškem jeziku; pregledne znanstvene članke samo po poprejšnjem dogovoru. Objavljamo tudi izbrane razširjene znanstvene prispevke s posvetovanj, vendar morajo taki prispevki zajeti najmanj 30 % dodatnih originalnih vsebin, ki še niso bile objavljene. O tovrstni predhodni objavi mora avtor obvestiti uredniški odbor. Če je prispevek del diplomske naloge, magistrskega ali doktorskega dela, navedemo to in tudi mentorja na dnu prve strani. Navedbe morajo biti v slovenskem in angleškem jeziku.
Prispevke sprejemamo vse leto.
Podrobnejša navodila:

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## INSTRUCTIONS FOR AUTHORS

(Odd-numbered volumes - plant production)

### Articles

The Journal *accepts original scientific* articles from the fields of agronomy, horticulture, plant biotechnology, plant-related food-and-nutrition research, agricultural economics, information-science, and related research - odd-numbered volumes (for example: 97, 99) - in Slovenian or English language. Review articles are published in advance agreement with the editorial board. Extended versions of selected proceedings-papers can also be considered for acceptance, provided they include at least 30 % of new original content, but the editorial board must be notified beforehand. If the article is based on a thesis or dissertation, the thesis-type must be indicated (BSc, MSc, PhD...), along with the role of the candidate and advisor, at the bottom of the first article page.
Manuscripts are accepted throughout the year.
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