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Ovitek: Poškodbe (bele pege) zaradi sesanja pisane stenice (*Eurydema ventralis* Kolenati) na listih cvetače (Foto: Stanislav TRDAN)
*Cover: Injuries (white spots) caused by sucking of the cabbage stink bug (*Eurydema ventralis *Kolenati) on the cauliflower leaf (Photo: Stanislav TRDAN)*

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Utilization of *Tithonia diversifolia* (Hemsl.) A.Gray compost and mycorrhiza on cultivation of *Allium ascalonicum* L. grown on post-mine sandpits soil

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Utilization of *Tithonia diversifolia* (Hemsl.) A.Gray compost and mycorrhiza on cultivation of *Allium ascalonicum* L. grown on post-mine sandpits soil

Abstract: Post-mine sandpits C soil has a potential to be used for vegetable cultivation, nevertheless needs improvement on its physical, chemical, and biological properties through the input of microbial application technology and organic matter. The purpose of this study was to examine the effect of Arbuscular Mycorrhiza Fungi (AMF) and compost (bokashi) of *Tithonia diversifolia* in improving soil physical properties and yield of *Allium ascalonicum*. The study used a two-factor Randomized Block Design. The first factor was AMF provision (control 0 g plant^{-1}, 4 g plant^{-1}, 6 g plant^{-1}, 8 g plant^{-1}, and 10 g plant^{-1}). The second factor was bokashi of *T. diversifolia* (control 0 t ha^{-1}, 3 t ha^{-1}, 6 t ha^{-1}, and 9 t ha^{-1}). The results showed that the application of AMF together with bokashi generated soil porosity and permeability that were suitable for the growth of shallot bulbs. The application of bokashi 9 t ha^{-1} increased bulbs diameter and bulbs fresh mass, although still below its potential due to unfavorable environmental factors. Thus, the successful application of AMF and organic materials need to pay attention on environmental factors in order to produce maximum effect.

Key words: AMF, compost (bokashi), shallot, T. diversifolia.

Uporaba biomase mehiške sončnice (*Tithonia diversifolia* (Hemsl.) A.Gray) in mikorize pri gojenju šalotke (*Allium ascalonicum* L.) na tleh nastalih iz jalovine po rudarjenju

Izvleček: Tla nastala iz jalovine po rudarjenju imajo potencial uporabe za gojenje zelenjave, a potrebujejo izboljšanje fizikalnih, kemijskih in bioloških lastnosti z dodatki mikrobov in organske snovi. Namen te raziskave je bil preučiti učinek dodatka mikorize in komposta iz biomase mehiške sončnice (bokashi) (*Tithonia diversifolia*) za izboljšanje fizikalnih lastnosti tal in pridelka šalotke. Poskus je bil izveden kot dvofaktorski naključni bločni poskus. Prvi factor je bil dodatek mikoriznih gliv (AMF) v odmerkih: kontrola 0 g rastlino^{-1}, 4 g rastlino^{-1}, 6 g rastlino^{-1}, 8 g rastlino^{-1}in 10 g rastlino^{-1}. Drugi factor so bili dodatki komposta v odmerkih: kontrola 0 t ha^{-1}, 3 t ha^{-1}, 6 t ha^{-1}, and 9 t ha^{-1}). Rezultati so pokazali, da sta dodatka mikoriznih gliv in komposta iz mehiške sončnice povzročila poroznost in propustnost tal, kar je bilo primerno za rast šalotke. Dodatek komposta iz mehiške sončnice v odmerku 9 t ha^{-1} je povečal premer čebulic šalotke in njihovo svežo maso, čeprav so bile vrednosti teh dveh parametrov še vedno pod potencialom za to vrsto zaradi neugodnih okoljskih dejavnikov. Iz tega sledi, da je za doseganje maksimalnih učinkov potrebno posvečati pozornost pri dodajanju odmerkov mikoriznih gliv in organske snovi.

Ključne besede: aurbuskularna mikoriza, compost iz biomas mehiške sončnice, šalotka, *T. diversifolia*.

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1 INTRODUCTION

The land-use change of agricultural land to non-agricultural occurs in several regions of Indonesia. In the highlands, there are many changes in land use from vegetable cultivation to hotels, housing or tourist areas. This leads to the narrowing of fertile agricultural land for the production of high economic value vegetables, therefore it requires search alternative soils even with not optimal carrying capacity, one of which is post-mine sandpits C soil.

Post-mine sandpits C soil can widely be found in various regions in Indonesia as a source of gravel and sand for building purposes and a source of regional income. The further activity of these minerals mining causes damage to the ecosystem and is vulnerable to erosion, consequently the Government Regulation of the Republic of Indonesia no. 78 of 2010 requires that mining C land be reclaimed to function again according to its allotment. Post-mine sandpits land reclamation using revegetation has a dual function, namely increasing the area of agricultural production land and remedying the ecology. Post-mine sandpits C soil has several obstacles when used for plant cultivation, such the low C-organic content, sand-dominating texture (Hidayat et al., 2020), low water binding capacity (Ginting et al., 2018), and has not yet formed aggregates so that sensitive to erosion.

Agricultural cultivation activities on post-mine sandpits C soil should be initiated by eliminating the inhibiting factors. The addition of organic matter is a necessity considering that post-mine sandpits C soil has very low organic C (0.86 %) (Hidayat et al., 2020). To support optimal plant growth requires a minimum of C-organic content > 2.5 % (Patrick et al., 2013). Organic matter has been proven to improve soil density, soil porosity, and soil permeability (Hidayat et al., 2020), maintain water availability and improve soil aeration (King et al., 2020). In addition, organic matter is an energy source to support the development of soil decomposer microorganisms (Yin et al., 2019) and as the main ameliorant (Maftu'ah et al., 2014). One source of organic matter that can be utilized is *Tithonia diversifolia* (Hemsl.) A.Gray which is a weed from the Asteraceae family. *Tithonia diversifolia* (Hemsl.) A.Gray was used as bokashi. Bokashi is compost fertilizer produced from a fermentation process using effective microorganisms 4 (EM4) technology so the time required to make it is relatively shorter. EM4 contains *Azotobacter* sp., *Lactobacillus* sp.. *T. diversifolia* (Hemsl.) A. Gray can grow in extreme environments, so that its availability is abundant because the adaptability of the plant is high, moreover, it can grow at various altitudes (Obiakara & Fourcade, 2018). This plant can also increase soil nutrients, improves soil physical properties, and leads to crops productivity enhancement (Hafifah et al., 2016).

The problem of fertilisation with organic matter is the high dose of organic matter, which ranges from 10 t ha^{-1} to 30 t ha^{-1} (Ginting et al., 2018). In this study, we tried to reduce it based on the principle that organic matter is not positioned as a source of nutrients, but as a source of carbon and energy for beneficial soil microbes, namely arbuscular mycorrhizal fungi (AMF).

AMF is a fungus that can be associated with almost all cultivated plants. This type of fungus has a special form of long external hyphae. According to Smith & Read (2008) the external hyphae of AMF can reach up to 30 meters per gram of soil, which is useful in post-mine sandpits C soil with high sand content to increase the bonding among the particles to make the soil more stable. Nurbaity et al. (2013) found that AMF increases the stability of andisol aggregates. Xiao et al. (2019) added that AMF can improve soil fertility in post-mine sandpits C soil.

The application of *T. diversifolia* organic matter together with AMF inoculation is expected to be synergistic. Organic matter improves soil physical properties and supplies carbon for AMF survival. Furthermore, AMF works in increasing aggregate stability, porosity, and soil permeability. AMF also plays a role in the decomposition of organic matter and releases high total P in the post-mine sandpits C soil so that the nutrients become available for the shallot plants that grow in porous conditions.

The purpose of this study was to determine the effect of *T. diversifolia* bokashi and AMF on the improvement of post-mine sandpits C soil properties and yield of shallot (*Allium ascalonicum* L.) Batu Ijo variety.

2 MATERIALS AND METHODS

The research was carried out in Kutamandiri Tanjungsari Village, Bandung Regency with an altitude of 800 m above sea level from February to May 2019. The materials used in this study were post-mine sandpits C soil from sandstone mining in Giri Asih Village, Batujajar District, West Bandung Regency, mixed AMF inoculum (*Gigaspora* sp., *Glomus* sp., and *Aclauspora* sp.), 60 % glucose solution, EM4, alcohol, HCl (2 %), KOH (10 %), blue writing ink, onion bulbs of Batu Ijo variety, plant parts (leaves and young stems) of *T. diversifolia*, bran, urea fertilizer, TSP, KCl, and water. The tools used during the research were sample rings, soil sifter, polybag of 30 x 40 cm, 500 ml rinse bottle, soil sample weighing paper, oven, tissue paper, 250 ml Beaker glass, hoe, knife, microscope, digital scale, watering bucket, scissors, analytical balance, spore clamp, Petridish, spore net, shovel, caliper, thermometer, pH meter, permeability unit, label, plastic rope, stationery, pest trap, paranet, and camera.

In this study, two factorial randomized block design (RBD) was used with 20 treatment levels and three replications. The first factor was the addition of bokashi *T. diversifolia* and the second factor was the addition of AMF.

Factor 1, addition of *Tithonia diversifolia* bokashi (b):

b0: Control (without bokashi)
b1: Bokashi doses of 3 t ha^{-1} (9.37 g polybag^{-1})
b2: Bokashi doses of 6 t ha^{-1} (18.75 g polybag^{-1})
b3: Bokashi doses of 9 t ha^{-1} (28.12 g polybag^{-1})

Factor 2, addition of arbuscular mycorrhizal fungi (m)

m0: control (without AMF)
m1: AMF inoculum of 4 g polybag^{-1}
m2: AMF inoculum of 6 g polybag^{-1}
m3: AMF inoculum of 8 g polybag^{-1}
m4: AMF inoculum of 10 g polybag^{-1}

Observation carried out in this research:

– The degree of AMF infection in plant roots at harvest time was calculated in units (%), using the grid line intersect method (Brundrett et al., 1996).

$$\text{Colonization (\%)} = \frac{\text{The number of infected roots}}{\text{The total number of observed roots}} \times 100\ \%$$

– Soil porosity was observed by calculating the total pore space of the soil with units of percent (%) and the formula (Kurnia et al., 2006):

$$\text{Total pore space (\%)} = 1 - \left[\frac{\text{Bulk density}}{\text{Particel density}}\right] \times 100\%$$

– Soil permeability in saturated solution in laboratory based on Darcy's law (Kurnia et al., 2006) in units (cm hour^{-1}):

$$\text{Permeability (K)} = \frac{QL}{AhL}\ \text{cm hour}^{-1}$$

Q: Water debit (cm3 hour^{-1})
L: Thickness of soil sample (cm)
hL: Water surface height of soil sample and soil thickness (cm)
A: Surface area of the soil sample (cm2)

– Number of bulbs per clump, observation was done after harvest on each plant.
– Bulb diameter (cm), the measurement was carried out using a caliper. This observation was done after harvest on each plant.
– Fresh mass of tubers per clump (g) observation was carried out at the end of the research by weighing the tubers harvested from each plant sample. Before weighing, the soil attaching the bulbs was cleaned.

Observation parameters were analyzed by Anova to determine the effect of treatment. If there was an effect of treatment, it was continued with Duncan's Multiple Range Test for further testing at the 5 % level.

The study started with making bokashi of *T. diversifolia* with 40 kg of plant material, 4 kg of bran, 25 g of glucose solution, and 100 ml of EM4 in December 2018 at UIN Sunan Gunung Djati Bandung campus. Soil from mining C was taken at a depth of 0-50 cm, and sieved with a 1 x 1 cm sieve diameter to separate the soil from the carried rock. The sifted soil was started additional bokashi according to the treatment. Preparation of planting media was carried out two weeks before planting. Moreover, the mixture of soil and bokashi was put into a polybag measuring 30 cm x 40 cm as much as 10 kg. Bokashi mixed with mining soil C one week before planting. The doses of bokashi used were 9.37 g polybag^{-1}, 18.75 g polybag^{-1}, and 28.12 g polybag^{-1}. Meanwhile, AMF inoculation was carried out at the same time as planting shallot bulbs at doses of 4 g polybag^{-1}, 6 g polybag^{-1}, 8 g polybag^{-1}, and 10 g polybag^{-1} bokashi with mixed AMF types. The inoculum was applied with the carrier medium fine zeolite.

The onion bulbs used were Batu Ijo variety of medium size (10^{-1}5 g), healthy and fresh, not wrinkled, dense, and bright in color. Before planting, the dried outer skin of the tubers was cleaned. The bulb seedling was cut at ¼ part of the end of the bulb. Furthermore, 2/3 part of the tuber was immersed into the ground and covered with soil. Each polybag was planted with one tuber.

The maintenance of shallot plants included watering, replanting, weeding, fertilizing and controlling of plant pests and diseases. Watering was carried out 2 times a day, if it rained then it was done according to the conditions. Replanting was done when the plant was 7 day after planting (DAP), weeding was done manually. Fertilization was carried out at the age of 21 DAP according to BALITSA (Vegetable Research Centre) recommendations i.e. 0.21 g polybag^{-1} urea, 0.14 g polybag^{-1} TSP, and 0.1125 g polybag^{-1} KCl.

3 RESULTS AND DISCUSSION

3.1 DEGREE OF ROOT INFECTION

There was no interaction effect of AMF inoculants and *T. diversifolia* bokashi on the degree of root infection in the late vegetative and generative observation times. At the end of vegetative phase observation, the value of the degree of infection was below 20 %, which was included to the low category. The value of the degree of infection increased to above 30 % in the AMF and *T. diversifolia* bokashi treatments during the late generative phase (Table 1).

AMF inoculation increased the degree of root infection in the late vegetative phase, though not significantly. Likewise, the addition of *T. diversifolia* bokashi increased the degree of infection not significantly. In the treatment without inoculants, it was observed that there was a root infection. This was because in mining soil C there were indigenous AMF (Dodd, 2000). The results of wet screening analysis of soil samples taken from the rhizosphere of weeds growing in the mining area of excavation C found AMF spore. Most of the spores were found in the rhizosphere of *T. diversifolia*, *Synedrella nodiflora* (L.) Gaertn., and *Impatiens balsamina* L.. The presence of AMF spores on *T. diversifolia* was the answer why under the independent influence of this weed bokashi generated a degree of infection. Suharno et al. (2014) also found indigenous AMF infecting several plants found on post-mine sandpits soil in Timika Papua, namely Bracharia sp. by 73.33 %, Setaria sp. by 23.33 % and Bidens pilosa L. by 63.33 %. This informs that AMF has the potential to improve the remedy post-mine sandpits soils. The root infection by AMF can be identified in the presence of mycorrhizal organs such as internal hyphae, vesicles, and spores observed under a microscope (Figure 1).

The low value of infection degree in onion plants (Table 1) was influenced by environmental factors. The research conditions during the vegetative phase were often raining which resulted in wet soil and a relatively low average temperature of 24 °C. In this condition, the development of AMF was hampered since AMF will be able to infect host plants effectively and produce mycelia in relatively dry soil conditions with a temperature range of 28-35 °C. This is in line with the research of Hifnalisa et al. (2018) who found a low degree of mycorrhizal infection in coffee seedlings due to high rainfall and a temperature range of 22 °C. Wu & Ying-Ning (2017) stated that in dry soil conditions, AMF can maintain contact with roots, so that AMF will infect plant roots. High soil moisture inhibits spore germination in line with Brundrett & Tedersoo (2018) which states that spore germination and AMF workability are closely related to environmental conditions, especially temperature and soil moisture levels.

Another factor that caused a low degree of infection was the pH of the soil. In this study, the soil pH

Table 1: Effect of *Tithonia diversifolia* bokhasi and AMF on infection degree, growth and yield *Allium ascolicum* L.

Treatments	Average of infection degree (%)		Average number of bulbs per clump (pcs)	Average of Bulbs Diameter (cm)	Average of fresh weight of bulbs per clump (g)
	Late vegetative	Late generative			
T. diversifolia bokhasi					
b0 (0 t ha^{-1})	13.97 a	27.75 a	3.93 a	1.02 a	11.63 a
b1 (3 t ha^{-1})	15.43 a	30.41 a	4.67 a	1.67 b	26.43 b
b2 (6 t ha^{-1})	16.83 a	31.41 a	5.13 a	1.41 ab	20.11 ab
b3 (9 t ha^{-1})	14.64 a	31.60 a	5.40 a	1.89 b	32.01 b
AMF					
m0 (0 g polybag^{-1})	10.09 a	16.05 a	4.83 a	1.37 a	21.13 a
m1 (4 g polybag^{-1})	13.39 a	28.39 b	4.75 a	1.43 a	18.53 a
m2 (6 g polybag^{-1})	18.35 a	34.39 c	4.67 a	1.52 a	23.11 a
m3 (8 g polybag^{-1})	16.90 a	35.13 c	5.08 a	1.56 a	24.68 a
m_4 (10 g polybag^{-1})	17.36 a	37.51 c	4.58 a	1.60 a	25.28 a

Explanation: The average numbers in each column followed by the same letter are not significantly different according to Duncan's

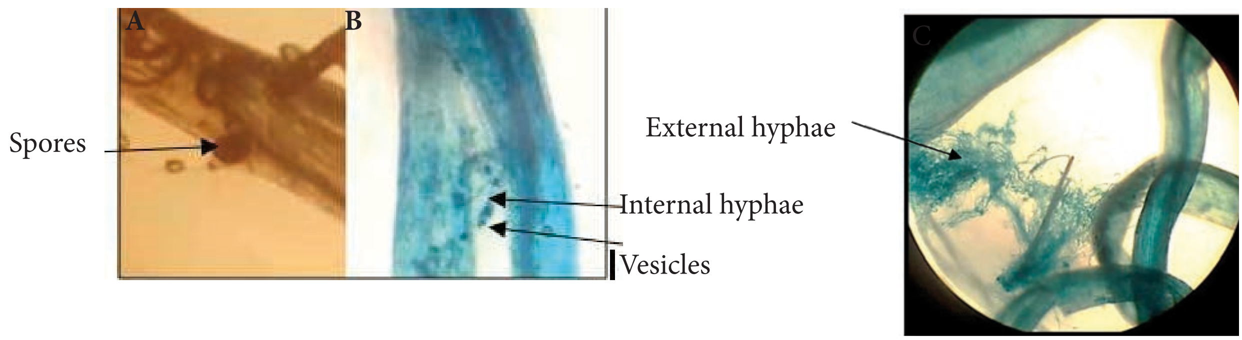

Figure 1: Shallot root infection degree (late vegetative phase); a) Spores that develop in the root tissue; b) AMF organs that are inside and outside the root tissue; c) Degree of generative infection due to 40x microscope magnification

was relatively neutral (6.9). According to Sudheer et al. (2021), AMF has “acidophilis” properties, means actively develops in acidic conditions. Likewise, spores are found in a greater numbers under acidic conditions. Entering the generative phase, the environmental conditions were undergoing to change. Rainfall decreased and temperature increased, so that environmental conditions began to match the conditions desired by AMF, which led to an increase in the degree of root infection to a moderate category (Jerbi et al., 2020).

3.2 SOIL POROSITY

The application of AMF and *T.diversifolia* bokashi had an effect on soil porosity. The lowest average porosity percentage was 24.67 % and the highest was 81.67 % (Figure 1). The provision of *T. diversifolia* bokashi starting at a dose of 3 t ha^{-1} to 9 t ha^{-1} and AMF 6 g $plant^{-1}$ was able to increase the average percentage of soil porosity compared to other treatments. This is in line with the research of Hidayat et al. (2017), namely the provision of AMF and various types of manure compost can improve soil porosity. According to Rahayu et al. (2018), the soil pore needed for shallots is around 60-75 % until the end of the generative period. The results of the study were treated with AMF 6 g $polybag^{-1}$ + *T. diversifolia* 6 t ha^{-1} achieved soil pore 74.00 %, AMF 8 g $polybag^{-1}$ + without *T. diversifolia* 65.33 %, and AMF 10 g $polybag^{-1}$ + *T. diversifolia* 3 t ha^{-1} 62.00 %. These values were sufficient for the pore needs of the shallot plant.

The addition of organic matter can determine the pore volume and size of the soil (Malik & Lu, 2015). The content of organic matter can improve the quality of soil’s physical properties through the stimulation of soil microbes, which makes the soil structure stable. Organic matter also helps the process of soil granulation, the more granulation of the soil formed, the more total of available soil pores.

The large amount of available organic C becomes a source of microbial food that makes the life of microfauna in the soil increase. According to Yang et al. (2017), the addition of AMF to the soil can help the formation of aggregates. AMF has hyphae that can release glomalin, which is able to make the soil particles patch to one another. In sandy soil, glomalin from AMF hyphae acts as an adhesive (binder) of soil particles so that the soil structure becomes granular and many pores are formed. In soils that have low porosity, AMF hyphae are also able to penetrate the soil layer to find sources of water and soil nutrients. When AMF-infected roots grow lengthwise, these roots will break down the soil layer and new pores will be formed inside the soil.

3.3 SOIL PERMEABILITY

The application of AMF together with *T. diversifolia* bokashi had an effect on soil permeability. The lowest average value of soil permeability was 0.52 cm $hour^{-1}$ and the highest was 2.57 cm $hour^{-1}$. The maximum point of soil permeability is found at *T. diversifolia* bokashi 3 t ha^{-1} and at AMF 10 g $polybag^{-1}$ (Figure 2).

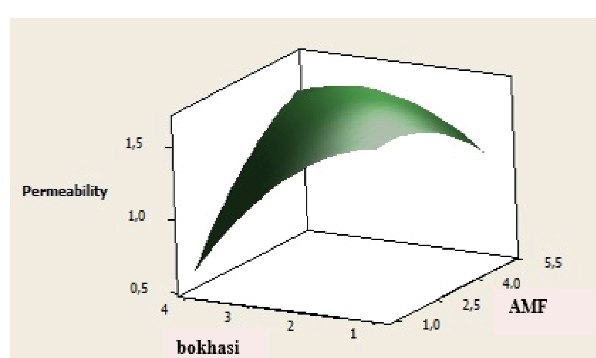

Figure 2: Maximm point AMF and *Tithonia diversifolia* bokashi on soil permeability (cm $hour^{-1}$)

The application of *T. diversifolia* bokashi of 3 t ha^{-1} without AMF and with AMF 10 g polybag^{-1} generated the highest permeability value and successfully entered the criteria for the medium permeability class (2.01-6.25 cm hour^{-1}) based on the permeability class criteria of Uhland and O'Neil (Kurnia et al., 2006). These data are consistent with the effect of AMF application and *T. diversifolia* bokashi on soil porosity (Figure 2) where AMF inoculation of 8 g polybag^{-1} without organic matter and lower AMF inoculation (6 g polybag^{-1}) with *T. diversifolia* bokashi resulted in higher soil porosity, which was sufficient for the soil pore needs of the onion.

The application of *T. diversifolia* bokashi affected the binding of soil particles into soil aggregates. As well as the formation of soil aggregates, it will produce pores that function as a way for water to enter the soil body (Nichols & Halvorson, 2013). According to King et al. (2020), organic matter that has undergone weathering has the ability to absorb water, which is twice higher than the mass. In addition, organic matter also helps in binding water in the soil so that it can be utilized by plants. Besides organic matter, AMF is involved in the process of aggregate formation (Nurbaity et al., 2013) which will affect the pores formed. When the application of *T. diversifolia* bokashi and AMF simultaneously, organic matter from *T. diversifolia* will provide carbon for AMF living needs and activity in binding soil aggregates carried out by external hyphae (Curaqueo et al., 2010) thereby improving the percentage of porosity which ultimately increases soil permeability.

3.4 NUMBER OF BULBS PER CLUMP

The application of AMF and *T. diversifolia* bokashi did not significantly affect the number of tubers (Table 1). The results showed that the average number of bulbs per clump was 3-5, appropriate to the potential for the number of bulbs per clump of 2-5 clumps.
The application of *T. diversifolia* increased the number of bulbs, though not significantly. Organic matter has a slow-release property which causes the availability of nutrients in the soil takes a long time to be absorbed by plants (El-Ramady et al., 2014), so tuber propagation did not increase significantly.

AMF inoculation also did not increase the number of bulbs significantly. This was related to unfavorable environmental conditions. According to Chandra (2018), AMF can develop at low soil moisture, which is 50-60 %. This research took place in conditions of high rainfall, which resulted in increased soil moisture so the AMF did not work optimally in increasing the uptake of P and K nutrients needed for bulbs formation. Yusriadi et al. (2017) Stated that AMF works well at acidic pH, while in this study the pH was neutral.

3.5 BULB DIAMETER

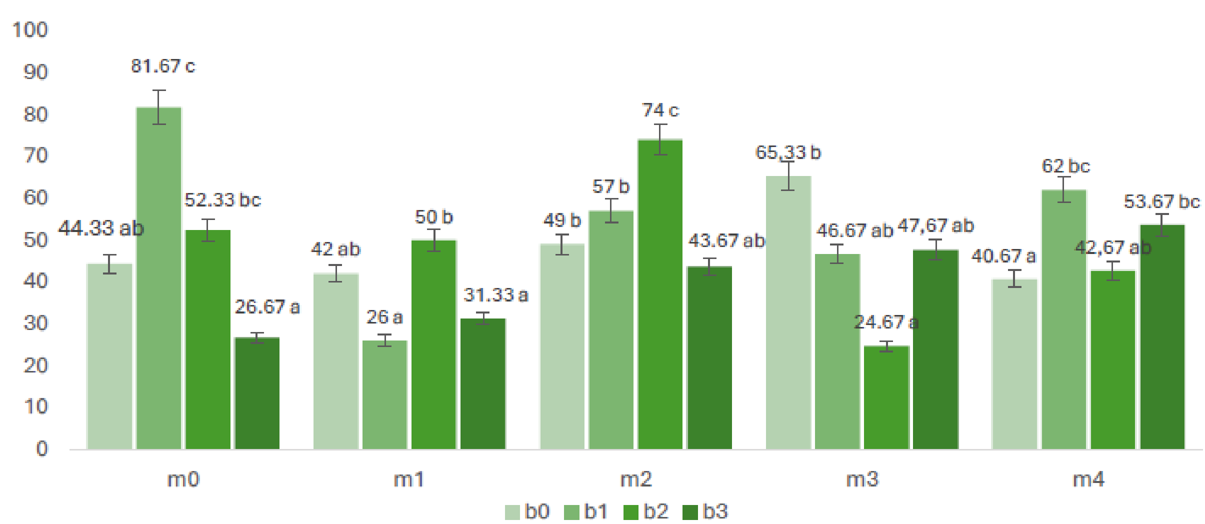

Figure 3: Effect of AMF and *T. diversifolia* bokashi on soil porosity (%)

The application of AMF and bokashi did not affect the diameter of shallot bulbs, but there was an independent effect from the provision of *T. diversifolia* bokashi. The increase of the doses increased the diameter of shallot bulbs and reached the highest value at a doses of 9 t ha^{-1}. Unlike the number of bulbs, *T. diversifolia* bokashi was able to provide the P and K elements needed for the formation of bulbs diameter, although it was not maximal, as seen the value was still below the plant description.

The formation of bulb diameter was also influenced by the planting medium used. Crumbly soil, lots of pores, and high nutrient content can help the tuber development process (Suprapto et al., 2018). The results of the research on the level of soil porosity, the combination of AMF inoculation and *T. diversifolia* bokashi resulted in the 60-75 % porosity required for bulbs enlargement (Table 1).

Referring to the description of the potential of the shallot bulbs diameter namely 3-4.5 cm, while the results of the study only produced an average tuber diameter of 1.2^{-1}.6 cm (Table 1), this indicates that the diameter of the onion bulbs produced in this study was low. The small diameter of the tubers was caused by the bokashi *T. diversifolia* which nutrients were not readily available for the plants. The slow-release nature of organic matter causes the supply of nutrients needed by plants during the generative period to be hampered (El-Ramady et al., 2014), hence the diameter of the tubers produced was not too large.

Based on the characteristics of the shallot plant, the bulbs will have a diameter of 3-4 cm in the optimal climate conditions, with a temperature of 25-32 °C and gets sunlight for more than 12 hours (Firmansyah & Bhermana, 2019). However, in the study, the average daily air temperature only reached 23.3 °C with high humidity (93.3 %), and ± 8 hours of sun exposure, thus the tuber growth was hampered.

3.6 FRESH MASS OF BULBS PER CLUMP

As in the observation of other tuber parameters, the application of AMF and *T. diversifolia* bokashi did not affect the fresh mass of shallots. The addition of bokashi *T. diversifolia* increased the fresh mass of shallot bulbs. The highest fresh mass was shown by giving 9 t ha^{-1} (Table 1). Bokashi *T.diversifolia* provides P and K that can be absorbed by plants (Isrun et al., 2018). The results of the bokashi *T.diversifolia* analysis showed that there was a P content of 1.89 % and K of 3.50 % which were able to support bulbs growth. Then the nutrients obtained by plants will be used for the formation of carbohydrates, proteins, and fats stored in the bulbs so that the fresh mass of the bulbs will increase (Jeptoo et al., 2013).

Referring to the description of the potential fresh mass of tubers per clump, it was ± 92 g $clump^{-1}$. While the results of the study only produced an average fresh mass of 20-25 g $clump^{-1}$, these results showed that the fresh mass of tubers per clump produced in this study was relatively low (Table 1).

4 CONCLUSIONS

The application of AMF and *Tithonia diversifolia* bokashi generated appropriate soil porosity and permeability for the formation of shallot bulbs, but both were not able enhance production of shallot bulbs yield compared to the potential yield. The adding of *T. diversifolia* bokashi 9 t ha^{-1} increased the bulbs diameter and fresh mass of shallots, but still below its potential. Environmental factors that were less supportive contributed to the achievement of results which below the potential.

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Določanje aromatičnih spojin v jabolkih z instrumentalnimi in senzoričnimi metodami

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Določanje aromatičnih spojin v jabolkih z instrumentalnimi in senzoričnimi metodami

Izvleček: Aroma je eden izmed parametrov kakovosti sadja, ki pomembno vpliva na sprejemanje sadja pri potrošnikih. Analiza hlapnih aromatičnih spojin je bistvenega pomena za raziskovanje sort jabolk, izbiro najprimernejše sorte za komercialno trženje ter za rutinsko preverjanje kakovosti. V ta namen se uporablja več metod, ki jih lahko razdelimo na senzorične in instrumentalne. Namen tega članka je predstaviti pregled raziskav, ki so se osredotočale na analizo hlapnih aromatičnih spojin v jabolkih, ki prispevajo k njihovi aromi. Plinska kromatografija je predstavnica instrumentalne metode, med katerimi je najbolj razširjena uporaba plinske kromatografije sklopljene z masno spektrometrijo, medtem ko se za senzorično analizo najpogosteje uporabljajo metode opisne senzorične analize. Senzorična analiza s potrošniki se najpogosteje izvaja z lestvicami všečnosti, kot uporabna metoda pa se je izkazala tudi kvalitativna opisna metoda CATA. Kombinacija instrumentalnih in senzoričnih metod omogoča natančno identifikacijo hlapnih aromatičnih spojin, ki prispevajo k aromi jabolka in njegovi sprejemljivosti.

Ključne besede: hlapne aromatične spojine, aroma jabolk, instrumentalne metode, senzorične metode, določanje arome

Determination of volatile aroma compounds in apples

Abstract: Aroma is one of the most important parameters of fruit quality and has a significant impact on consumer acceptance of fruits. The analysis of volatile aroma compounds is essential for the research of apple varieties, the selection of the most suitable variety for commercial marketing, and routine quality control. Several methods have been used for this purpose, and can be divided into sensory and instrumental methods. In this review article, we present an overview of studies focused on the analysis of volatile compounds in apples that contribute to their aroma. Gas chromatography is a representative instrumental method, among which the most widespread application is gas chromatography coupled with mass spectrometry, while descriptive sensory methods are the most commonly used for sensory analysis. In sensory analysis with consumers, hedonic scales are most often used, however the qualitative descriptive CATA method has also proven to be useful. A combination of instrumental and sensory methods allows accurate identification of the volatile aroma compounds that contribute to apple aroma and its acceptance.

Key words: volatile aroma compounds, aroma of apples, instrumental methods, sensory methods, aroma determination

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1 UVOD

Leta 2021 je bilo globalno pridelanih več kot 93 milijonov ton jabolk (*Malus domestica* Borkh.), kar jih uvršča na tretje mesto po količini največ pridelanega sadja na svetu (FAO, 2023). Vendar podatki kažejo, da poraba jabolk na prebivalca v Evropi upada zaradi spreminjajočih se preferenc potrošnikov do okusa in arome (Bossi Fedrigotti in Fischer, 2020). S prehranskega vidika je jabolko živilo z majhno vsebnostjo maščob (pod 1 %) in beljakovin (pod 1 %), medtem ko sladkorji predstavljajo približno 10 % njegove celotne mase. Prisotna so tudi mikrohranila, kot so minerali (predvsem kalij, kalcij in magnezij), vitamini (predvsem vitamin C) ter sekundarni metaboliti, kot so fenolne spojine (Di Matteo in sod., 2021).

Aroma je, poleg teksture in okusa, eden izmed pomembnejših parametrov kakovosti sadja in pomembno vpliva na sprejemanje sadja pri potrošnikih (El Hadi in sod., 2013). Hlapne spojine, ki določajo aromatični profil sadja, neposredno prispevajo k zaznavnim lastnostim vonja in okusa (Yang in sod., 2021a). Analiza hlapnih aromatičnih spojin je bistvenega pomena za raziskovanje sort jabolk, izbiro najprimernejše sorte za komercialno trženje ter za rutinsko preverjanje kakovosti. Slednje je še posebej pomembno po obiranju jabolk, saj lahko razmere med transportom in skladiščenjem pomembno vplivajo na rok uporabnosti in zaznano »svežino« končnega pridelka (Roberts in Spadafora, 2020). Zaradi vse večjih zahtev trga po zagotovitvi količin pridelka izven pridelovalne sezone in kompleksnosti dobavnih verig, vse večje količine sadja skladiščimo v hladilnicah s kontrolirano atmosfero.

Glede na dihanje plodov, lahko sadje razdelimo v dve skupini: klimakterijsko in neklimakterijsko sadje. Pri klimakterijskem sadju, katerega predstavnik je tudi jabolko, je za postopek zorenja značilen porast dihalne aktivnosti takoj po obiranju in proizvodnja etilena, ki maksimum doseže v obdobju klimakterija (Boeckx in sod., 2019, Gvozdenović, 1989). Z upočasnjevanjem pojava klimakteričnega maksimuma oziroma z zmanjševanjem dihanja, je mogoče zmanjšati intenzivnost dihanja in podaljšati življenjsko dobo plodov. Na to pa lahko v največji meri vplivamo z zniževanjem temperature in zmanjševanjem količine O_2 (Gvozdenović, 1989). Možnost aktivnega nadzora in vzdrževanje majhne vsebnosti kisika je ključna komponenta skladiščnih sistemov za upočasnitev zorenja in ohranjanje kakovosti sadja ter omogoča, da se jabolka skladišči tudi do 11 mesecev (Butkeviciute in sod., 2022). Vendar po drugi strani, majhna vsebnost kisika omejuje količino prekurzorjev, pomembnih za nastanek sadnih estrov, kar vodi v izgubo sortnih arom sadja (Espino-Díaz in sod., 2016; Yang in sod., 2021a).

Za spojine, ki tvorijo aromo živila, je značilna njihova interakcija z vohalnim sistemom človeka, ki tvori posebno občutenje vonja. Hlapne spojine se vežejo na proteinske receptorje, ki so prisotni v vohalnem epiteliju nosne votline. Aktivirani receptorji (t.j. s proteinom G sklopljeni receptorji) nato sprožijo zaporedje dogodkov za pretvorbo kemičnih informacij v senzorični dražljaj. Da hlapne spojine dosežejo vohalne receptorje, morajo običajno izpolnjevati dva ključna pogoja. Prvič, imeti morajo visok parni tlak, in drugič, njihova molekulska masa ne sme biti večja od 300 daltonov (Egea in sod., 2021).

Za snovi, ki prispevajo k aromi sadja, je pomembna njihova najmanjša koncentracija, ki jo človek lahko še zazna. Prav zato le del hlapnih snovi prispeva k aromi, ki jo tudi dejansko zaznamo (Chambers in Koppel, 2013). Čeprav je bilo v jabolkih identificiranih že več kot 300 hlapnih organskih spojin, vključno z alkoholi, aldehidi, kislinami, ketoni, terpenoidi in estri, podskupina, ki jo sestavlja običajno le 20–30 spojin, pomembno prispeva k tipični aromi jabolka. Med njimi glavnino aromatičnega profila jabolk tvorijo estri, zlasti tisti s sodim številom ogljikovih atomov, vključno s kombinacijami ocetne, butanojske, in heksanojske kisline z etilnim, butilnim in heksilnim alkoholom (El Hadi in sod., 2013; Yang in sod., 2021b). ′Granny Smith′ in ′Jonagold′ sta dve sorti jabolk z izrazitim okusom in aromo ter značilno barvo olupka. Ti dve sorti predstavljata dve fenotipski skrajnosti pri proizvodnji in sproščanju hlapnih aromatičnih spojin pri jabolkih. Za ′Granny Smith′ kot pozno zorečo sorto jabolk je značilno, da proizvaja majhne vsebnosti etilnih in propilnih estrov ter višjih alkoholov, kot je 2-metilbutanol. Za sorto ′Jonagold′ pa je značilen hlapni profil z veliko heksil acetata, heksil butanoata, butil heksanoata in α-farnezena. ′Jonagold′ velja za eno najbolj aromatičnih sort jabolk, medtem ko ′Granny Smith′ velja za eno najmanj aromatičnih sort jabolk (Yang in sod., 2022). V jabolkih sorte ′Golden Delicious' je bilo identificiranih in kvantificiranih skupno 65 hlapnih aromatičnih spojin, med katerimi so estri in alkoholi predstavljali več kot 90 % vseh hlapnih spojin. Največji delež so predstavljali butil acetat, 2-metil-^{1}-propanol (izobutanol) in n-butanol. V sorti ′Fuji′ so najbolj prevladovale spojine 2-metil-^{1}-butanol n-butanol in butil acetat. K aromi jabolk sorte ′Honeycrips′ pa domnevno v največji meri prispevajo spojine heksil 2-metilbutanoat, α-farnezen in (E)-2-heksenal (Liu in sod., 2021). Pri sorti ′Gala′ največ k profilu arome prispevajo butil 2-metil acetat, butil acetat in heksil acetat, medtem ko so za sorto ′Pink Lady′ značilni estri etil butanoat, etil 2-metilbutanoat in 2-metilbutil acetat (Yang in sod., 2021b). V preglednici 1 so za spojine, ki prispevajo največ k aromi različnih sort jabolk, predstavljeni pragi vohalnih zaznav in senzorični opisniki.

Preglednica 1: Prag vohalne zaznave in senzorični opisniki hlapnih aromatičnih spojin v jabolkih
Table 1: Olfactory threshold and sensory descriptors for volatlie aroma compounds in apples

Spojina	Prag vohalne zaznave (mg l^{-1})	Opisnik
(E)-2-Heksenal	0,017[b]	po travi, po zeliščih[b]
Butil acetat	0,066[a]	po rdečem jabolku, po banani[a]
Butil 2-metil acetat	0,011[a]	po jabolku, sadno[a]
Butil heksanoat	0,7[a]	travnato, po zelenem jabolku[a]
Etil 2-metilbutanoat	0,001[a]	po jagodah, sadno[a]
Etil butanoat	0,001[a]	po jabolku, po ananasu, sadno[a]
Heksil 2-metilbutanoat	0,022[a]	po jabolku, po grozdju[a]
Heksil acetat	0,002[a]	po rdečem jabolku, po hruški[a]
Heksil butanoat	0,25[a]	po zelenem[a]
Izobutanol	5,3[a]	sladko, plesnivo, patoč
n-Butanol	0,5[a]	močni patoki sladko, po banani[a]
α-Farnezen	NP	po zelenem, po olju, po maščobi[c]

NP: ni podatka, [a]Espino-Díaz in sod. (2016), [b]Yang in sod. (2021c), [c]Yang in sod. (2021)

V zadnjih letih je opravljenih vse več raziskav, ki namenjajo pozornost analizi hlapnih aromatičnih spojin, ki določajo aromo jabolk (Yang in sod., 2021a). Te tradicionalno temeljijo na senzoričnih metodah, ki merijo, analizirajo in interpretirajo reakcije na tiste značilnosti živil, ki jih zaznamo s človeškimi osnovnimi čuti (Lawless in Heymann, 2010). Uporaba senzoričnih metod je lahko včasih draga in dolgotrajna ter ne nudi vedno takojšnih povratnih informacij. V ta namen so se razvile različne instrumentalne metode za proučevanje arome, ki podajajo informacije o posameznih spojinah, povezanih z aromo živila (Chambers in Koppel, 2013).

Namen tega članka je bil pregledati raziskave, ki vključujejo instrumentalne in senzorične metode za analizo hlapnih aromatičnih spojin ter povzeti glavne ugotovitve.

2 INSTRUMENTALNE METODE

Instrumentalne metode omogočajo proučevanje hlapnih spojin. Čeprav so si med seboj različne, pa v osnovi vse temeljijo na ločevanju, določanju in kvantifikaciji spojin iz plinske faze nad živilom ali iz same matrike živila (Sipos in sod., 2011).

Instrumentalne metode za analizo spojin, ki prispevajo k aromi živila, imajo zaradi kompleksnosti področja kar nekaj omejitev. Med njimi so (Rocha in sod., 2022):

- velika raznolikost živil in živilskih izdelkov,
- kemijska raznolikost hlapnih in polhlapnih molekul,
- variabilnost vzorca, ekstrakcijskih in analiznih metod, reagentov …,
- pomanjkanje analitskih standardov,
- potrebe po robustni in zanesljivi obdelavi podatkov ter bioinformatiki,
- težave pri pripravi in analizi velikega števila vzorcev,
- pomanjkanje informacij o opisnikih arome in pragu vohalne zaznave,
- pomanjkanje informacij, povezanih z antagonističnimi in sinergističnimi interakcijami med molekulami ter vplivom matrike živila.

K aromatičnemu profilu jabolk najbolj prispevajo hlapni estri. Celotne arome mešanice hlapnih spojin ne moremo povezati s seštevkom značilnosti arome posameznih komponent, saj imajo hlapne spojine različne prage senzorične zaznave. Aroma ni le vsota posameznih sestavin, temveč je rezultat kompleksnih interakcij med velikim številom kemijskih spojin. Spojine, ki prispevajo k aromi jabolka, lahko medsebojno delujejo in imajo antagonistične ali sinergistične učinke. Vonj je mogoče zaznati tudi kot rezultat sinergizma različnih molekul, čeprav so njihove koncentracije pod pragom detekcije instrumentalnih metod (Niu in sod., 2019).

2.1 PRIPRAVA VZORCA IN EKSTRAKCIJA HLAPNIH SNOVI

Za natančno identifikacijo in kvantifikacijo spojin, odgovornih za aromo v matrikah živil, je pred samo instrumentalno analizo ključna priprava vzorca. Ti koraki običajno vključujejo vzorčenje in homogenizacijo, sledi priprava, čiščenje in koncentracija ekstrakta. Vsi ti koraki morajo biti prilagojeni specifičnim lastnostim matrike živila, ki je predmet analize. Izolacija aromatičnih spojin iz živil običajno temelji na njihovi hlapnosti ali topnosti v določenem topilu. Vsaka metoda pa ima za rezultat drugačen profil arome (Egea in sod., 2021). Na primer, metode ki temeljijo na hlapnosti, bodo predvsem identificirale najbolj hlapne aromatične spojine v živilu, medtem ko bo na ekstrakcijske metode vplivala topnost aromatičnih spojin v izbranem topilu. Pri analizi živil, ki vsebujejo encime, je potrebno zagotoviti, da se sestava arome ne spremeni med samim postopkom priprave vzorca (Regueiro in sod., 2017).

Za ekstrakcijo hlapnih aromatičnih spojin iz živil so bile razvite številne metode, med njimi so: ekstrakcija tekoče-tekoče (angl. Liquid/Liquid Extraction), ekstrakcija na trdni fazi (angl. Solid-Phase Extraction, SPE), superkritična tekočinska ekstrakcija (angl. Supercritical Fluid Extraction, SFE), ekstrakcija na osnovi sorptivnega mehanizma s pomočjo mešalnih paličic (angl. Stir Bar Sorptive Extraction, SBSE), ekstrakcija z destilacijo v visokem vakumu (angl. Solvent Assisted Flavour Evaporation, SAFE), mikroekstrakcija na trdnem nosilcu (angl. Solid-Phase Microextraction, SPME), toplotna desorpcijska enota (angl. Thermal Desorbtion Unit, TDU) in tehnika vzorčenja nadprostora (angl. Headspace, HS) (Starowicz, 2021, Werrie in sod. 2021).

2.1.1 Mikroekstrakcija na trdnem nosilcu (angl. Solid-Phase Microextraction, SPME)

Splošno sprejeto je, da je tehnika vzorčenja iz plinske faze nad živilom (angl. Headspace, HS) najučinkovitejša metoda za vzorčenje hlapnih spojin živila, saj doseže ravnovesje med parno fazo in matriko živila. Zato se v analizi živil najpogosteje uporablja HS-SPME, tudi pri vzorčenju hlapnih aromatičnih spojin v jabolkih (Starowicz, 2021).

SPME je razmeroma nova tehnika vzorčenja, pri kateri je v enem koraku mogoče doseči ekstrakcijo in koncentriranje vzorca. Osnovni princip SPME je izpostavitev trdnega nosilca (vlakna), prevlečenega z določenim materialom, matriki preiskovanega vzorca. Ko je vlakno izpostavljeno vzorcu, se analiti iz matrice vzorca porazdelijo v stacionarno fazo, dokler se ne vzpostavi ravnotežje. Prevleka na vlaknu ekstrahira spojine iz vzorca z absorpcijo (tekoči premazi) ali adsorpcijo (trdni premazi). Po predpisanem času ekstrakcije se vlakno odstrani iz matrike vzorca ter se ga neposredno vstavi v kromatografski instrument, kjer se analit z desorpcijo odstrani iz vlakna (Balasubramanian in Panigrahi, 2011; Billiard in sod., 2020).

Navedeno metodo ekstrakcije so uporabili Qin in sod. (2017) pri analizi hlapnih spojin 43 vzorcev jabolk sorte 'Fuji', z namenom primerjave vsebnosti aromatičnih spojin v jabolkih med različnimi regijami pridelave na Kitajskem. Yang in sod. (2021a) so s HS-SPME vzorčili hlapne spojine olupkov 40 različnih sort jabolk ter vzorce uporabili za identifikacijo, primerjavo in klasifikacijo aromatičnih spojin. Za vzorčenje hlapnih spojin v jabolkih so HS-SPME uporabili tudi Waghmode in sod. (2021), Yang in sod. (2021b), Contreras in sod. (2016), Pontesegger in sod. (2023) ter Yang in sod. (2022).

2.2 PLINSKA KROMATOGRAFIJA (ANGL. GAS CHROMATOGRAPHY (GC))

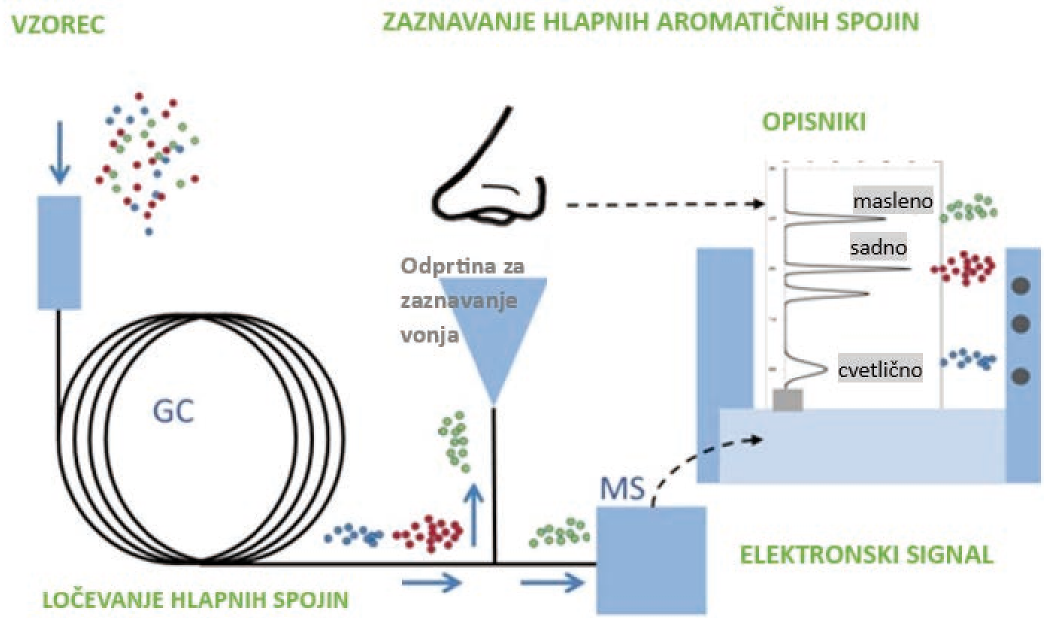
Plinska kromatografija (angl. Gas Chromatography, GC) je separacijska tehnika, pri kateri se posamezne spojine iz vzorca med seboj ločijo na podlagi različne afinitete oziroma interakcij s stacionarno in z mobilno fazo, ki je plin (Coskun, 2016; Pihlar in Prosen, 2019). Z njo je mogoče opraviti tako kvali- kot kvantitativno analizo, pri čemer so potrebne le majhne količine vzorca. Da lahko molekule učinkovito analiziramo z GC, morajo imeti dve pomembni lastnosti: biti morajo hlapne in termično stabilne. Hlapnost je odvisna od parnega tlaka in vrelišča molekul. Termostabilnost pa je pomembna zaradi izvajanja postopka pri visokih temperaturah (50–300 °C), kar lahko povzroči, da analiti spremenijo svojo sestavo (Angeli in sod., 2021; Roberts in Spadafora, 2020).

GC aparat je v osnovi sestavljen iz šestih osnovnih komponent: (Kaur in sod., 2018; Pihlar in Prosen, 2019):

- injektorja: sistem za vnos vzorca v kolono,
- nosilnega plina oziroma mobilne faze, ki prenaša vzorec po koloni,
- kolone,
- ogrevanega prostora ali pečice kjer se nahaja kolona,
- detektorja za zaznavanje analitov,
- sistema za kontrolo kromatografa, prikaz in obdelavo podatkov.

2.2.1 Detektorji

Pri instrumentalnem merjenju hlapnih spojin živilih z GC sta v uporabi običajno plinska kromatografija sklopljena z masno spektrometrijo (angl. Gas Chromatography-Mass Spectrometry, GC-MS) in plinska kromatografija z olfaktometrijo (angl. Gas Chromatography-Olfactometry, GC-O), kjer je plinski kromatograf sklopljen z odprtino za zaznavanje vonja (slika 1). GC-MS združuje dve tehniki, in sicer plinsko kromatografijo za ločevanje hlapnih spojin v vzorcu in masno spektrometrijo za



Slika 1: Shema GC-O (Odour Observatory, 2023)
Figure 1: Schematic of GC-O (Odour Observatory, 2023)

njihovo karakterizacijo. Masni spektrometer je naprava, ki ločuje molekule ali dele molekul glede na razmerje med njihovo maso in nabojem ter nato izmeri množino posameznih ionov. Rezultat je masni spekter: množina posameznih ionov kot funkcija razmerja med maso in nabojem *(m/z)* ter omogoča kvalitativno in kvantitativno vrednotenje (Angeli in sod., 2021; Pihlar in Prosen, 2019). V kolikor je sistem opremljen še z vohalnim priključkom, je mogoče določanje spojin v mešanici hlapnih spojin, ki dejansko prispevajo k aromi, ki jo zaznava človek (Chambers in Koppel, 2013). Za analizo hlapnih spojin v jabolkih je v uporabi tudi plamensko ionizacijski detektor (angl. Flame Ionization Detector, FID) (Altisent in sod., 2008).

HS-SPME v kombinaciji z GS-MS so uporabili Yang in sod. (2022) za analizo hlapnih spojin v jabolkih sorte ′Granny Smith′ in ′Jonagold′ z namenom proučitve regulativnih mehanizmov izražanja genov, ki so povezani s sintezo hlapnih aromatičnih spojin. Enako metodo so uporabili tudi Waghmode in sod. (2021) za vrednotenje hlapnih spojin jabolk sorte ′Golden Delicious′ med skladiščenjem pri nizki temperaturi. Aromo jabolk je mogoče analizirati tudi z uporabo elektronskega jezika in elektronskega nosu. Elektronski nos je analitični instrument, ki posnema človeški vohalni sistem in omogoča preprosto in hitro odkrivanje hlapnih spojin, ki izzovejo občutek vonja (Marx in sod., 2021). Zhu in sod. (2020) so elektronski jezik in elektronski nos v kombinaciji s SPME/GC-MS uporabili za analizo razlik v aromi petih različnih sort jabolk z namenom ovrednotenja kazalnikov kakovosti jabolk. Vonj jabolk so analizirali s prenosnim elektronskim nosom. Sistem so sestavljali enota za vzorčenje, detektorska enota z nizom desetih senzorjev plina, ki zaznajo hlapne spojine ter programska oprema za snemanje in analizo podatkov. Analizo okusa so izvedli z elektronskim jezikom, ki ga je sestavljalo sedem senzorjev: pet testnih in dva referenčna. Testni senzorji so zaznali snovi, ki jih zaznamo kot kisle, slane, sladke, grenke ter trpke. Ekstrakcija hlapnih spojin so izvedli s SPME in nato analizirali z GC-MS. V petih sortah jabolk so skupno kvantificirali 45 hlapnih spojin, med katerimi so bili količinsko najbolj zastopani estri. S pomočjo analize glavnih komponent (PCA, angl. Principal Component Analysis) so ugotovili, da k aromi jabolk najbolj prispevajo spojine heksil butanoat, (E)-2-heksanal in α-farnezen. V raziskavi so prišli tudi do zaključkov, da je SPME/GC-MS v kombinaciji z elektronskim nosom in elektronskim jezikom učinkovita metoda za ocenjevanje razlik v aromi jabolk.

3 SENZORIČNA ANALIZA

Senzorična analiza ja znanstvena disciplina, ki meri, analizira in interpretira reakcije na tiste značilnosti živil, ki jih zaznamo s petimi osnovnimi čuti: z vidom, okusom, vohom, sluhom in tipom oz. dotikom (Stone in Sidel, 1993). Obsega niz različnih tehnik in načinov, ki omogoča natančno merjenje človekovega odziva na hrano ter hkrati minimizira zunanje dejavnike, ki vplivajo na preiskuševalčevo oz. potrošnikovo zaznavo (Golob in sod., 2006). V splošnem lahko senzorične metode razdelimo na analitične in afektivne preskuse. Analitične preskuse izvajajo šolani preskuševalci, ki analizirajo posamezno senzorično lastnost izdelka, medtem ko afektivne preskuse uporabljamo za ocenjevanje všečnosti ali sprejemljivosti izdelka pri potrošniku (Golob in sod., 2005).

3.1 PRESKUŠEVALCI

V senzorični analizi se senzorični preskuševalec obravnava kot instrument za merjenje senzoričnih lastnosti (Kim in sod., 2023). Senzorični preskuševalci delujejo v senzoričnem panelu, ki ga vodi vodja panela in je odgovoren za splošno spremljanje panela in usposobljenost senzoričnih preskuševalcev. Senzorično analizo lahko izvaja več vrst senzoričnih preskuševalcev: senzorični preskuševalec, izbrani senzorični preskuševalec in izvedeni senzorični preskuševalec (ekspert). V prvo skupino, med senzorične preskuševalce, uvrščamo vse, ki sodelujejo v senzoričnem ocenjevanju in so lahko »laiki« brez senzoričnega šolanja, ki imajo povprečne senzorične sposobnosti, ali ocenjevalci začetniki, ki so že sodelovali v senzoričnih testih. Izbrani preskuševalci so izbrani glede na njihovo sposobnost zaznav in izvajanja senzoričnega testa. Izvedeni senzorični preskuševalci so izbrani preskuševalci z izkazano senzorično občutljivostjo, z veliko usposobljenostjo, imajo veliko izkušenj iz senzoričnih ocenjevanj ter so sposobni dosledno in ponovljivo senzorično ocenjevati različne izdelke. Usposobljenost in sposobnosti preskuševalcev je potrebno redno preverjati (Sipos in sod., 2021). Tradicionalna opisna senzorična analiza se izvaja s senzoričnim panelom, ki ga sestavlja večje število senzoričnih preskuševalcev (Kim in sod., 2023). Raziskava Brookfield in sod. (2011) je pokazala, da je za ocenjevanje senzoričnih lastnosti jabolk primerna tudi uporaba manjšega senzoričnega panela (štirje člani), v primeru ko se raziskava osredotoča na majhno število senzoričnih lastnosti. Vendar pa obstaja več težav povezanih z usposabljanjem, s ponovljivostjo in z vzdrževanjem senzoričnega panela za opisno analizo. Usposabljanje omogoča, da se preskuševalci seznanijo z besediščem (opisniki) in ga usvojijo, da izboljšajo sposobnost razlikovanja ter izboljšajo soglasje znotraj panela (Kim in sod., 2023).

Izbira in usposabljanje preskuševalcev je odvisna od nalog, ki jih bodo imeli izbrani in strokovni senzorični preskuševalci (Sipos in sod., 2021). Odvisno od namena senzorične analize, šolanje oz. usposabljanje preskuševalcev traja od nekaj ur do nekaj mesecev. Corollaro in sod.(2012) so z namenom razvoja metode za senzorično profiliranje jabolk po skladiščenju za senzorični panel izbrala 13 preskuševalcev: 6 oseb moškega spola in 7 oseb ženskega spola. Glede na uspešnost na preliminarnem izboru so prvotno izbrali 28 kandidatov. Usposabljanje kandidatov je potekalo v 6 sklopih, po 1,5 h. Za ocenjevanje sposobnosti kandidatov za prepoznavanje in merjenje intenzivnosti osnovnih okusov ter več običajnih vonjav so izvedli 9 posameznih testov. Dražljaje okusa in vonja so predstavili v vodi in v komercialnih motnih raztopinah jabolčnega soka. Pri vsakem testu je kandidat za vsak pravilni odgovor dobil 1 točko, rezultati testa pa so različno ponderirali za predstavljene dražljaje. Končni rezultat je predstavljal kumulativno število doseženih točk z upoštevanjem ponderjev. Pri izboru kandidatov so upoštevali rezultate testov, pri čemer so morali kandidati pravilno prepoznati vsaj 60 % oziroma 80 % dražljajev, in pogostost udeležbe kandidata na usposabljanjih. Kim in sod. (2023) so z uporabo kombinacije metabolomske analize in senzorične ocene želeli ugotoviti povezavo med senzorično oceno in kemijsko sestavo jabolk. Senzorično oceno so izvedli s potrošniki in s panelom usposobljenih preskuševalcev.

3.2 OPISNA ANALIZA

Opisna analiza (angl. Descriptive Analysis) je ena izmed glavnih metod v senzorični analizi za opisovanje senzoričnih lastnosti izdelkov (Charles in sod., 2019). Opisne metode vključujejo usposobljene preskuševalce za kvantitativno vrednotenje senzoričnih lastnosti v vzorcu ali bolj običajno v izboru vzorcev. Preskuševalci so usposobljeni za merjenje različnih senzoričnih lastnosti ocenjevanega izdelka. Besedišče je opisno in objektivno, saj ocenjevalcev na primer ne sprašujejo ali jim je ocenjevan izdelek všeč (O'Sullivan, 2017).

Opisna analiza je uporabna za karakterizacijo izdelkov, opis in spremljanje sprememb senzoričnih lastnosti izdelkov, kot tudi razvoj novih izdelkov ali za primerjavo s konkurenčnimi. Dobljene rezultate je mogoče z uporabo statističnih tehnik, kot sta regresija in korelacija, primerjati z rezultati senzoričnih testov s potrošniki in z rezultati instrumentalnih meritev (Golob in sod., 2006; Lawless in Heymann, 2010).

3.2.1 Opisniki

Posebno pozornost pri vrednotenju senzoričnih lastnosti je potrebno nameniti njihovemu poimenovanju (Chambers in Koppel, 2013). V zadnjih letih je bilo objavljenih več senzoričnih leksikonov, tudi za jabolka (Corollaro in sod., 2013). Leksikon je nabor standardiziranih opisnikov, ki jih izberejo visoko usposobljeni senzorični preskuševalci, in se uporablja za opis senzoričnih lastnosti, prisotnih v analiziranem živilu oziroma izdelku (Suwonsichon, 2019). Za opisnik je značilno, da je jasno definiran, tako besedilno kot z navedbo referenčne snovi in, v primeru raztopine, njene koncentracije. Standardizirani opisniki so še posebej pomembni pri primerjavah rezultatih senzoričnih analiz z rezultati instrumentalnih analiz (Chambers in Koppel, 2013).

3.2.2 Označi vse, kar ustreza (angl. Check-All-That-Apply, CATA)

Ob zavedanju pomena razvoja izdelkov, ki ustrezajo preferencam potrošnikov, je bilo v zadnjih letih razvitih več metod opisne analize, ki se osredotočajo na senzorično ocenjevanje s potrošniki. Ena izmed njih je tudi metoda Označi vse, kar ustreza (angl. Check-All-That-Apply, CATA). Metoda temelji na vprašalniku, ki preskuševalcem predstavi seznam vnaprej določenih opisnikov ali besednih zvez, med katerimi so lahko tudi hedonski, funkcionalni in podobni. Preskuševalci označijo vse izraze, ki se jim zdijo primerni za opis ocenjevanega izdelka. Metoda omogoča pridobitev podobnih rezultatov tistim, ki se jih pridobi s kvalitativno opisno analizo, izvedeno z usposobljenim senzoričnim panelom (Sinesio in sod., 2021; MacKenzie in sod., 2022).

3.2.3 Ovrednoti vse, kar ustreza (angl. Rate-All-That-Apply, RATA)

Metoda CATA ne omogoča neposrednega merjenja intenzivnosti zaznanih opisov senzoričnih lastnosti, kar pa omogoča njena izpeljana različica, metoda Ovrednoti vse, kar ustreza (angl. Rate-All-That-Apply, RATA). Pri tej potrošniki intenzivnost vsakega zaznanega opisa senzorične lastnosti (opisnika) ovrednotijo s pomočjo 3- ali 5-stopenjske lestvice (Vidal in sod., 2018). Primer vprašalnika RATA s 3-stopenjsko lestvico in 15-imi opisniki za senzorično ocenjevanje jabolk prikazuje preglednica 2. Vidal in sod. (2018) so primerjali metodi CATA in RATA v sedmih raziskavah s potrošniki, med katerimi sta dve vključevali tudi jabolka. Metoda RATA se je izkazala za nekoliko bolj uporabno za razlikovanje med vzorci. Potrošniki so pri tej metodi označili več zaznanih lastnosti, kar je lahko morda posledica tega, da so vprašanja RATA vzpodbudila večje razmišljanje in pozornost. Vendar ti rezultati niso nujno boljši, saj CATA omogoča bolj spontano ocenjevanje, ki je bolj podobno situacijam naravnega prehranjevanja. Avtorji raziskave navajajo, da je izbira metode odvisna od značilnosti vzorcev in cilja raziskave. V primerih, ko so razlike med vzorci odvisne od odsotnosti ali prisotnosti opisnikov na seznamu, naj ima prednost CATA, saj je manj analitična in zato bolj primerna naloga za potrošnike. Metoda RATA se priporoča le, če je cilj študije oceniti serijo vzorcev, ki se razlikujejo po relativni intenzivnosti izstopajočih senzoričnih lastnosti, ki so potrošnikom znane in se uporabljajo za opis večine vzorcev.

Preglednica 2: Primer vprašalnika RATA za senzorično ocenjevanje jabolk
Table 2: Example of a RATA questionnaire for the sensory evaluation of apples

Opisnik	Označi, če velja	Označi intenzivnost		
		Nizka	Srednja	Visoka
Sladko				
Kislo				
Grenko				
Trpko				
Aromatično				
Aroma po jabolku				
Nearomatično				
Sočno				
Hrustljavo				
Sveže				
Mehko				
Mokasto				
Grobo				
Trdo				
Vonj po jabolku				
Brez vonja				

3.2.4 Metoda ′Trenutno prevladujoče zaznave′ (angl. Temporal Dominance of Sensations, TDS)

Potrebno se je zavedati tudi, da se med uživanjem živil v ustih odvijajo različni procesi, kot so žvečenje in izločanje sline, ki lahko privedejo do znatnih sprememb sestave živila, kar se lahko kaže tudi v drugačnem profilu arome. Zato je pri zaznavanju arome v ustih smiselno upoštevati tudi časovno enoto (Charles in sod., 2017). Metoda ′Trenutno prevladujoče zaznave′ (angl. Temporal Dominance of Sensations, TDS) je senzorična metoda, ki upošteva dinamično naravo prehranjevanja in vrednoti zaznavanje sprememb senzoričnih lastnosti živil, ki se spreminjajo med samim uživanjem (Oliver in sod., 2018). Charles in sod. (2017) so TDS uporabili v kombinaciji z opisno metodo, da bi bolje razumeli povezavo arome, okusa in teksture v kompleksni in znani matriki jabolka.

4 KOMBINACIJA INSTRUMENTALNIH IN SENZORČINIH METOD

Človeški čuti lahko včasih zaznajo hlapne spojine v manjših koncentracijah od tistih, ki jih zazna instrument, poleg tega pa instrument ne more izmeriti všečnosti. V nasprotju z instrumentalnimi metodami, ki omogočajo merjenje samo ene vrste spojin ali lastnosti (npr. hlapnih spojin, sladkorjev, teksture …), senzorična analiza velja za celosten pristop, ki upošteva vse senzorične lastnosti hkrati ali v zaporednem načinu (Charles in sod., 2019).

Instrumentalne metode pridejo še posebej v poštev, ko uporaba senzoričnih metod ni primerna ali je omejena, kot je pri iskanju in identifikaciji neželenih spojin in spojin, ki povzročajo spremembo okusa. Poleg tega lahko instrumentalne metode delujejo neprekinjeno in omogočajo hitre povratne informacije o analiziranem vzorcu (Sipos in sod., 2021).

Uporaba kombinacije instrumentale in senzorične metode pri analizi arome živila, omogoča identifikacijo spojin, ki so povezane z določeno aromo ter izboljšajo interpretacijo senzoričnih podatkov s prikazom kako se fizikalne in kemijske lastnosti živila odražajo v senzoričnih občutkih (Regueiro in sod., 2017). V preglednici 3 je predstavljen pregled instrumentalnih in senzoričnih metod, uporabljenih v raziskavah hlapnih spojin v jabolkih.

Yan in sod. (2020) so proučevali aromo jabolk sorte ʹHoneycrispʹ s kombinacijo GC-MS, GC-O in opisne senzorične analize. Kombinacija navedenih metod se je izkazala učinkovita za prepoznavanje značilnih vonjev in arome jabolk ʹHoneycrispʹ. K aromi jabolk je najbolj prispeval 2-metilbutanoat, z značilno sadno noto. α-farnezen in 1,3-oktandiol so opredelili kot spojini z najbolj neprijetnim vonjem. Za α-farnezen je značilna nota po zelenem in mastnem in se uvršča med opisnike »po rastlinah«. V raziskavi sicer niso prepoznali nobene specifične spojine, ki bi prispevala k medeni aromi jabolk, vendar se predvideva, da je za to odgovorna spojina estragol, za katero so Souleyre in sod. (2014) poročali, da ima cvetlično aromo. Druga možnost je, da je medena nota posledica mešanice več različnih spojin v matriki jabolka. Yauk in sod. (2015) navajajo, da daje estragol tudi pikanten/aromatičen okus nekaterim sortam jabolk, kot so ʹEllison's Orangeʹ, ʹD'Arcy Spiceʹ in ʹFenouilletʹ, ter noto janeža svežemu jabolku ʹRoyal Galaʹ.

Kim in sod. (2023) so z uporabo kombinacije metabolomske in senzorične analize ugotavljali povezavo med senzoričnimi lastnostmi jabolk ter njihovo kemijsko sestavo. Senzorično analizo so izvedli s potrošniki in z usposobljenim senzoričnim panelom. Potrošniki so stopnjo ugajanja ocenili na lestvici od $^{-1}$00 do +100, pri čemer je $^{-1}$00 pomenila najmočnejšo nenaklonjenost, 0 nevtralno ter +100 najmočnejšo naklonjenost. Za oceno intenzivnosti okusa (sladko in kislo), arome in teksture so uporabili 100-točkovno lestvico (0 = ni zaznavno, 100 = zelo intenzivno). Usposobljeni preskuševalci so intenzivnost deset predhodno izbranih opisnikov ocenili na 15-stopenjski lestvici. Ekstrakcija in analiza hlapnih spojin so izvedli s HS-SPME in GC-MS ter GC-MS/O. Za sorto ʹGamhongʹ (najbolj priljubljena med potrošniki) so bili značilni predvsem prijetni vonji (po jabolku, ananasu, medu, hruški, cvetlični, sadni), ki so bili povezani s hlapnimi aromatičnimi spojinami, predvsem različni estri: derivati acetata in butanoata, vključno s heksil acetatom (po jabolku, sadni), heksil heksanoatom (po jabolčni lupini), heksil butanoatom (po jabolku, ananasu, sadni), butil 2-metilbutanoatom (po ananasu, sadni), butil butanoatom (po ananasu, sadni), butil acetatom (po hruški), 2-metilbutil acetatom (sadni), 2-metilbutil izovaleratom (po jabolku, sadni), izobutil butanoatom (po jabolku, ananasu, sladek), izoamil izobutanoatom (sadni, sladek), amil butanoatom (po ananasu, sladek) in 2-metilbutil butanoatom (sadni, sladek). Po drugi strani pa je bila za sorto ʹHongraʹ (najmanj všečna sorta med potrošniki) značilna neprijetna aroma (večinoma po kumari), ki je bila povezana s hlapnimi snovmi, kot so (E)-2-nonenal (podobni kumari), 2-metil- 1-butanol (po patočnem olju), izobutanol (po vinu), furfuril alkohol (po zažganem) in furfural (po mandlju). Kombinacijo instrumentalnih in senzoričnih metod za analizo aromatičnega profila jabolk so uporabili tudi Altisent in sod. (2011), Charles in sod. (2017), Chitarrini in sod. (2020), Pontesegger in sod. (2023), Roberts in Spadafora (2020) ter Yan in sod. (2020).

4.1 ANALIZA PODATKOV

Za povezovanje podatkov, pridobljenih z instrumentalnimi in s senzoričnimi metodami je ključna zanesljiva analiza podatkov. Poleg osnovnih opisnih statistik je pogosto v uporabi analiza variance (ANOVA), ki ugotavlja ali obstajajo statistično pomembne razlike med različnimi vzorci ali skupinami (Roberts in Cozzolino, 2016). Proučevanje korelacij med različnimi podatki lahko prikaže ali med njimi obstajajo določene povezave. Eden najpogosteje uporabljenih korelacijskih koeficientov je Pearsonov koeficient korelacije. Različne multivariatne tehnike, kot sta analiza glavnih komponent (angl. Principal Component Analysis, PCA) in metoda razvrščanja v skupine (angl. Cluster Method), pomagajo pri prepoznavanju odnosov med spremenljivkami (Chambers in Koppel, 2013).

PCA zmanjša razsežnost podatkov tako, da identificira glavne komponente, ki so najbolj odgovorne za variabilnost med vzorci. Navedena metoda se pogosto

Preglednica 3: Pregled instrumentalnih in senzoričnih metod za analizo hlapnih aromatičnih spojin v jabolkih
Table 3: A review of instrumental and sensory methods for the analysis of volatile aroma compounds in apples

Sorta jabolk	Vir	Geografsko poreklo	Metoda ekstrakcije	Instrumentalna metoda za analizo hlapnih spojin	Senzorična metoda
′Fuji′	Qin in sod. (2017).	40 različnih okrajev Kitajske	HS-SPME	GC-MS	/
40 različnih sort (′Golden Delicious′, ′Fuji′, ′Jonagold′ …)	Yang in sod. (2021a)	Shaanxi Kitajska	HS-SPME	GC-MS	/
′Golden Delicious′	Waghmode in sod. (2021)	Srinagar, Jammu & Kašmir, Indija	HS-SPME	GC-MS	/
9 različnih sort (′Braeburn′, ′Gala′, ′Fuji′…)	Chitarrini in sod. (2020)	Južna Tirolska, Italija	HS-SPME	GC-MS	Kvantitativna opisna analiza
′Honeycrisp′	Yan in sod. (2020)	Shaanxi, Kitajska	Ekstrakcija s topili	GC-MS, AEDA in GC-O	Kvatitatina opisna analiza
′Orin′	Yang in sod. (2021c)	Shaanxi, Kitajska	HS-SPME	GC-MS	/
′Fuji′	Altisent in sod. (2008)	Leida, Španija	TDU	GC-FID	Potrošniški test z 9-točkovno hedonsko lestvico
′Crimson Crisp′	Pontesegger in sod. (2023)	Južna Avstrija	HS-SPME	GC-MS	CATA
′Gamhong′, ′Yangwang′, ′Hongro′, ′Fuji′	Kim in sod. (2023)	Južna Koreja	HS-SPME	GC-MS, GC-MS/O	Hedonsko ocenjevanje na lestvici od $0^{-1}00$, kvantitativna opisna analiza
′Gala′, ′Smitten′, ′Rubens′, ′Granny Smith′	Roberts in Spadafora (2020)	Cardiff, Wales	Ekstrakcija z mikrokomornem toplotnem ekstrakcijskem sistemom	GC-MS	7-točkovna hedonska lestvica
′Golden Delicious′	Charles in sod. (2017)	Lavis, Italija	HS-SPME	GC–MS	TDS in kvantitativna opisna analiza
18 različnih sort (′Braeburn′, ′Topaz′, ′Red Delicious′ …)	Aprea in sod. (2012)	Trento, Italija	HS-SPME	GC-MS	Kvantitativna opisna analiza
′Ralls′, ′Jonagold′, ′Orin′, ′Indo′, ′Hanfu′	Zhu in sod. (2020)	Liaoning, Kitajska	HS-SPME	GC-MS, elektronski nos, elektronski jezik	/
35 različnih sort (′Jonagold′, ′Golden Delicious′, ′Miguo′…)	Wu in sod. (2022)	Liaoning, Kitajska	HS-SPME	GC-MS	/
85 različnih sort (′Granny Smith′, ′Jonagold′, ′Huashuo′…)	Yang in sod. (2021b)	Shaanxi, Kitajska	HS-SPME	GC-MS	/
′Granny Smith′, ′Jonagold′	Yang in sod. (2022)	Shaanxi, Kitajska	HS-SPME	GC-MS	/

uporablja tudi pri analizi podatkov kombinacije instrumentalnih in senzoričnih rezultatov analize arome jabolk (Chitarrini in sod., 2020; Aprea in sod., 2021). Klasifikacijski modeli, katerih primer je linearna diskriminacijska analiza (angl. Linear Discriminant Analysis, LDA), pa omogočajo oblikovanje skupin s podobnimi značilnostmi (Charles in sod., 2017).

5 ZAKLJUČEK

Raziskav, ki se osredotočajo na analizo hlapnih aromatičnih spojin v jabolkih, je veliko, vendar pa je za natančen opis arome najprimernejša kombinacija več metod. Najbolj razširjena instrumentalna metoda je GC-MS, med senzoričnimi metodami pa prevladujejo opisne metode. Ekstrakcijo hlapnih spojin iz jabolk je učinkovito mogoče doseči s tehniko HS-SPME. GC-O omogoča hkratno ločevanje in zaznavanje različnih hlapnih spojin ter identificiranje ključnih spojin, ki prispevajo k aromi jabolk. Pri senzorični analizi s potrošniki so najpogosteje v uporabi hedonske lestvice, primerna pa je tudi kvalitativna opisna metoda CATA. Prednost metode TDS je v tem, da upošteva dinamično naravo prehranjevanja in ocenjuje zaznavanje sprememb senzoričnih lastnosti živil, ki se spreminjajo med samim uživanjem. Pomembno je, da so opisniki v senzorični analizi standardizirani, saj to omogoča medsebojno primerjavo rezultatov senzoričnih in instrumentalnih analiz. Različne statistične multivariatne metode, kot sta PCA in metoda razvrščanja v skupine, omogočajo prepoznavanje povezav med spremenljivkami in podatki, pridobljenih z različnimi metodami. Kombinacija instrumentalnih in senzoričnih metod lahko nudi celostno sliko o povezavi hlapnih spojin v jabolkih ter njihovo aromo.

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Land productivity of root crop farmers amid pesticide application in Southeast Nigeria

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Abstract: The study evaluated the land productivity of root crop farmers amid pesticide application in Southeast Nigeria. A sample of 358 root crop producers was chosen using a multi-stage sampling process. Information on the objectives of the research was obtained using primary instruments. The analysis of the data included the use of mean, frequency, percentage, total factor and partial factor productivity, analysis of variance, multiple regression model, and local average treatment effect (LATE). The results show that root crop growers were mostly women (76.9 %), married(85.1 %), educated (mean=12.0), and in their prime working age (51 years). Estimate of total factor productivity (TFP) and partial factor productivity (PFP) were 7.69 and 177.25, which indicates higher land productivity values across Imo, Abia, and Ebonyi State. Education, access to farm inputs, soil/land improvement practices, size of farm, and extension visits were significant determinants of land productivity at1 % and 5 % levels. Use and application of pesticides according to specified recommendation increased land productivity by (727.07 %) and (880.28 %). Erosion problems (99.7 %), pests and disease (96.9 %), high cost of inputs (99.1 %), climate change (99.4 %) and land fragmentation (93.0 %) constrained land productivity in the states. The study recommends farmers to practice more of soil and land improvement practices and adhere strongly to specified pesticide use and application to increase land productivity.

Key words: land productivity, root crops, household farmers, pesticide application

Produktivnost kmetov pridelovalcev korenovk in gomoljnic ter uporaba pesticidov v jugozahodni Nigeriji.

Izvleček: V raziskavi sta bili ovrednoteni produktivnost kmetov, ki pridelujejo gomoljnice in korenovke ter uporaba pesticidov v jugozahodni Nigerijio. Izbran je bil vzorec 358 pridelovalcev v večstopenjskem procesu vzorčenja. Informacije o predmetih raziskave so bile pridoblljene s primarnimi postopki. Analiza podatkov je vsebovala uporabo poprečij, frekvenčne in odstotkovne analize delne in skupne faktorske produktivnosti, analizo variance, multipli regresijski model in učinek poprečja lokalne pridelave (LATE). Rezultati kažejo, da so pridelovalci teh poljščin pretežno ženske (76,9 %), ki so poročene (85,1 %), izobražene (poprečje = 12,0), z najpogostejšo starostjo 51 let. Izračuna produktivnosti glede na vse (TFP) in posamezne dejavnike (PFP) sta znašala 7,69 in 177,25, kar kaže na večjo produktivnost zemljišč v državah Imo, Abia in Ebonyi. Izobrazba, dostop do pomoči kmetijam, izboljšane tehnike obdelave tal, velikost kmetij in obiski kmetijskih svetovalcev so bili značilni določevalci produktivnosti zemljišč na1 % in 5 % ravni. Uporaba pesticidov glede na priporočila je povečala produktivnost zemljišč za 727,07 % in 880,28 %. Problemi z erozijo(99,7 %), s škodljivci in boleznimi (96,9 %), velikimi stroški pridelave (99, 1 %), s klimatskimi spremembami (99, 4 %) in razdrobljenostjo zemljišč (93,0 %) so omejevali produktivnost v vseh državah. Raziskava priporoča kmetom, da uporabljajo boljše načine obdelave tal in zemljišč in, da se bolj posvetijo k primerni rabi in pripravi pesticidov, kar bo vse povečalo produktivnost.

Ključne besede: produktivnost zemljišč, korenovke in gomoljnice, kmetje, uporaba pesticidov

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1 INTRODUCTION

Despite the strategic significance of the petroleum industry, agriculture remains a substantial sector of the Nigerian economy (FAO, 2020a). In addition to fostering economic growth, it has the capacity to lessen hunger and poverty. The industry employs a sizable labor force and contributes more than 30 % of Gross Domestic Product in Nigeria (FAO, 2021, World Bank, 2022). Agricultural cultivation in Nigeria is still domiciled at the subsistence level with the cultivation of root/tuber crops, legumes, cereals and vegetables. Root crops are majorly grown because they are excellent source of carbohydrates and includescassava (Manihot esculenta Crantz), yam (Dioscorea spp.), cocoyam (Colocassia spp.), and sweet potato (Ipomoea batatas Lam.) are categorized as primary root crops in Africa (FAO, 2020b). These crops are categorized as the primary root crops in Africa. They are important source of income for rural household farmers who make a living from it. They are mostly used in the production of grain, alcohol, fermented drinks, and contain nutrients for humans and animals (Ukeje et al., 2022).

The land productivity of root crops is an indicator of production efficiency. According to Muhammad et al. (2022), it is a measurement of the relationship between output and inputs during the process of production. Umar et al. (2021) define productivity as the total output divided by the total input. It deals with the conversion of specific inputs into outputs. Land productivity can be calculated using both partial and total factor productivity. Partial factor productivity, or PFP, is the ratio of output to each individual input used in the manufacturing process, while total factor productivity TFP, is the ratio of a farm's total output to its entire input used in production (Fuente et al., 2020). Thus, farm output and land productivity could be improved through the application of high yielding inputs. However, root crop production has recently being under attack from pests and diseases, lowering its yield and productivity and thus necessitating pesticide use and application (Đokić et al., 2022). Consequently, to prevent the damaging impact of insects and other pests' attacks on crops, household farmers employ pesticide as a damage control input. Its use is considered a cost-effective, labor-saving, and effective method for controlling insects and other pests (Ladapo et al., 2020). Despite its detrimental effects on both human health and the environment, pesticides provide competitive advantage in agriculture. This is because the usage of pesticides is necessary for maintaining the current levels of production yield and crop quality (Prihandiani et al., 2021). It is on record that pest can reduce yield and productivity of arable land due to its excessive application, and can equally increase crop yield and land productivity of farmers when applied correctly (FAO, 2021).

In Nigeria, several researches have been conducted on crop production, agricultural growth and productivity (Alemu et al., 2017, Montfort et al., 2020, Kurdyś-Kujawska et al., 2021, Đokić et al., 2022, and Hemathilake and Gunathilake, 2022), while other studies have looked at pesticide application on crop production (Yadav et al., 2015, Lozowicka et al., 2015, Al-Wabel et al., 2016, and Tudi et al., 2021). The above studies examined the generality of farmers' land productivity and pesticide application without consideration on the principal root crop or crops; hence, this creates a lacuna that is a wide gap in knowledge and literature.

A priori, the study hypothesized that the land productivity of root crop farmers performed well amid the application of pesticides. This study differs from previous studies in that it is the first study in Sub-Saharan Africa to examine the land productivity of three major root crops (cassava, yam and sweet potatoes) amid pesticide application. Again, the complexity of induced alterations of pesticide use and application on root crop production at the farm level have not been explored in previous studies but was empirically and objectively analyzed in this study, thereby contributing to new knowledge in science and literature. Thus, the study accessed the land productivity of root crop farmers amid pesticide application in Southeast Nigeria.

2 MATERIALS AND METHODS

The study was conducted in southeast Nigeria. The states of Abia, Anambra, Ebonyi, Enugu, and Imo make up this region. The region has an estimated population of 22 million residents, representing 10 % of the entire nation's population (NPC, 2022). Its land area is approximately 41,440 square kilometers. The location of the region lies between latitudes 4 and 7 degrees north and longitudes 7 and 9 degrees east of the equator. The region's native vegetation is that of the tropical rainforest, with sandy-loamy soil predominating. This study employed a multi-stage sampling technique. First, three of the five states that make up the region were chosen at random. In the subsequent phase, two local government areas (LGAs) were chosen from the states, totaling six LGAs. Two communities were chosen at random in the third stage, to make 12 communities. From these communities, two villages were chosen bringing the total to 24 villages. In the last stage, 16 farmers who grow root crops were randomly chosen, creating a sample size of 384 persons that participated in the study. The sample frame was created using a list of registered growers of root crops that was obtained from the State Agricultural Development Program. The study made use of primary data collected using the survey tool (questionnaire). Only 358 of the questionnaires were considered useful for data analysis based on its verified contents. Descriptive statistics, total factor and partial factor productivity, analysis of variance, multiple regression model, and local average treatment effect (LATE) were used to analyze the data. Analysis of the land productivity of the principal root crops grown throughout the states was conducted using total factor and partial factor productivity models and was expressed as follows;

TFP = TO/TI ------------------- eqn. 1

PFP = TO/ IthIPU……………….. eqn. 2

Where;
TFP = Total factor productivity
PFP = Partial factor productivity
TO = Total output
TI = Total input
Ith IPU = Individual inputs used by ith farmer

Analysis of variance (ANOVA) was used test the significant difference in land productivity of major root crops cultivated across the states and was expressed as follows;

$$F = \frac{MSSB}{MSSW} = \frac{SSB/(n-k)}{SSW/(k-1)} \text{-----------} \quad \text{eqn. 3}$$

$$SSB = \sum_{j=1}^{k} nj\ (x-x)^2$$

$$SSW = \sum_{i=1}^{nj} \sum_{j=1}^{k} (Xij - \ddot{X})$$

$$SST = SSB + SSW$$

Where:
F = the number that will be used to determine the statistical significance of the mean difference.
SSB = Sum of square variations between the principal root crops grown throughout the states
SSW = Sum of squares variations from the mean land productivity of the main root crops grown in the states.
SST = Sum total of squares of the land productivity of major root crops cultivated across the states.
Xi = Mean level of land productivity of major root crops cultivated
 = Mean level grand of land productivity of major root crops cultivated across the states.
Xij = ith level of land productivity of major root crops cultivated
nj = Size of the farmers
n = Nominal observances in the 3 states.
k^{-1} = Freedom of degree between samples.
n-k = Freedom of degree within samples.
k = No. of state.
x = Land productivity of major root crops cultivated across the states.

Multiple regression technique isolated the land productivity determinants of the root crop growers and was specified;
$Y = f (b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_5X_5 + b_6X_6 + b_7X_7 + b_8X_8 + b_9X_9 + b_{10}X_{10}) + e$

Where
Y = Land productivity (Naira)
X_1 = Education (schooled years)
X_2 = Age (no. of years)
X_3 = Access to subsidized farm inputs (Accessed = 1, Otherwise = 0)
X_4 = Soil/land improvement practices (Practiced = 1, Otherwise = 0)
X_5 = Farm size (ha)
X_6 = Farming experience (Years)
X_7 = Access to credit (Accessed =1, Otherwise = 0)
X_8 = Labour supply (manday)
X_9 = Land tenure patterns (Inheritance = 1, Otherwise = 0)
X_{10} = Extension contacts (No of visits)

To examine the impact of pesticide use and application on the land productivity of growers of root crops, the LATE model was utilized:

$$E\left(y_1 - \frac{y_0}{d_1} = 1\right) = LATE = \frac{cov(y,z)}{cov(d,z)} \text{--- --- --} \quad \text{eqn. 4}$$

$$= \frac{E\left(\frac{y}{z}=1\right) - E\left(\frac{y}{z}=0\right)}{E\left(\frac{d}{z}=1\right) - E\left(\frac{d}{z}=0\right)}$$

$$= \frac{E(y_i*(z-E(z_i)}{E(d_i*(z-E(z_i)}$$

$$= \left(\frac{\sum_{i=1}^{n} y_i z_i}{\sum_{i=1}^{n} z_i} - \frac{\sum_{i=1}^{n} y_i(1-z_i)}{\sum_{i=1}^{n}(1-z_i)}\right) X \left(\frac{\sum_{i=1}^{n} d_i z_i}{\sum_{i=1}^{n} z_i} - \frac{\sum_{i=1}^{n} d_i(1-z_i)}{\sum_{i=1}^{n}(1-z_i)}\right) \text{-----} \quad \text{eqn. 5}$$

Specifying LATE model components,

$$ATE = \frac{1}{n} \sum_{i=1}^{n} i \frac{(d_i - p(X_i)y_i}{p\,(X_i)(1-p(X_i)}$$

$$ATE1 = \frac{1}{n1} \sum_{i=1}^{n} i \frac{(d_i - p(X_i)y_i}{(1-p)(X_i)}$$

$$ATE0 = \frac{1}{1-n1} \sum_{i=1}^{n} i \frac{(d_i - p(X_i)y_i}{p(X_i)}$$

Let z (yi) be a binary outcome variable with the value 1 when a farmer uses pesticides and 0 otherwise. We have d0 = 0 for all farmers and the observed outcome is given by d = zd1. As a result, the sub-populations of Eyi* and Edi* are described by the condition d1 = 1 and d = 1 (which is equivalent to the condition z = 1 and d1 = 1), respectively. We suppose that the possible outcomes d1, y1, and y0 are unrelated to z. ATE = ni = (I = 1) and i is the total number of farmers employing pesticides, where n is the sample size. ATE1 represents the typical treatment outcome for farmers who use pesticides in accordance with suggested specifications, while ATE0 represents the typical treatment outcome for farmers who do not use pesticides in accordance with recommended specifications. Propensity score matching (PSM) and inverse propensity score weighing (IPSW) are represented as P(Xi)

3 RESULTS AND DISCUSSION

3.1 SOCIO-DEMOGRAPHIC FEATURES OF ROOT CROP PRODUCERS

The socio-demographic features of root crop producers are presented in Table 1. The average length of education for growers of root crops was 12 years, which suggests that the farmers at least finished their secondary education. Crop productivity increases with increase in educational attainment. This is because education increases farmers' knowledge and comprehension of agricultural production principles (Ayi, 2022).The root crop farmers were 51 years old, which suggests that they are actively engaged in root crop production. Age of farmers is a symbol of extensive farming expertise, which aid crop productivity. The household size was 6, indicating that the farmers had a sizable household to deal with root crop production. Large household size guarantees large-scale farm cultivation. Gender of the root crop farmers' shows that more females, 77 %, were involved in root crop cultivation relative to the male folk. Typically, studies have reported engagement of more female root crop farmers than their male counterparts (Fanelli, 2022). The percentage of married root crop farmers is 85.1; this shows that the married farmers dominated the states. Marriage contributes immensely to family labour utilized in crop cultivation. The mean extension contact of the root crop farmers was 3.6, this shows that the farmers had up to 4 visits within the cropping season. Extension visits impact positively on the knowledge of the farmers and inculcate practical experiences required for improved farm production (Issa, 2021). The root crop farmers were experienced in agricultural practices with 22 years of farming experience. Farming experience enhances farmers' skills and helps in overcoming inherent farm production challenges (Ladapo et al., 2019). The percentage of credit access was 21.0, indicating that just a small portion of root crop farmers used agricultural loans. This could be due to collateral demands of financial institutions. Membership of farmer group indicated a percentage of 56.6; implying that about 57 % of the root crop farmers belongs to farmer groups. Cooperative association support crop farmers and provides farm incen-

Table 1: Socio-demographic features of root crop producers

Variable	Mean / %	Std. Deviation
Education (schooled years)	12.01	8.01
Age (no. of years)	50.6	0.82
Household size (people living together)	6.02	0.08
Gender (% of female)	76.9	12.4
Marital status (percentage married)	85.1	11.2
Extension contact (no. of extension visits)	3.6	9.50
Years of experience	22.3	12.8
Credit access (% of access)	21.0	7.01
Membership of farmer groups (% of members)	56.6	8.10
Farm size (hectare cultivated)	3.70	0.49
Income from off farm activity	72,345.8	4.06

Source: Field survey data, 2022

Table 2: Land productivity of major root crops cultivated in the State

State	Mean output of cassava	Mean inputs used	Mean output of yam	Mean inputs used	Mean output of sweet potatoes	Mean inputs used	Total mean outputs	Total mean inputs	TFP	PFP
Abia	91673.27	42178.02	88363.46	37732.83	69935.36	19832.52	249972.09	99743.37	2.51	67.80
Ebonyi	87055.35	38982.49	93452.93	45832.04	59546.56	18834.50	240054.84	103649.03	2.32	58.04
Imo	94936.53	33982.83	90336.63	31834.73	62634.56	20935.45	247907.72	86753.01	2.86	51.41
Total	273665.15	115143.34	272153.02	115399.60	192116.48	59602.47	737934.65	290145.41	7.69	177.25

Field survey data, 2022. TFP = Total factor productivity; PFP = Partial factor productivity

tives. Farm size cultivated was 3.70 hectares; this is synonymous with rural lands and implies small-scale land cultivation which affects land productivity. Income from off farm activity gave ₦72, 345.8; this could support the root crop farmers in their cultivation in terms of inputs accessibility.

3.2 PRODUCTIVITY OF LAND OF PRINCIPAL ROOT CROPS CULTIVATED ACROSS STATES

The land productivity of principal root crops cropped in the state is presented in Table 2. The land productivity of the farmers was isolated into mean outputs, mean inputs and total factor and partial factor productivity across the states. The result shows that Imo state had an estimated mean cassava output, (94936.53 kg) which is higher than that of Abia and Ebonyi state. This connotes a 104 % and 109 % increase above Abia and Ebonyi states respectively. This could imply optimal efficiency in cassava production in the state (Esiobu, 2019). The mean inputs used in cassava production across the states shows that Abia state seemingly had about 108 percentage increases in mean inputs over Ebonyi and 124 percentage increases over Imo state. This could imply high usage of inputs in cassava production in Abia state relative to Ebonyi and Imo state. The low input usage from other states could result in high costs of inputs in southeast states (Okorie et al., 2021). Again, mean outputs in yam cultivation across the states shows that Ebonyi state had an estimated value of 93452.93 kg, which implies about 106 percentage increases in mean output over Abia and 1.03 percent increase over Imo. This could result from efficient utilization of inputs in Ebonyi state as depicted in the mean inputs used, which was relatively higher compared to Abia and Imo state. (See Table 2). Consequently, Abia state recorded a higher estimate of 69935.36 kg in sweet potato production, implying about 1.2 percentage increases over Ebonyi state and a whopping 112 percent increase over Imo state. The mean inputs in sweet potato production indicate that Ebonyi state had the least input relative to other two states. Furthermore, TFP estimate shows higher value in Imo state relative to Abia and Ebonyi state. This implies that Imo state had the highest TFP compared to other two states. More so, the PFP estimates across the states indicate that Abia state had the highest PFP in comparison with other two states. It is important to note that the differences in estimates of land productivity across the three states may be related to both internal and external production factors (Vibeke et al., 2020; Wang et al., 2021). However, the principal root crops produced total TFP and PFP values of 7.69 and 177.25indicating a high land productivity of the crops across the three states.Source:

3.3 TEST OF SUBSTANTIAL DIFFERENCES IN LAND PRODUCTIVITY OF THREE MAIN ROOT CROPS GROWN IN THE STATES USING ANALYSIS OF VARIANCE

In Table 3, the test of considerable variation in land productivity of key root crops grown in the states is shown. The outcome demonstrates that the ANOVA

Table 3: Test of substantial differences in land productivity of main root crops grown in the states using analysis of variance

Sources of variation	Sum of squares	Degrees of freedom	Mean squares	Fcal	Ftab
Between groups	899330712	2	61760210	4.09	2.17
Within groups	659608143	355	54075137		
Total		357			

Source: Field survey data, 2022 Fcal; Significant at 1 % level model generated an F-cal. value of 4.09, which was higher than the F-tab. value of 2.17 and significant at the 1 % level. This suggests that there are large regional variations in the productivity of the main root crops grown in the three states. One could add that there are statistical differences and inequalities in the land productivity of the main root crops grown in the states. Table 2 above further supported the conclusion.

3.4 DETERMINANTS OF LAND PRODUCTIVITY OF ROOT CROP PRODUCERS

Table 4 discussed the factors affecting the land productivity of the root crop growers. The four functional forms of the multiple regression model were fitted to produce the lead function. Judging from the results the Double-Log function's have high F-value, high number of significant variables, and high coefficient of multiple determinations (R^2), and was chosen as the lead model. The variance in the dependent variable was explained by the analyzed independent variables, while the model's fitness was indicated by the F-value. Positive and significant results for education imply that land productivity among root crop farmers rises with higher educational

Table 4: Determinants of land productivity of root crop producers

Variable	Linear	Semi-log	Double-log	Exponential
Constant	403.172 (0.514)	0.3315 (4.502)***	2.6608 (4.071)***	15.9022 (3.902)***
Education (X_1)	8.8951 (0.134)	3.1043 (2.701)**	8809.61 (4.117)***	0.7758 (1.305)
Age (X_2)	-902.433 (-4.302)***	-12.0409 (-1.401)	-948.118 (-1.050)	-10.6155 (-4.402)***
Access to farm inputs (X_3)	45.9040 (2.103)**	1.5566 (0.711)	4405.15 (3.100)***	7.1943 (2.007)**
Soil & land improvement practices (X_4)	4112.90 (0.219)	13.1950 (3.010)***	7.2478 (4.000)***	0.9095 (1.021)
Farm size (X_5)	880.051 (3.311)***	4.0121 (1.401)	2854.19 (1.591)*	21.7701 (3.712)***
Farming experience (X_6)	5667.89 (0.942)	6892.01 (1.589)*	0.5467 (4.735)***	8921.34 (0.951)
Access to credit (X_7)	7.9642 (0.735)	6389.03 (0.839)	19.6371 (2.834)**	0.7488 (2.835)**
Labour supply (X_8)	0.7448 (0.563)	0.7346 (0.982)	18.8456 (0.747)	0.9454 (0.943)
Land tenure patterns (X_9)	0.5467 (0.734)	0.6488 (2.834)**	9.0001 (0.456)	0.7457 (0.745)
Extension contact (X_{10})	1789.603 (4.913)***	0.0203 (1.306)	18901.7 (2.661)**	0.7735 (1.041)
R2	0.7814	0.7751	0.8991	0.8182
F- ratio	17.109***	12.001***	21.642***	8.482***

Source: Field survey data, 2022. Significant at ***1 %, **5% and *10 %

attainment. Education enhances knowledge acquisition of the farmers and helps them adopt soil management practices targeted at increasing land productivity (Ukeje et al., 2022). Access to farm inputs was significant and positive; indicating that access to farm inputs increases the productivity of the land. Accessibility of farm inputs such as improved seedlings, fertilizers, pesticides, etc. improves crop yield and aid the productivity of the land (Ullah et al., 2020). Soil and land improvement practices became positively significant; this implies that a 1 % increase in soil and land improvement practices will cause a corresponding increase in land productivity by 725 %. Soil management and land improvement practices such as erosion control, weeding, crop rotation, mulching, organic manure, irrigation and good drainage systems enhance crop yield and thus the productivity of farmlands (Gizaw et al., 2021). The impact of farm size was significant and favorable, which suggests that any increment in farm size will result in a comparable rise in farmers' land productivity. Large hectares of land aid large-scale cultivation and allow the practice of sustainable soil management and improvement practices, which aid productivity of the land (Dereje et al., 2021). Farming experience was substantial and favorable. This suggests that a 1 % improvement in the root crop farmers' farming experience will result in a commensurate increase in land productivity of 54.5 %. Farming experience helps farmers in evaluating, understanding and adoption of land management measures targeted at improving crop yield and land productivity of the farmers. Access to credit was significant and positive; this indicates that increase in credit access increases the productivity of the land. Credit is a veritable tool in farm production in that it enables farmers to acquire essential and enhanced agricultural inputs like improved seeds, agro-fertilizer, agro-pesticides, labor, and lease land rent (Amanullah et al., 2020), this improves crop yield and land productivity at large. However, requirement and demand for collaterals in most cases limits credit accessibility of the farmers. Positive and meaningful extension contact suggests that an increase in the number of extension visits to farmers will result in a proportionate rise in the farmers' land productivity. Extension contacts impact positively on the crop farmers in terms of on-hand practical knowledge and encourage adoption of land management and soil sustainability techniques (Osuji et al., 2023). These techniques improve crop yield, income and productivity of the land.

3.5 IMPACT OF PESTICIDE APPLICATION ON LAND PRODUCTIVITY OF ROOT CROP GROWERS

Table 5 shows how pesticide use and application affect root crop growers' land productivity. The table shows that the estimates using propensity score matching (PSM) and inverse propensity score weighing (IPSW) were 62.5501 and 42.0177. These estimation falls short of identifying the true incidental impact of pesticide use and application on farmers' land productivity. As a result, they are deemed insufficient to paint a complete picture of the impact of pesticides on land productivity. This suggests that non-compliance may be present or at the very least taken into account when dealing with impact of pesticide use and application on root crops. The lack of compliance in this case indicates that some farmers will never follow the instruction for applying and use of pesticides as indicated in the instruction manual. Furthermore, the lack of compliance effectively explains the hidden bias in pesticide application and usage problems, which can only be eradicated through an impact parameter known as the local average treatment effect (LATE) (Choi, 2021). The LATE (WALD) and LATE (IV) estimation, which were highly significant, produced results of 7.2707 and 8.8028 respectively. In the event of non-compliance, LATE assessed either way indicates the genuine causal impact of pesticide use and application on farmers' land outputs (Choi, 2021). This suggests that the use and application

Table 5: Impact of pesticide application on land productivity of root crop growers

PARAMETER	LATE (WALD)	LATE (IV)	ATE (IPSW)	PSM
ATE	7.2707	8.8028	42.0177	62.5501
	(45.02)***	(26.40)***	(19.06)***	
ATE 1			7.9094	
			(4.17)***	
ATE 0			-3.0250	
			(-2.75)**	

Source: Field survey data, 2021. Significant at ***1 %, **5 % and *10 %

of pesticides in accordance with the stipulated recommendations enhanced the productivity of the land by 72.7 % and 88.0 %, respectively. This further implies that the higher the use and application of pesticides as recommended, the higher the land productivity of the growers of root crops, meaning that a unit increase in the use and application of recommended pesticides would result in a unit increase in yield and land productivity of the growers of root crops (Prihandiani et al., 2021). Again, the ATE 1, estimate was positive and significant, implying that the use and application of pesticides according to stated usage and specification yielded a positive increase of 79.0 % in land productivity. While the ATE 0 was negative though significant, implying the wrong use and application of pesticides on planted root crops. This further indicates that some of the farmers did not adhere strictly to pesticide manual instruction as recommended and this caused a decrease in land productivity of about 30.2 % (Anthony et al., 2021). The adherence and non-adherence to pesticides manual instruction could be associated with the farmers' literacy levels, exposures and other related socio-economic variants. This is to say that pesticides is targeted at controlling root crop insects, diseases and pest attacks in a bid to improve crop output and land productivity; however its usage and application most times could be detrimental as it could either increase or mar yield and land productivity per cropping season.

3.6 PERCEIVED CONSTRAINTS TO LAND PRODUCTIVITY OF ROOT CROP GROWERS

Table 6 discussed the root crop growers' perceived barriers to land productivity. The result shows that erosion problems constituted about 99.7 %. Erosion destroys arable farm lands causing land denudation and disintegration which seriously affects crop yield and productivity of the land (Joseph et al., 2020). Poor drainage menace was indicated by 86.3 % of the farmers. Poor drainage causes flooding and water percolation on farmlands suffocating crop yield and in turn reducing land productivity. About 84.4 % of the farmers attested to a high cost and a limited quantity of workers, this severely impedes land productivity owing to the increasing labour wages which is in short supply (Umar et al., 2021). Ignorance on soil/land improvement practices was observed by 77.9 % of the growers suggesting no knowledge on soil and land improvement practices. This poses serious constraints to land productivity. Limited farming lands were reported by 81.3 % of the farmers. No doubt inadequate and/ or shortage of farm land are great disadvantage to land productivity. Large farmlands support large scale production and vice versa (Dokic et al., 2022). Poor extension access and services was attested by 80.7 % of the farmers. Extension service and access increases land productivity by exposing farmers to new ideas and practices, whereas lack of access or restricted access limits land productivity (FAO, 2021). About 93.0 % of the farmers indicated land fragmentation. Land fragmentation refers to small land holdings or fragment which may not be sustainable for improved crop yield and land productivity. Climate change issues were reported by 99.4 % of the crop growers. Nowadays, issue of climate change has altered cropping calendars and cropping systems causing havoc to

Table 6: Perceived constraints to land productivity of root crop farmers

Perceived Constraints	*Frequency	Percentage
Erosion problems	357	99.7
Poor drainage menace	309	86.3
High cost and limited supply of labor	302	84.4
Ignorance on soil/land improvement practices	279	77.9
Limited farming lands	291	81.3
Poor extension access and services	289	80.7
Land fragmentation	333	93.0
Climate change issues	356	99.4
Inadequate capital	350	97.8
Low access to credit facilities	299	83.5
Pests and disease attacks	347	96.9
High cost of input materials	355	99.1

Source: Field survey data, 2022.

crop production and productivity of the land (Osuji et al., 2023). Issues of high temperatures, unpredictable rainfall patterns, high humidity, etc. worsen land productivity at large. Inadequate capital was indicated by 97.8 % of the farmers. Capital is a major incentive and necessary tool for crop production, because it is essentially needed to purchase farm inputs. Its inadequacy demoralizes farmers and impedes their cultivation plans thereby affecting productivity of the land (Anthony, 2021). Pests and disease attacks was reported by 96.9 % of the farmers. The attack planted root crops reducing their yield and land productivity. High cost of input materials was attested by 99.1 % of crop growers. Inability of the growers to access farm inputs could limit the productivity of the land.

4 CONCLUSION AND RECOMMENDATION

Land productivity of root crop farmers has been a source of concern due to variant internal and external factors associated with crop production. Findings show that Imo state had an estimated mean cassava output, 94936.53 kg, which is higher than that of Abia and Ebonyi states. Mean outputs in yam cultivation across the states shows that Ebonyi state produced a high value of 93452.93 kg, which is higher than the values obtained in Abia and Imo states. Again, Abia state produced a higher, value 69935.36 kg in sweet potato production over Ebonyi and Imo states. Furthermore, TFP estimate shows higher value in Imo state relative to Abia and Ebonyi state. More so, the PFP estimates across the states indicate that Abia state had the highest PFP in comparison with other two states. Education, access to farm inputs, soil and land improvement practices, size of farms, and extension contacts were important determinants of land productivity across the states. LATE estimates show that use of pesticides increased land productivity by 72.7 % and 88.0 %. Inadequate capital, pests and disease attacks, climate change issues, and erosion problems were perceived as land productivity constraints. Farmers were recommended to embrace land and soil improved practices and adhere strictly to recommended pesticide use and application for increased crop yield and land productivity.

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Phaeoacremonium hungaricum, a species causing grapevine wood necroses in Iraq

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Phaeoacremonium hungaricum, **a species causing grapevine wood necroses in Iraq**

Abstract: This paper describes the symptoms caused by *Phaeoacremonium hungaricum* Essakhi, Mugnai, Surico & P.W. Crous on grapevine seedlings observed in pathogenicity tests. The tested isolate was obtained from a 15 to 20-year-old grapevine in the Duhok province, Kurdistan region, Iraq. It was identified based on morphological characters and phylogenetic inferences using ITS sequences. Disease symptoms included interveinal chlorosis progressing to necrosis, defoliation, wilting, and shoot-tip dieback. Additionally, dark brown to black streaking became evident in artificially wounded shoots approximately two months post-inoculation. This is the first report of *Phaeoacremonium hungaricum* causing young vine decline in Iraq.

Key words: molecular identification, ITS, *P. hungaricum*, grapevine.

Phaeoacremonium hungaricum, **vrsta, ki povzroča nekroze lesa na žlahtni vinski trti v Iraku**

Izvleček: Članek opisuje simptome ki jih povzroča gliva *Phaeoareminium hungaricum* Essakhi, Mugnai, Surico & P.W. Crous na sejankah žlahtne vinske trte v testih patogenosti. Preiskušani sev je bil dobljen iz 15 do 20 let starih trt, v provinci-Duhok na območju Kurdistana v Iraku. Sev je bil določen na osnovi morfoloških znakov in filogenetskih razmerij z uporabo ITS zaporedij. Simptomi bolezni so obsegali medžilne kloroze, ki so prehajale v nekroze, odpadanje in venenje listov ter odmiranje vršičkov poganjkov. Dodatno so bile na umetno ranjenih poganjkih v dveh mesecih po inokulaciji vidne temnorjave do črne proge. To je prvo poročilo o pojavu te glive, ki povzroča propadanje mladih žlahtnih vinskih trt v Iraku.

Ključne besede: molekularna identifikacija, ITS, *P. hungaricum*, žlahtna vinska trta

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1 INTRODUCTION

Grapevine trunk diseases represent a significant economic challenge in all grape-growing regions, profoundly affecting grape production and long-term sustainability. Eutypa and Botryosphaeria diebacks, black foot, and Esca diseases are primary culprits, causing substantial financial losses in the sector (Billones-Baaijens and Savocchia, 2019; Wicks and Davies, 1999; Whitelaw-Weckert et al., 2013). These issues are particularly prominent in older vineyards, typically over 10 years old, where diseases that affect the grapevine trunk are pervasive. However, even young grapevines in newly established vineyards exhibit signs of decline, a condition commonly referred to as Petri disease or young esca (Scheck et al., 1998). *Phaeoacremonium* species contribute significantly to this complex of diseases, with grape wood necrosis being a primary symptom. This necrosis manifests as brown internal streaking, foliar discoloration, and desiccation (Haleem et al., 2013). The early detection of grapevine trunk diseases presents a growing challenge, given that symptoms typically manifest over years. Fungi primarily infiltrate through pruning wounds, as noted by Gramaje et al. (2018). There is a suspected pivotal role in preventing pathogen infections affecting grapevine trunks by treating pruning wounds (Mondello et al., 2018). As the pathogen progresses, it breaks down the wood, ultimately leading to the vine's demise. The genus *Phaeoacremonium* was established by Crous et al. (1996), but identifying *Phaeoacremonium* species remains arduous. Traditional techniques such as isolation, culturing, and subsequent morphological trait analyses are employed for identification. Despite the existence of several morphological identifying keys (Moster et al., 2005; Crous et al., 1996; Dupont et al., 2000), distinguishing characteristics can be challenging, resulting in misidentifications. Furthermore, *Phaeoacremonium* species are slow-growing, often requiring more than 20 days to develop on a microbiological media. This slow growth allows other microorganisms to overgrow *Phaeoacremonium*, complicating identification and prolonging the process. Molecular tools have proven invaluable in identifying *Phaeoacremonium* species. For instance, *Phaeoacremonium parasiticum* Ajello, Georg & Wang was differentiated from *Phaeoacremonium* inflatipes W. Gams, Crous & M.J. Wingf. using the ITS region, certain *Phaeoacremonium* species associated with diseased grapevines were identified using partial protein-encoding genes such as the β-tubulin gene (Dupont et al., 2002; Tegli et al., 2000). Species-specific primers based on the ITS region, actin, and β-tubulin genes from rDNA have facilitated the detection and identification of *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingf. & Mugnai, and *Phaeoacremonium chlamydosporum* W. Gams, Crous, M.J. Wingf. & Mugnai from various locations worldwide (Dam and Fourie, 2005; Groenewald et al., 2000; Retief et al., 2005, Mostert et al. 2006, Saccà et al. 2018).The two important fungal diseases of grapevines belonging to *Phaeoacremonium* species are Petri disease of young vines and Esca disease of adult vines (Moster et al. 2006). By using the ITS regions 1 and 2, including the 5.8S rDNA (Dupont et al. 2000), and the β-tubulin gene (Groenewald et al. 2001, Mostert et al 2005), sixteen species of *Phaeoacremonium* have been identified on the grapevine. Furthermore, Multiplex PCR tests based on the use of TUB and ACT primers, twenty-two Phaeoacermonium species associated with grapevine showing esca diseases identified (Gramaje et al.,2009; Essakhi et al., 2008; Mostert et al, 2006). The *Phaeoacremonium* isolates studied here are derived from all over the Dohuk province in Iraq, mostly from Sendori, Badi, and Baribuhar, where local grape varieties are grown. We aimed at identifying *Phaeoacremonium* species isolated from grapevines showing disease symptoms and pruning wounds of trees not yet showing disease symptoms.

2 MATERIALS AND METHODS

2.1 FUNGAL ISOLATION

During the summer season, fifteen-year-old Reshmew local cultivar of *Vitis vinifera* L. branches (10 cm diameter) and trunks exhibiting symptoms such as brown streaking, necrosis, and brown-red wood were sampled from five distinct grapevine yards in the Duhok governorate of Iraq. To ensure proper surface disinfection, small pieces of tissue from the margin between necrotic and healthy tissue were immersed in 70 % ethanol for 30 seconds, followed by a 1-minute treatment in 1 % NaOCl solution, and then rinsed again in 70 % ethanol for an additional 30 seconds. Subsequently, the surface disinfected pieces were dried on filter paper and then plated on Potato Dextrose Agar (PDA) supplemented with 0.25 mg ml^{-1} chloramphenicol (Himedia Laboratories Pvt. Ltd., India). The growing hyphae from the plated tissue pieces were carefully transferred and subcultured onto fresh PDA plates. The plates were then incubated at 25 °C, following the method described by Van Niekerk et al. (2004). To stimulate sporulation, the isolates were cultured on autoclaved grapevine cane pieces embedded in 2 % water agar, and maintained at 25 °C under a 12/12 hour photoperiod, as outlined by Luque et al. (2005). This process aimed to facilitate the growth and development of the isolates for further analyses and identification.

2.2 MORPHOLOGY IDENTIFICATION

In this study, morphological characteristics such as conidia, conidiophore length, mycelial texture, and phialide shape (Type I, II, and III) were utilized to differentiate between various species of *Phaeoacremonium* isolated during a sampling that was carried out in five grapevines yards of Duhok governorate. The central point of the grape vineyard was selected in each location, four directions were determined from this point, and then 15–20 samples were taken randomly from each one to ensure identification, 40 conidia and phialides were measured for length and width for each isolate. All isolates were cultivated on potato dextrose agar (PDA) at 25 °C, either in the absence of light or under NUV + fluorescent illumination with a 12-hour photoperiod using Philips 36W bulbs. This cultivation process aimed to stimulate the sporulation of the isolates for further examination. Identification of the isolated fungus was carried out based on morphological characters outlined by Crous et al. (1996, 2009) and Essakhi et al. (2008).

2.3 DNA EXTRACTION AND POLYMERASE CHAIN REACTION

Pure culture of fungal isolate was done by hyphal tip method by cutting out the tip of a single hyphae growing from a single spore colony. Fungal isolates from the single spore were sub-cultured in potato dextrose broth and then incubated at 25 °C for six days to extract genomic DNA. Under aseptic conditions, mycelia were purified and then frozen at –20 °C. The manufacturer's instructions were followed when extracting DNA using a Jena (Jena Bioscience, Germany) kit for extracting yeast DNA. PCR was done with primer set ITS1/ITS4 (White,

Table 1: Species name, Isolate number and NCBI GenBank accession numbers included in this study (*Phaeoacremonium hungaricum* Isolate PHD 45).

Species	Isolate ID	GenBank accession number
Phaeoacremonium aleophilum W. Gams, Crous, M.J. Wingf. & Mugnai	CBS 631.94	AF266647.1
Phaeoacremonium aleophilum W. Gams, Crous, M.J. Wingf. & Mugnai	strain 30	DQ404355.1
Phaeoacremonium aleophilum W. Gams, Crous, M.J. Wingf. & Mugnai	STE-U 3080	AF197996.1
Phaeoacremonium aleophilum W. Gams, Crous, M.J. Wingf. & Mugnai	PVFi 157	AF266654.1
Phaeoacremonium hungaricum Essakhi, Mugnai, Surico & Crous	PHD45	MW355028.1
Phaeoacremonium hungaricum Essakhi, Mugnai, Surico & Crous	JAMA5	OK649973.1
Phaeoacremonium hungaricum Essakhi, Mugnai, Surico & Crous	IHBF 2229	MF326630.1
Phaeoacremonium prunicola L. Mostert, Damm & Crous	STE-U5967	NR_135938.1
Phaeoacremonium viticola J. Dupont	CBS 101738	MH862747.1
Phaeoacremonium viticola J. Dupont	LCP 93 3886	AF118137.1
Phaeoacremonium alvesii L. Mostert, Summerb. & Crous	KMU10268	LC508975.1
Phaeoacremonium sp. W. Gams, Crous & M.J. Wingf	MRHf10	MK120896.1
Phaeoacremonium italicum A. Carlucci & M.L. Raimondo,	ColPat-676	MT022463.1
Phaeoacremonium parasiticum (Ajello, Georg & C.J.K. Wang) W. Gams, Crous & M.J. Wingf.	UZ577 17	MF363156.1
Phaeoacremonium parasiticum (Ajello, Georg & C.J.K. Wang) W. Gams, Crous & M.J. Wingf.	KMU 9954	LC203479.1
Phaeoacremonium scolyti L. Mostert, Summerb. & Crous	voucher DUCC404	KC013299.1
Phaeoacremonium hispanicum Gramaje, Armengol & L. Mostert	Y549-09-3b	JF275865.1
Phaeoacremonium inflatipes W. Gams, Crous & M.J. Wingf	CBS 391.71	MH860178.1
Phaeoacremonium sphinctrophorum L. Mostert, Summerb. & Crous	51561	KT989682.1
Ulocladium atrum Preuss, Linnaea	ATCC 18040	AF229486.1

et al. 1990), Ex-Taq PCR Master Mix (Jena Bioscience, Germany), 1 µl of each forward and reverse primer (20 pmol), 6 µl of template DNA (30-100 ng μl^{-1}), and 12 µl RNase-free water to reach a final volume of 40 µl. Reactions took place on an Applied Biosystems ABI Geneamp 9700 PCR-thermal cycler. PCR was carried out using a denaturation step of 95 °C for 4 min; 10 cycles with 94 °C for 30 s, then 60 °C for 45 s, 72 °C for 1:30 s: 25 cycles with 94 °C for 30 s, 55 °C for 45 s, 72 °C of 1:30 s; and finally, an extension step of 72°C for 10 min. A 1 % agarose gel was used to visualize PCR products with EvaGreen ® Fluorescent Gel Stain.+

2.4 SEQUENCING AND MOLECULAR PHYLOGENY

Sequence data was edited with BioEdit sequence alignment editor (BioEditv7) before depositing the sequence in GenBank. The NCBI's BLAST method was employed to assess the degree of similarity between the generated sequence to others stored in GenBank (Table 1). Selected sequences were aligned using ClustalW. The evolutionary history of *Phaeoacremonium* spp. was inferred with the maximum likelihood method and implementation of the Tamura-Nei model (Tamura and Nei, 1993) by using MEGA11 Software (Tamura et al., 2021). The tree that has the highest log likelihood is displayed (-2569.41). Bootstrap support values were derived from 1000 replicates and were printed below branches.ITS sequences from 20 taxa were compared. Branches with less than 50 % bootstrap support collapsed. The final dataset included 859 positions in total.

2.5 PATHOGENICITY TEST

Pathogenicity tests were done at the College of Agricultural Engineering Sciences at Duhok University in Iraq by using rooting cuttings of Reshmew and Taefi cultivars of *Vitis vinifera* L., planted in 15 kg pots with autoclaved sandy loam: peat moss (3:1) soil. Trunks were inoculated above the fourth shoot internode with *Phaeoacremonium* sp., isolate PHD 45. Grapevine shoots were wounded to a depth of 8 mm using a sterilized and sharp blade. Plugs of an agar culture (4 mm in diameter) were taken from the edge of a two-week-old PDA culture and inserted into the wound with the mycelium facing inward. Inoculated wounds were sealed with parafilm. After 48 hours, the parafilm was taken off. Negative control plants were equally treated but inoculated with a plug from a sterile PDA plate. Pieces of tissue from the lesions' margins were placed on PDA plates at the end of the experiment to check for the presence of the pathogen (Philips, 1998), After four months, the length of the canker on the inoculated shoot was measured. A Complete Randomized Design (CRD) was applied by using 3 inoculated trees and 3 negative control plants per cultivar.

3 RESULTS AND DISCUSSION

3.1 MORPHOLOGY IDENTIFICATION

Phaeoacremonuim hungaricum Essakhi, Mugnai, Surico & P.W. Crous (Isolate PHD 45) was isolated from grapevine wood tissues exhibiting internal wood discoloration in vineyards aged between 15–20 years in the Duhok province. On potato dextrose agar (PDA), the colonies displayed a flat, felt-like texture with entire edges. After 16 days of culture, the colony's color was whitish-grey. The mycelium hyphae were observed to be branched and septate, appearing singly or bundled, sometimes exceeding 14 in number. They exhibited a width ranging from 1–3.5 µm, with a smooth texture varying from subhyaline to medium brown, occasionally presenting verruculose characteristics. Conidiophores typically bore a single terminal phialide, ranging from subhyaline to pale brown, becoming paler towards the tip, and displaying a smooth to verruculose surface. They measured 27 to 30 millimeters in length and 2.5 to 3.5 millimeters in width. These conidiophores were unbranched, short, erect, and simple in structure. Phialides, measuring approximately 1 µm in length and 1.6 µm in width, were predominantly subhyaline and located laterally. They exhibited a smooth to verruculose texture and were monophialidic. Type I phialides, the most prevalent, displayed an elongated ampulliform shape, often with a constricted or attenuated base, while others exhibited a cylindrical form, measuring 7–14 × 2.5–3 µm. Type II phialides were navicular or subulate, sometimes appearing subcylindrical. The hyaline conidia were typically subcylindrical or cylindrical, occasionally allantoid, with dimensions of 3–5 × 1.5–2 µm. Accordingly, the isolate was identified as *Phaeoacremonuim hungaricum*. (Figure 1).

3.2 TAXONOMY

Taxon name: *Phaeoacremonium hungaricum* Essakhi, Mugnai, Surico & Crous*, Persoonia, 21*, 127 (2008) [MB#506948]

Basionym: *Phaeoacremonium hungaricum* Essakhi, Mugnai, Surico & Crous, Persoonia, *21*, 127 (2008) [MB#506948]

Material examined: Iraq, Kurdistan region, Dohuk province, College of Agricultural Engineering sciences, Plant Protection Department, from grapevine trunk, cv. Rashmew, collected and isolated by R. Haleem (Isolate No. PHD 45; GenBank MW355028).

3.3 SEQUENCING AND PHYLOGENIC ANALYSIS

Blast searches based on obtained ITS sequence identified isolate PH45 as *Phaeoacremonium hungaricum* by the unique universal primer pairs ITS1/ITS4 region ITS sequences of the isolate from Iraq and CBS 123036 (ex holotype strain of *Phaeoacremonium hungaricum*, NR_135953, were identical. ITS rDNA blast analysis backed up the morphological analysis. Maximum likelihood analysis (ML) was used to generate a phylogenetic tree for *Phaeoacremonium hungaricum* that was divided into eleven clades using 18 *Phaeoacremonium* reference strains, by using *Ulocladium atrum* Preuss, Linnaea as outgroup (Fig. 2). The isolate of this study (No. PH45) clade together with *Phaeoacremoinum hungaricum* JAMA 5 strain with 96 % ML value.

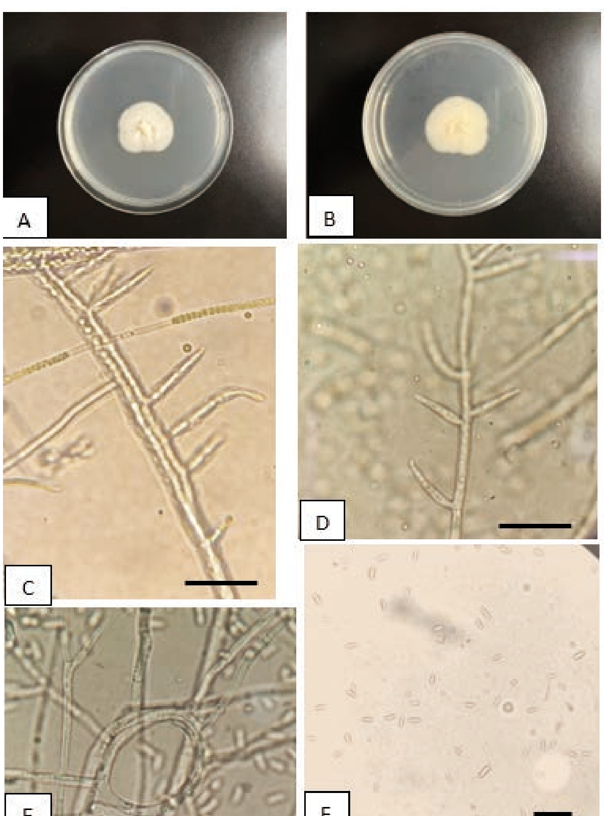

Figure 1: *Phaeoacremonuim hungaricum*. A, B = Colony growth on PDA. C, D = Lateral monophialides. E = Hyphae sometimes forming coils. F = Hyaline subcylindrical conidia Scale bar C, D = 15µm, E,F = 10µm..

3.4 PATHOGENICITY TEST

The inoculation of *Phaeoacremonium hungaricum* on shoots (Fig. 3) resulted in the characteristic symptoms of vascular fungal infection in plants, evidenced by brownish to black vascular discolorations. Evaluation of canker lengths on shoots revealed that *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingf. & Mugnai induced significant canker formation on 'Taefi' shoots after four months of inoculation, with an average length of 12.67 mm. Within 20 days of inoculation, symptoms of *P. hungaricum* were observed on 'Selemani' leaves as chlorotic spots between veins and along margins. These initial spots later progressed to necroses. These observations align with previous reports by Mugnai et al. (1999), Sands et al. (1997), Harrington et al. (2000), Edwards et al. (2001), and Feliciano et al. (2004), who documented similar signs and symptoms associating *Phaeoacremonium* infections.

Through the morphological and cultural characteristics, coupled with DNA sequence data analysis, the

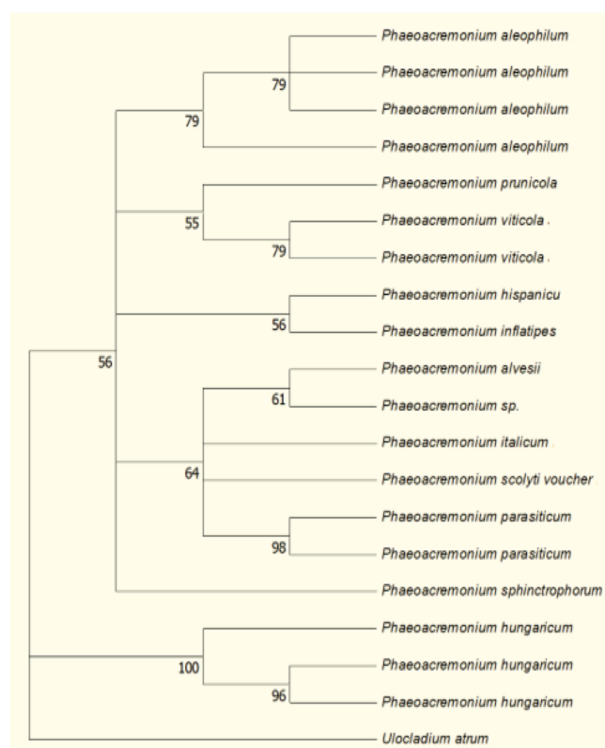

Figure 2. Phylogenetic maximum likelihood analysis based on ITS sequences. The tree identifies PHD 45 isolate from Iraq as *Phaeoacremonium hungaricum*.

presence of *Phaeoacremonium hungaricum* in the sampled region was recorded in five grapevine orchards of the Duhok governorate. Key morphological features such as conidia and phialide size and shape, along with the structure of conidiophores, proved instrumental in identifying one of the obtained isolates as *P. hungaricum*. Particularly, distinguishing characteristics included short, typically unbranched conidiophores arising from aerial or sunken hyphae, erect and simple in structure, often with a single terminal phialide, and displaying a smooth to verruculose surface. Despite the importance of morphological and cultural characteristics, challenges persist in species determination solely based on these traits. The overlapping nature of morphological features among species complicates accurate identification. Hence, DNA sequence data analysis remains crucial for comprehensive and reliable species description. Mostert et al. (2006) developed a polyphasic identification key incorporating multiple aspects of *Phaeoacremonium* species, including DNA sequence data, and morphological, and cultural characteristics. This tool combines various data sources to differentiate between *Phaeoacremonium* species effectively.

Due to the diverse range of species found on grapevines, including some that are infectious to humans, accurate identification poses a significant challenge. While morphological identification has limitations, molecular-based approaches offer valuable tools for detecting and identifying these species effectively. It is essential to generate sequences of protein-encoding genes especially when describing new species. Especially the variable introns from the actin and beta-tubulin gene could be used for the generating of species-specific primer pairs and as species-specific molecular detection tools. By leveraging these genes, researchers can develop robust molecular assays capable of accurately identifying *Phaeoacremonium* species, aiding in research and disease management efforts.

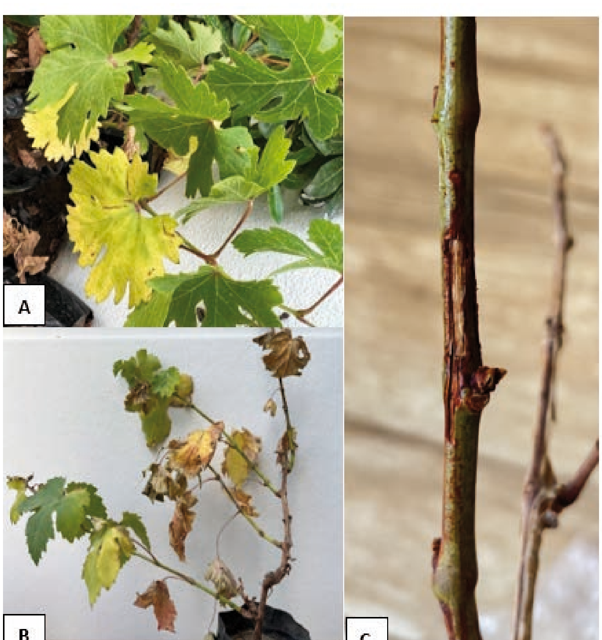

Figure 3: A.B = Chlorosis and necrotic symptoms on inoculated, two-year-old grapevine seedlings, xxx d after inoculation. C- Dark brown canker on young shoot x d after it was artificially infected with *Phaeoacremonium hungaricum* isolate PHD 45.

PCR offers significant advantages in the detection of *Phaeoacremonium*, notably rapidity and sensitivity, both crucial factors in managing this pathogen effectively. However, the time required for identification is often prolonged when using enriched growth media, as *Phaeoacremonium* species exhibit slow growth on such media. Consequently, *Phaeocremonium* may often be obscured or intermingled with other pathogens or saprophytic organisms, potentially leading to ambiguous detection results. Comparative studies between traditional and PCR methods for *Phaeocremonium* detection have consistently demonstrated the superior sensitivity of PCR. The vascular discoloration observed at inoculation sites during this investigation likely stems from the oxidation and transfer of various breakdown products of plant cells, resulting from fungal enzymes attacking the plant. Indeed, *P. hungaricum*, like other phytopathogenic fungi, produces numerous enzymes that degrade macromolecules within plant tissues, including polysaccharides. Valtaud et al. (2009) corroborated this observation, noting xylanase and glucosidase activity in the culture medium of this fungus. Accurate identification of the causal organism, as facilitated by PCR-based strategies, holds promise for enhancing Iraq's capacity to mitigate grapevine trunk diseases effectively.

In summary, the PCR-based approach outlined in this study enables sensitive, rapid, and reliable identification of *Phaeoacremonium* species associated with grapevine decline.

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Application of fluorescence spectroscopy as a field method in the determination of varietal differences radish (*Raphanus sativus* L.var. *sativus*) accessions after harvesting

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Application of fluorescence spectroscopy as a field method in the determination of varietal differences radish (*Raphanus sativus* **L.var.** *sativus*) **accessions after harvesting**

Abstract: The aim of the study is to establish the application of fluorescence spectroscopy as a field method for determining post-harvest varietal differences in radish accessions (*Raphanus sativus* L.var. *sativus*). The proposed method includes studies of radish root crops from standard varieties and those from first-generation hybrids by means of fluorescence spectra. They will be compared in terms of determining the spectral distribution due to the varietal differences of a given genotype. Specimens were grown under uncontrolled field conditions. This will allow the approach to be practiced non-invasively in the quality control of radish production on undefined premises and outdoors. The experimental studies were carried out locally on the farm. The spectral installation for generating emission fluorescence spectra is mobile. In its setup (optical setup), a system engineering approach based on the classical principles of modern optoelectronics was applied. The results of the experiment can be used to optimize the time for the analysis of the varietal difference of radish genotypes after harvest under uncontrolled conditions. The stability of the breeding variety and its similarities with an established variety of the same species can be observed by monitoring the intensity of the signal. This will assist the process of determining the belonging of a studied plant to a certain variety or breeding line, even for samples of unknown origin, when it is necessary to qualify the result of the samples in a short time.

Key words: radish accessions (*Raphanus sativus* L.var. *sativus*), varietal differences, uncontrolled conditions; field method; fluorescence spectroscopy

Uporaba fluorescenčne spektroskopije kot poljske metode za določanje sortnih razlik akcesij redkvice (*Raphanus sativus* **L. var.** *sativus*) **po spravilu**

Izvleček: Namen raziskave je bil vzpostaviti uporabo fluorescenčne spektroskopije kot poljske metode za določanje sortnih razlik akcesij redkvice (*Raphanus sativus* L. var. sativus) po spravilu. Predlagana metoda obsega preučevanje standardnih sort redkvice in križancev prve generacije na osnovi fluorescenčnih spektrov, ki bodo primerjani glede na razlike med sortami znotraj določenega genotipa. Preiskušane rastline so bile gojene na prostem v kontroliranih razmerah. Metoda bo omogočala neinvazivno kontrolo kakovosti v pridelavi redkvice, pridelane tudi v neznanih okoljih na prostem. Poskusi so bili izvedeni na posameznih kmetijah. Naprava za generiranje emisijskih fluorescenčnih spektrov je mobilna. Pri njeni postavitivi je bil uporabljen klasični inženirski pristop za postavitev modernih optično-elektronskih naprav. Rezultati raziskave bodo lahko uporabljeni za optimizacijo časa pri analizah sortnih razlik genotipov redkvice po spravilu v nenadzorovanih razmerah. Stabilnost gojenih sort in njihova odstopanja bodo določena na osnovi jakosti signala uveljavljene sorte. To bo pripomoglo pri določitvi, če določena preučevana rastlina pripada določeni sorti ali le gojitveni liniji, celo za vzorce neznanega izvora, še posebej, ko je potrebno hitro opredeliti kakovost vzorcev.

Ključne besede: akcesije redkvice (*Raphanus sativus* L. var. *sativus*), sortne razlike, nenadzorovane razmere, poljska metoda, fluorescenčna spektroskopija

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1 INTRODUCTION

Radish (*Raphanus sativus* L.var. *sativus*) is an annual root vegetable plant from the Cruciferous family. It originates in Central Asia (Kaneko et al., 2007). And has been cultivated as a vegetable crop since about 1000 BC in China, Japan, Egypt, Rome, and Greece (Perez Gutierrez et al., 2004). There are two groups of varieties: European and Chinese.

Radishes are for fresh consumption (Kyung-Mi et al., 2015).Their widest application is in making fresh vegetable salads. They are not suitable for heat treatment. Radishes are easy-to-grow root vegetables. They tolerate most soil types and grow rapidly (Hyde et al., 2012).

Optoelectronic methods, including fluorescence spectroscopy, are well-established techniques for the determination of chemical components in foods (Butzet al., 2005; Nicolaï et al., 2007). The advantages of optoelectronic methods include the rapidity of the analysis, the low cost of the test, and the possibility of simultaneous evaluation of several markers from one spectrum (Dakin and Brown, 2006; Mitchke, 2010). These advantages make them suitable for application in breeding programs and quality assessment when many samples need to be analyzed.

Optoelectronic methods are also used as rapid and non-destructive techniques to measure the intrinsic quality of various biological objects. They are successful techniques in the determination of dry matter and soluble solids content (SSC) of fruits and vegetables due to the absorption of sugar and water. Studies are available on the determination of SSC in apples (Lu et al., 2011) and tomatoes (Ecarnot et al., 2013).

In connection with the demands of consumers for high food quality, the conducted research can serve as a basis for the creation of mobile detecting devices for instant analysis of warehouse production of radish in uncontrolled conditions, both in processing plants and in food retail outlets.

The present study aims to establish the feasibility of fluorescence spectroscopy as a field method in the determination of varietal differences after radish harvesting. The differential parameter, utilized in our study is the spectral distribution due to the varietal differences of a particular genotype. The specimens were grown under uncontrolled field conditions. This will allow the application of the technique to the non-invasive quality control of radish production in unspecified rooms and outdoors.

2 MATERIALS AND METHODS

2.1 MATERIAL

Accessions of three standard radish varieties and one first generation hybrid variety were investigated:

- 'French Breakfast': The variety is suitable for spring and autumn field production. The hypocotyl tubers are single and oblong, with a white five around the tail. The fleshy part is white and crispy. The vegetation period is 30 days. The sowing rate is 1.5-2.5 kg per hectare
- 'Nacional 2': The variety is an early field variety that produces large, round, red roots with white tips. Its flesh is white, delicate in taste and crunchy.
- Red large: The variety is medium early. It is suitable for spring and autumn field production. The fruits are single, deep red, the fleshy part is white with excellent taste qualities. The variety is resistant to cracking.
- 'Espresso F1': A very early variety with round root tubers colored red. It is hardy with a fine root, a strong bond with the foliage. It has very good transportability and storage. It is recommended for growing in winter and very early in open areas

Radishes are grown according to standard technology. The seeds are sown in evenly spaced rows on an area of 1.6 m^2. The distance between the rows is 8 cm and the depth of the furrow is 2 cm. The seeds should be 3 cm apart in the row itself. The seeds are sown in moist soil.

2.2 FLUORESCENCE SPECTROSCOPY

The mobile fiber-optical spectral installation for the study of fluorescence signals is designed specifically for

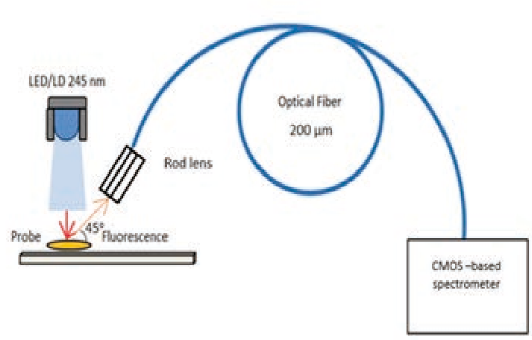

Figure 1: Mobile experimental installation used by fluorescence spectroscopy .

the rapid analysis of plant biological samples. The mobile experimental setup used by fluorescence spectroscopy includes the following components:

- Laser diode (LED) with an emission radiation of 245 nm with a supply voltage in the range of 3V. It is housed in a hermetically sealed TO39 metal housing. The emitter has a voltage drop from 1.9 to 2.4 V and a current consumption of 0.02 A. The minimum value of its reverse voltage is – 6 V.
- Rod lens of the achromatic doublet type. It is composed of two bonded lenses with different Schott and Corning dispersion coefficients with an anti-reflective coating. The radii of the two lenses are selected so that the chromatic aberration of one lens compensates that of the other. The tolerance of the diameter of the forming optics is -0.005 mm.
- The multimode optical fiber is FG200LEA. It has a core diameter of 200 µm and a step index of refraction.
- Quartz plate: area 4 cm^2. Its optical properties are to be transparent to visible light and to ultraviolet and infrared rays. This allows it to be free of inhomogeneities that scatter light. Its optical and thermal properties exceed those of other types of glass due to its purity. Light absorption in quartz glasses is weak.
- CMOS detector with photosensitive area 1.9968 × 1.9968 mm. Its sensitivity ranges from 200 nm to 1100 nm. Its spectral resolution is $\delta\lambda = 5$. The profile of the detector sensor projections along the X and Y axes is also designed for very small amounts of data, unlike widely used sensors.

The sample is irradiated by the LED, after which it fluoresces. The emission signal is received at 45 ° by the rod lens, which transmits it through the optical fiber to the detector. The three unique advantages of this scheme are:

- Inclusion of the rod lens in the construction of the system. Due to its increased light transmission efficiency by almost completely filling the air gaps between the individual lenses.
- Unique design of optical fiber coupling from a headquarters lens in a duralumin housing. In this way, the most optimal for compiling with optical fibers and forming images from laser diodes with low levels of intense losses is achieved.
- The sample fluoresces after being irradiated by the LED. The emission signal is received at 45 ° from Rod Len, and the emission signal is generated. It is then transmitted through the optical fibre to the detector.

3 RESULTS AND DISCUSSION

Radishes contain specific fluorophores/chromophores/ scatterers. The most important of these are myrosinase, glucosinolate and isothiocyanate. They determine their spectral properties.The possible transitions between these energy levels, as a function of photon energy, are specific to radishes, resulting in spectra and

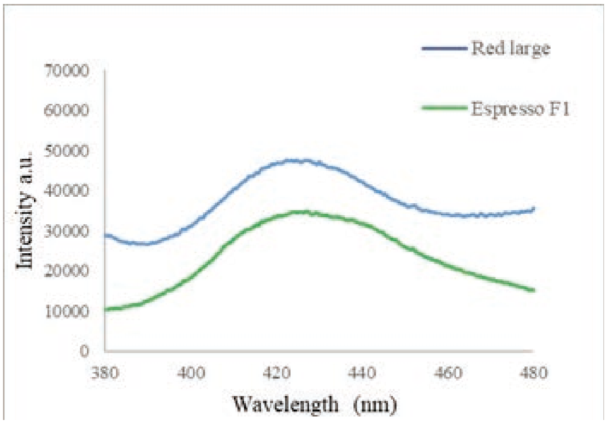

Figure 2: Emission wavelengths of 'Red Large' and 'Espresso F1'

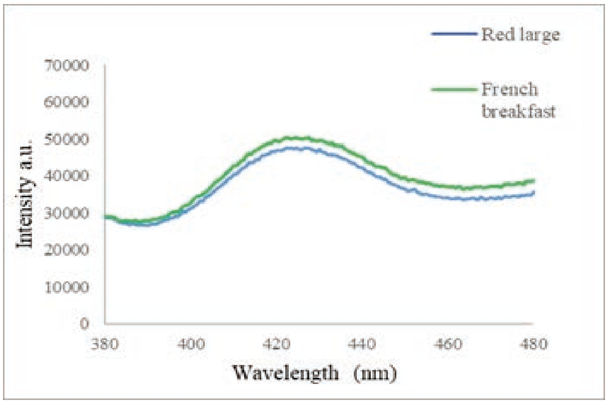

Figure 3: Emission wavelengths of 'Red Large' and 'French Breakfast'

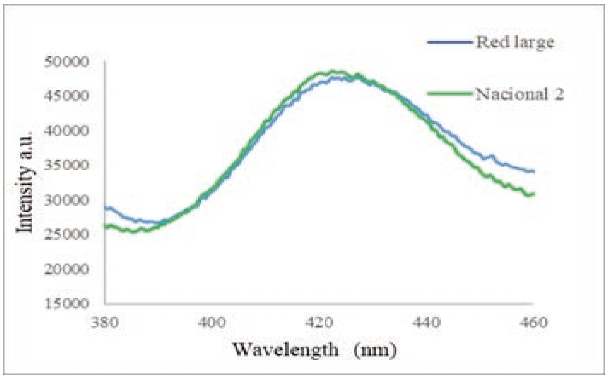

Figure 4: Emission wavelengths of Red Large and Nacional 2..

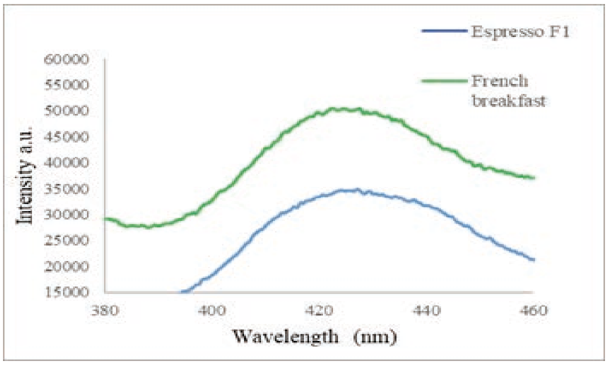

Figure 5: Emission wavelengths of 'Espresso F1' and 'French Breakfast'.

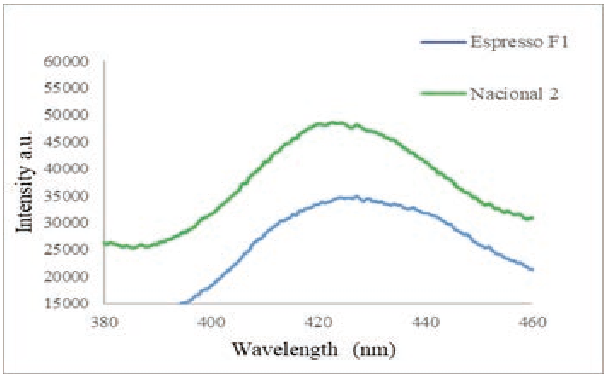

Figure 6: Emission wavelengths of 'Espresso F1' and 'Nacional 2'.

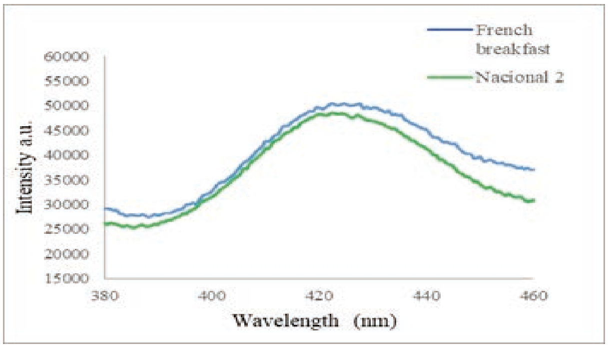

Figure 7 Emission wavelengths of 'French Breakfast' and 'Nacional 2'.

optical properties unique to them. Radishes contain structures smaller than the wavelength of visible light. When specific fluorophores are irradiated in the radishes, light with a wavelength also greater than that of the exciting radiation is emitted, but with this difference that the afterglow with is too short-lived. In this process, an electron absorbs a high-frequency photon, makes at least one emissionless transition to a lower energy level, then undergoes a relaxation process to its ground level and re-emits a low-frequency photon Radishes contain non-uniform particles smaller than the wavelength of visible light, and their orientation also affects the degree of polarization. In turbid media such as radish fluorophore compounds act as independent light sources, emitting incoherently, causing their visible fluorescence. The presence of fluorophores (myrosinase, glucosinolate and isothiocyanate) in radishes allows their qualification by fluorescence spectroscopy. Therefore, fluorescence spectroscopy finds application for analysis of this vegetable crop.

The optical parameters and spectral properties also change as a function of temperature, pressure, external electric and magnetic fields, etc., which allows obtaining essential information about changes in the chemical and cellular morphological composition of radishes. This gives us reason to claim that, for the first time, to the best of our knowledge, fluorescence spectroscopy has been applied to analyze radishes regarding their varietal differences under uncontrolled conditions, to the best of our knowledge. A difference in the emission fluorescence signal of the different varieties was clearly observed.

The results give reason to conclude that fluorescence spectroscopy can be successfully applied as a rapid tool to establish the origin of unknown root radishes in the presence of a rich library of spectra. This will be an applied tool in selection programs. For processing from the fluorescence study, 10 averaged plots from 4 different radish cultivars are presented. Graphs are averaged after the 15th measurement of each sample. Emission spectral data are decomposed into relations in such a way that only primary facts are stored in each relation. In this way, the truth of the real sample plot is preserved by tracking signal intensity, one can monitor the stability of a cultivar and its common blacks with other varieties. The emission fluorescence signals of 'French Breakfast' and 'Nacional 2' (Figure 7), 'Red Large' and 'Nacional 2' (Figure 4), and 'Red Large' and 'French Breakfast' (Figure 3) are close in terms of wavelength localization and signal intensity level. This is expected from the fact that the varieties have a similar cell-morphological composition when grown in open air. The emission fluorescence signals of radish root tubers were analyzed. As they are located at 45 degrees to the receiving multimode fiber. Radishes are placed on a quartz substrate, with their root part oriented downwards. Radishes are available with their root part below 45 degrees. This placement was chosen because it was found that the most informative emission fluorescence signals were obtained at this position of the irradiating signal. Experiments were conducted for the most informative placement of the radish tubers, in order to choose the most optimal placement However, the method of fluorescence spectroscopy can be applied to distinguish the root tubers of these two varieties because the correlation in the spectral distribution is sufficiently distinct and distinguishable to determine practically qualitatively the belonging of the roots to a given variety. The method of fluorescence spectroscopy can practically be used to qualitatively determine the belonging of a root radish to a given variety.

It turned out that we couldn't find any previous applications of the described experimental approach for the field method in the determination of varietal differences after radish harvesting has not been applied internationally. This gives us reason to claim that for the first time, fluorescence spectroscopy was applied to the application of fluorescence spectroscopy as a field method in the determination of varietal differences after radish harvesting under uncontrolled conditions. The method is successfully applied to distinguish root radishes from different varieties. Fluorescence spectroscopy can be applied to analyze radish root crops of unknown cultivars and establish its origin with a sufficiently well-structured data library. Because it can be applied topically to test specimens. It eliminates sample damage during transport and provides a highly sensitive assay.

4 CONCLUSIONS

The fluorescence spectroscopy method is fast-acting in application as a field method in the determination of varietal differences after radish harvesting locally under uncontrolled conditions.

It has been proven that fluorescence spectroscopy will successfully apply as a rapid tool to establish the origin of unknown radish accessions in the presence of a rich library of spectra. This will be an applied tool in selection programs. By monitoring the signal intensity, the stability of a breeding line and its common blacks with an established variety of the same species can be monitored.

The differentiation of related varieties is a laborious and time-consuming task. For these reasons, the development of techniques that could assist in the early, quick, and accurate differentiation of related varieties is of utmost importance.

It has been established that the system engineering approach for adjustment (optical adjustment) of a specialized installation for applied research with fluorescence spectroscopy is applicable in the determination of varietal differences during radish breeding.

5 ACKNOWLEDGMENTS

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Enhancing drought tolerance in common bean by plant growth promoting rhizobacterium *Bacillus amyloliquefaciens*

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Enhancing drought tolerance in common bean by plant growth promoting rhizobacterium ***Bacillus amyloliquefaciens***

Abstract: The study was set to explore the implications of plant growth promoting rhizobacterium in common bean while acclimating to drought stress. *Bacillus amyloliquefaciens* Priest et al., 1987 (PPB6)-inoculated plants showed better morphological attributes and pod yield as compared to uninoculated drought-stressed plants. PPB6 enhanced photosynthesis efficiency under drought stress by means of improving photosynthetic pigments and photochemical efficiency (*Fv/Fm*) when compared to uninoculated drought-stressed plants. PPB6 increased leaf relative water content (59.67 %) and reduced proline accumulation (0.63 μmol g^{-1} FM) under drought stress, compared to uninoculated drought-stressed plants (43.67 % and 1.42 μmol g^{-1} FM respectively). However, during drought stress, PPB6 led to produce comparatively lower level of hydrogen peroxide (66.60 μmol g^{-1} FM), and lipid peroxidation product; malondialdehyde (0.05 μmol g^{-1} FM), and electrolyte leakage (30.20 %) as compared to uninoculated drought-stressed plants (136.25 μmol g^{-1} FM, 0.08 μmol g^{-1} FM and 55.93 % respectively). However, as compared to uninoculated drought-stressed plants, significant reduction of total phenolics and flavonoids, and activity of enzymatic antioxidants such as ascorbate peroxidase, glutathione S-transferase and peroxidase in both non-stressed uninoculated and PPB6-inoculated drought affected plants, suggesting the lowering of drought-induced oxidative damage by this bacterium. Here, we suggest PPB6 had the ability to mitigate drought effects in common bean plant. Besedilo izvlečka

Key words: drought, common bean, stress, antioxidant

Pospeševanje odpornosti na sušo pri navadnem fižolu z rizobakterijo ***Bacillus amyloliquefaciens*****, ki pospešuje rast rastlin**

Izvleček: Raziskava je bila izvedena za preučevanje uporabe rizobakterij, ki pospešujejo rast rastlin pri prilagajanju navadnega fižola sušnemu stresu. Z rizobakterijo Bacillus amyloliquefaciens Priest et al., 1987 (PPB6) inokulirane rastline so pokazale boljše morfološke parametre rasti in večji pridelek strokov v primerjavi z neinokuliranimi. PPB6 je povečala učinkovitost fotosinteze v sušnem stresu z izboljšanjem stanja fotosinteznih barvil in večjo fotokemično učinkovitostjo fotosinteze (Fv/Fm) v primerjavi z rastlinami, ki v sušnem stresu niso bile inolkulirane. PPB6 je povečala relativno vsebnost vode (59,67 %) in zmanjšala akumulacijo prolina (0,63 μmol g-1 FM) v razmerah sušnega stresa v primerjavi z neinokuliranimi rastlinami (43,67 % in 1,42 μmol g-1 FM). V sušnem stresu inokulirane rastline so imele manjšo vsebnost vodikovega peroksida (66,60 μmol g-1 FM), manjšo vsebnost produktov peroksidacije lipidov kot je malondialdehid (0,05 μmol g-1 FM) in manjše puščanje elektrolitov (30,20 %) v primerjavi z neinokuliranimi rastlinami rastočimi v razmerah sušnega stresa (136,25 μmol g-1 FM, 0,08 μmol g-1 FM in 55,93 % respectively). V neinokuliranih rastlinah, ki so rastle v razmerah sušnega stresa je bilo ugotovljeno značilno zmanjšanje v vsebnosti celokupnih fenolov in flavonoidov kot tudi v aktivnosti antioksidacijskih encimov kot so askorbat peroksidaza, glutation S-transferaza in peroksidaza. Isti trend je bil opažen v neinokuliranih rastlinah, rastočih v nestresnih razmerah kot tudi v inokuliranih rastlinah, rastočih v sušnem stresu, kar vse nakazuje zmanjševanje od sušnega stresa povzročenih oksidacijskih poškodb z omenjeno rizobakterijo. Prepostavljamo, da ima rizobakterija PPB6 sposobnost blaženja učinkov suše pri navadnem fižolu.

Ključne besede: suša, navadni fižol, stres, antioksidant

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1 INTRODUCTION

The unprecedented and constant threats of global warming have been greatly involved in reducing crop yield worldwide by triggering the negative consequences of abiotic stresses. Among the abiotic stresses, drought is the most dangerous one, having tremendous impacts on crop productivity. It is anticipated that drought will likely to affect the crop yield of about 50 % of arable lands by 2050 (Akhtar et al., 2021). Due to the fact of aberrant nature of world's climate, more severe and persistent droughts accompanied by less rainfall, are expected in the 21st century across many regions of the world (Schwalm et al., 2017; Trenberth et al., 2014). Many plant attributes such as growth, photosynthetic pigments, water and nutrient use efficiency, cellular and biochemical changes including enzymatic activities are greatly affected by drought stress which is responsible for ultimate reduction in crop yield (Rezayian et al., 2018; Seleiman et al., 2021). The reactive oxygen species (ROS)-induced damage to biomolecules is one of the major factors that limit plant growth under drought stress (Nadeem et al., 2020). Hence, management of drought is a crucial challenge for increasing crop production to ensure food security.

Common bean (*Phaseolus vulgaris* L.), an annual crop belonging to the family Fabaceae, is one of the important grain legume crops in tropical and temperate countries for direct human consumption and comprises 50 % of the grain legumes consumed worldwide (McClean et al., 2002). It is a dual-purpose crop that is grown as pulse (grain) and also consumed in the immature stage as a tender vegetable (Nazrul & Shaheb, 2016). Common bean cultivation has been drastically affected by drought stress across the globe (Assefa et al., 2015). Management of drought by altering physicochemical properties and developing drought tolerant varieties should be very imperative for enhancing yield. Conventional plant breeding techniques have allowed the development of high-yielding and drought-tolerant crop varieties but that is very time consuming and may lead to the loss of other desirable traits from the host's gene pool (Eisenstein, 2013). Agronomic management and exogenous application of several growth regulators such as melatonin, abscisic acid (ABA), methyl jasmonate, salicylic acid have been investigated to mitigate drought stress (Arnao & Hernandez-Ruiz, 2018; Mohi-Ud-Din et al., 2021). Though these strategies have a potential scope, the application of such expensive regulators in farmers' field and performances of those is still questionable. Hence, drought management strategy by natural and less expensive way might cause suitable solution for this issue. Currently, plant-associated microbial communities including plant growth promoting rhizobacteria (PGPR) have received increased attention for enhancing crop productivity and stress resistance (Glick, 2012). PGPR are soil born bacteria living in association with the plant roots (rhizosphere) which are involved in promoting plant growth and development through the secretion of various regulatory molecules (Vocciante et al., 2022). PGPR have been implicated in increasing abiotic stress tolerance including drought in different crop plants (Gowtham et al., 2022; Chieb & Gachomo, 2023). PGPR inoculation in plants can enhance the productivity of crops under drought stress through a variety of mechanisms (Bouremani et al., 2023). For instance, PGPR-treated plants maintained relatively higher relative water content (RWC) compared to non-treated plants by enhancing synthesis of phytohormones and osmoprotectants, leading to the survival of plants under drought stress (Kudoyarova et al., 2019; Chieb & Gachomo, 2023;). Besides, PGPR have been reported to increase the stomatal conductance, photosynthetic rate, photochemical efficiency of pigment system II; PSII (*Fv/Fm*), and decrease the transpiration rate in plants, which simultaneously affect the structural and functional characteristics of photosynthetic apparatus (Martins et al., 2018; Khan & Bano, 2019). Improvement of drought tolerance has also been reported in wheat plant by using growth promoting bacterium *Stenotrophomonas maltophilia* Palleroni & Bradbury 1993 (Kasim et al., 2021). Furthermore, PGPR were investigated to increase the activity of ROS scavenging enzymatic and non-enzymatic antioxidants, thereby decreasing the excess ROS accumulation in drought affected plants (Chandra et al., 2020; Ansari et al., 2021). *Bacillus subtilis* (Ehrenberg 1835) Cohn 1872 and its sister species *Bacillus amyloliquefaciens* have been widely used as the beneficial agents for plant growth promotion, suppression of soil-borne diseases in agriculture and industrial purposes (Chen et al., 2007; Fan et al., 2012). *B. subtilis* and *B. amyloliquefaciens* have been investigated to improve drought tolerance in maize (Vardharajula et al., 2011), rice (Tiwari et al., 2017), wheat (Sood et al., 2020), tomato (Gashash et al., 2022) and soybean (Braz et al., 2022). Although having such potentials, the role of *B. amyloliquefaciens* in the drought tolerance of common bean has been less clarified. For instance, *B. amyloliquefaciens* was investigated in abiotic stress tolerance of common bean by bacterial biofilm formation (Martins et al., 2017). Another study using *B. amyloliquefaciens* as biostimulant did not confer tolerance to moderate level of drought stress in common bean (Galvão et al., 2019). Although having this progress, the alteration of morpho-physiological and biochemical processes by this bacterium is yet to be elucidated in drought-stressed common bean plants. Therefore, the investigation of which is of utmost importance for sustainable crop yield in climate resilient agriculture. However, regulation of drought-induced oxidative stress by this bacterium is still to be clarified in common bean. Considering the facts, the present investigation was set to find out the implications of plant growth promoting rhizobacterium in common bean while acclimating to drought stress by means of morpho-physiological and biochemical analyses. The efforts suggest that *B. amyloliquefaciens* has the ability to mitigate the negative consequences of drought effects in common bean that might help in sustainable crop yield and future food security.

2 MATERIALS AND METHODS

2.1 EXPERIMENTAL MATERIALS, PREPARATION OF BACTERIAL SUSPENSION, INOCULATION AND SCREENING OF SUITABLE BACTERIAL STRAIN

Seeds of common bean (variety BARI Jharsheem-1) were collected from Bangladesh Agricultural Research Institute (BARI), Bangladesh. The bacterial strains such as *Bacillus subtilis* (PPB2), *Stenotrophomonas maltophilia* (PPB3), and *B. amyloliquefaciens* (PPB6) were collected from the department of Plant Pathology, Bangabandhu Sheikh Mujibur Rahman Agricultural University, where the strains were isolated from the rhizosphere of different crop plants and found to be promising for having higher efficacy for the synthesis of indole acetic acid (IAA), and secondary metabolites (Islam et al., 2015; Masum et al., 2018). PPB2, PPB3 and PPB6 strains were cultured in yeast extract peptone dextrose agar media for 48 hours at 28 °C and stored in 4 °C temperature. From the stock culture, bacteria were grown in liquid culture for 48 hours at 28 °C on a rotary shaker (80-100 rpm). The cultured bacteria were centrifuged at 4000 rpm at 4 °C for 15 minutes. Then, the supernatant was removed and the pellet was re-suspended in distilled water and the volume was adjusted. Before sowing, the sterilized common bean seeds were inoculated with the bacterial suspensions and kept overnight in petri dishes. Then, seeds were sown in pots containing sterile soils with proper fertilization by maintaining three replicates. Treatment combinations at this level were uninoculated control, plants inoculated with three bacterial strains like PPB2, PPB3 and PPB6. When plants got three trifoliate leaves (at 21 days after germination), the bacterial suspensions were again injected into the root zones and they were allowed to grow under ambient condition with proper fertilization and irrigation. PPB6 was screened out as the best bacterial strain based on the effects of those bacterium on the growth and development of common bean.

2.2 IMPOSITION OF DROUGHT TREATMENTS AND MORPHOLOGICAL DATA COLLECTION

The treated and non-treated common bean seeds with PPB6 were allowed to grow in pots containing sterile soil with proper fertilizers by maintaining three replications. After 15 days of germination, some plants were thinned out and only five plants were kept to grow furtherly and PPB6 was inoculated at the root zone at 21 days after germination. The uninoculated plants were injected by same amount of deionized water. After 2^{nd} injection (at 32 days after germination) with PPB6 suspension, the plants were supposed to either normal irrigated or drought-stressed conditions by stopping irrigation and maintaining 50 % field capacity as followed by Mohi-Ud-Din et al. (2021). The treatment combinations followed at this level were non-stressed uninoculated plant (Control), uninoculated drought-stressed plants (Drought) and Drought stressed plants inoculated with PPB6 (D+PPB6). The morpho-physiological data were collected upon getting the drought effects on plant (after 12 days of drought imposition). The experiments were done by maintaining at least three replications. During screening of bacteria, the phenotypic data were collected after harvesting. During drought treatment, the morphological data such as shoot length, number of leaflets, leaflet length, number of flowers and pods were collected after 12 days of drought imposition. The tri-foliate leaves were numbered as per development as 1, 2, 3 etc. and data were collected consistently according to the number. The root, shoot and pod fresh and dry mass were noted after harvesting of common bean.

2.3 ANALYSIS OF PHYSIOLOGICAL DATA

The percentage (%) of leaf relative water content (LRWC) was determined by following the procedure of Shivakrishna et al. (2018)., Briefly, leaves were collected from each treatment and weighed immediately to record the fresh mass (FM) and then placed in petri dishes containing distilled water for 4 h, at room temperature to record the turgid mass (TM). The leaves were dried in an oven at 80 °C for 24 h to obtain dry mass (DM). Then LRWC (%) was calculated using the formula; ($RWC = FM - DM/TM - DM$) x 100. Chlorophylls and carotenoids content were estimated using the method described by Porra et al. (1989). Briefly, leaf pigments were extracted in 80 % (v/v) acetone followed by filtered with

Whatman filter papers. The absorbance of the supernatants was taken with a UV-visible spectrophotometer at 663, 646, and 470 nm. The quantification of the pigments was done according to the formula of Lichtenthaler & Welburn (1983). SPAD values of the leaves were also recorded with a Chlorophyll Meter (Model: SPAD-502, Minolta Co., Ltd., Tokyo, Japan) after 12 days of drought imposition. Maximum photochemical efficiency of PSII in terms of Fv/Fm in the fresh leaf samples was determined according to the method of Yaghoubian et al. (2016) with slide modification. Briefly, after dark acclimation of leaves for 15 minutes, the minimum (F_0) and maximum fluorescence intensity (Fm) were measured in leaves using a portable fluorometer. The variable fluorescence (Fv) and maximum photochemical efficiency of PSII (Fv/Fm) were calculated using equation I ($Fv = Fm - F_0$) and II ($Fv/Fm = (Fm - F_0)/Fm$) respectively. The proline content of fresh leaf was measured spectrophotometrically at 520 nm using the acid ninhydrin assay according to Bates et al. (1973).

2.4 MEASUREMENT OF ELECTROLYTE LEAKAGE, HYDROGEN PEROXIDE (H_2O_2) AND MALONDIALDEHYDE (MDA) CONTENT

Electrolyte leakage of the damaged tissue in the leaf was measured by following the protocols as followed by Ghosh et al. (2021). The fresh leaf tissues (0.04 g) were homogenized in 1.5 ml of 5 % (w/v) trichloroacetic acid (TCA). After centrifugation at 11,500 × g for 10 minutes, the supernatant was used to determine H_2O_2 and malondialdehyde (MDA). The H_2O_2 content was determined spectrophotometrically at 390 nm according to the procedure of Ghosh et al. (2021). The H_2O_2 concentration was calculated using the extinction coefficient of 0.28 μM^{-1} cm^{-1} and expressed as μmol g^{-1} FM. The MDA content was determined spectrophotometrically at 532 nm and 600 nm according to the procedure of Ghosh et al. (2021). The MDA content was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as μmol g^{-1} FM).

2.5 DETERMINATION OF NON-ENZYMATIC ANTIOXIDANTS ACTIVITY

Phenolic content of the methanolic extracts was determined spectrophotometrically according to the Folin-Ciocalteu method (Singleton et al., 1999). The absorbance of reaction solutions was measured spectrophotometrically at 765 nm and quantification was done according to the formula of Abdul-Hafeez et al. (2014). Then, the results were compared to a standard curve of gallic acid solutions and expressed as micrograms of gallic acid equivalent per gram fresh leaf mass (μg g^{-1} FM). The flavonoids content was measured using the aluminium-chloride colorimetric assay (John et al., 2014). The absorbance of the extracts and standard solutions was measured at 510 nm using a spectrophotometer. The measurements were expressed as micrograms of quercetin equivalent (QE) per gram of fresh leaf mass (μg g^{-1} FM).

2.6 ANALYSIS OF ENZYMATIC ANTIOXIDANTS

0.3 g of leaf tissue was crushed well with an ice-cold mortar and pestle in 1 ml of the extraction buffer containing 50 mM ice-cold K-P buffer (pH 7.0), potassium chloride (100 mM), ascorbate (1 mM), β- mercaptoethanol (5 mM) and glycerol (10 % v/v). The homogenized plant materials were then centrifuged at 11,500 rpm at 4 °C for 12 minutes and the supernatant was used as a soluble protein solution for enzyme activity. The protein concentration of the samples was determined by the method of Bradford (Bradford, 1976) using BSA as a protein standard. The activity of ascorbate peroxidase (APX) was determined according to the protocol used by Nakano & Asada (1981). APX activity was estimated with the extinction coefficient of 2.8 mM^{-1} cm^{-1} and determined as μmol $min^{-1}mg^{-1}$protein. The activity of catalase (CAT) was determined by using the procedure of Hasanuzzaman et al. (2014). CAT activity was calculated with an extinction coefficient of 39.4 M^{-1} cm^{-1} and determined as μmol $min^{-1}mg^{-1}$protein. Glutathione S-transferase (GST) activity was measured by following the procedure of Hossain et al. (2010). GST activity was calculated with an extinction coefficient of 9.6 mM^{-1} cm^{-1} and determined as nmol $min^{-1}mg^{-1}$protein. Peroxidase (POD) was measured by the method followed by Hemeda and Klein (1990). POD activity was estimated with an extinction coefficient of 26.6 mM^{-1} cm^{-1} and determined as nmol $min^{-1}mg^{-1}$protein protein.

2.7 STATISTICAL ANALYSIS

All the experiments were conducted by following CRD (Completely Randomized Design) with three replications. Statistical analysis was performed using Statistix 10 software. The least significant difference (LSD) was analyzed at 5 % level of significance.

3 RESULTS AND DISCUSSION

3.1 EFFECTS OF GROWTH PROMOTING RHIZOBACTERIA ON GROWTH AND YIELD OF COMMON BEAN

Three bacterial strains, PPB2, PPB3, and PPB6 were applied for showing their effects on the growth and yield performances of common bean. Among the bacterial strains, plants inoculated with PPB6 showed best performances on all the morphological characteristics studied here such as shoot length, leaflet length, number of flowers, number of pods, and pod fresh mass and dry mass (Table 1). Although plants inoculated with PPB6 showed more or less similar performances to uninoculated control plants, the length of leaflets were significantly increased by PPB6. The leaflet length of plants treated with uninoculated control, PPB2, PPB3, PPB6 was 14.67 cm, 14.67 cm, 16.00 cm, 16.67 cm respectively (Table 1). Previous investigations with *Bacillus* species produced improved phenotypes in rice seedlings as compared to uninoculated control (Awlachew & Mengistie, 2022). This was because of the improvement of the plant's growth by *Bacillus* spp by means of enhancing availability of nitrogen, phosphorus, potassium and iron in soil (Xue et al. 2021). In addition, plant growth could be promoted by *Bacillus* species by engaging several essential phytohormones like auxin; indole acetic acid (IAA), cytokinins (CKs) and gibberellic acids (GAs) (reviewed in Tsotetsi et al. 2022). The PGPR also promote volatile organic carbon (VOCs) which can modify the hormonal signal and enhance cell division (reviewed in Tsotetsi et al. 2022). Moreover, upon inoculation, PGPR induce spermidine synthesis which was claimed for plant growth via expansin and reduction of ethylene levels (Xie et al. 2014). In our studies, as compared to uninoculated control, plants inoculated with PPB6 produced better phenotypes particularly in the enhancement of leaflet length which should have impacts on the better pod yield of the plant. The larger leaflet by PPB6 may be due to the accumulation of higher phytohormones, and VOCs and spermidine in the leaf. Therefore, for having relatively better performances of PPB6 (Table 1), we screened out this bacterium for further drought studies in common bean.

3.2 IMPROVEMENTS OF PLANT'S PHENOTYPE AND POD YIELD BY PPB6 UNDER DROUGHT STRESS

The phenotypic parameters of common bean plants have been significantly altered by drought stress. Plant's height in terms of shoot length was found to be the highest (48.67 cm) in non-stressed uninoculated control (Control) plants followed by the drought-stressed plants inoculated with PPB6; D+PPB6 (44.67 cm) and uninoculated drought-stressed (Drought) plants (32.33 cm) (Fig. 1A & Fig. 1B). Likewise, others phenotypic data such as shoot fresh and dry mass, root fresh and dry mass, number of flowers, number of pods, and fresh and dry mass of pods were recorded as best in the control plants followed by the plants of D+PPB6 and drought. (Figs. 1C-1J).

Plants inoculated with effective PGPR strains could maintain near normal shoot growth rates, resulting in increased crop productivity. For instance, Vardharajula et al. (2011) inoculated growth-promoting *Bacillus* spp in drought-stressed maize and found that all the PGPR treated plants showed significantly greater shoot compared to non-treated plants. Similarly, Lim & Kim (2013) showed that pepper plants treated with *Bacillus licheniformis* (Weigmann 1898) Chester 1901 K11 produced higher biomass than non-treated plants. PGPR were reported to enhance shoot growth under drought stress of mungbean (Sarma & Saikia, 2014), and maize (Naseem & Bano, 2014). Alongside, root is also vital organ for plant which could perceive and senses the changes in soil moisture (Amtmann et al., 2022). Naveed et al. (2014) reported that maize plants inoculated with *Burkholderia phytofirmans* (Sessitsch et al. 2005) Sawana et al. 2015 significantly increased root biomass in Mazurka and Ka-

Table 1: Effect of different growth promoting bacterial strains on the growth and yield performances of common bean plants treated with uninoculated control and *Bacillus subtilis* (PPB2), *Stenotrophomonas maltophilia* (PPB3), and *Bacillus amyloliquefaciens* (PPB6).

Treatment	Shoot Length (cm)	Leaflet Length (cm)	No. of Flowers	No. of Pods	Pod Fresh Mass (g)	Pod Dry Mass (g)
Control	32.00 a	14.67 b	23 ab	18.67 ab	29.60 ab	2.36 a
PPB2	28.67 b	14.67 b	19 b	15.33 b	23.40 b	2.27 a
PPB3	31.33 a	16.00 a	21 ab	14.67 b	32.35 ab	2.80 a
PPB6	33.00 a	16.67 a	25 a	20.33 a	39.93 a	3.36 a

Different letters in the table show significant differences at $p < 0.05$.

leo cultivars, respectively under drought stress. Besides, *Pseudomonas aeruginosa* (Schröter 1872) Migula 1900 was found to increase the number of pods per plant in *Vigna radiata* (L.) R. Wilczek under drought stress (Uzma et al., 2022). Our results regarding less compromization of shoot and root biomass, and pod yield in common bean plants inoculated with PPB6 under drought stress are very consistent to those findings (Fig. 1). Although, *Bacillus* species are well documented to show response to abiotic stress tolerance, the molecular mechanism and signaling of those are still remains enigmatic. Enhanced synthesis of IAA in plants should be vital for approaching drought tolerance issue. For instance, *Pseudomonas tolaasii* Paine 1919 and *Pseudomonas fluorescens* (Flügge 1886) Migula, 1895 maintained better plant growth under cadmium stress by engaging IAA (Dell'Amico et al. 2008). However, *Bacillus* species could enhance expression of several genes related to the synthesis of IAA, phytase and siderophores which have tremendous roles in stress acclimation, and nutrient availability in soil respectively (reviewed in Luo et al. 2022). The species also conferred the expression of transcripts of late embryogenesis abundant (LEA) and dehydrin (DHN) under salt and heat stresses (Tiwary et al. 2017) suggesting to have roles in drought stress. Therefore, based on those findings, it is assumed that upon inoculation, PPB6 may enhance the expression of those genes in common bean while acclimating to drought stress and lead to better phenotypes than that of uninoculated drought-stressed plants.

3.3 AMELIORATION OF DROUGHT-INDUCED PHOTOSYNTHETIC DAMAGE BY PPB6

Because maintenance of chlorophyll content is crucial for plants while acclimating to drought stress, we determined Chl a, Chl b, total Chl content of common bean under different treatment conditions. Chl a content of plants of control, drought and D+PPB6 was 0.45, 0.16 and 0.30 mg g^{-1} FM respectively and Chl b of those treatments was 0.09, 0.003 and 0.07 mg g^{-1} FM respectively by D+PPB6 and drought (Fig. 2C). We also measured non-destructive chlorophyll index (SPAD) values of common bean and highest SPAD values was recorded in control followed by D+PPB6 and drought (Fig. 2D). We found varied level of carotenoids content under control (0.16 mg g^{-1} FM), drought (0.07 mg g^{-1} FM) and D+PPB6 (0.11

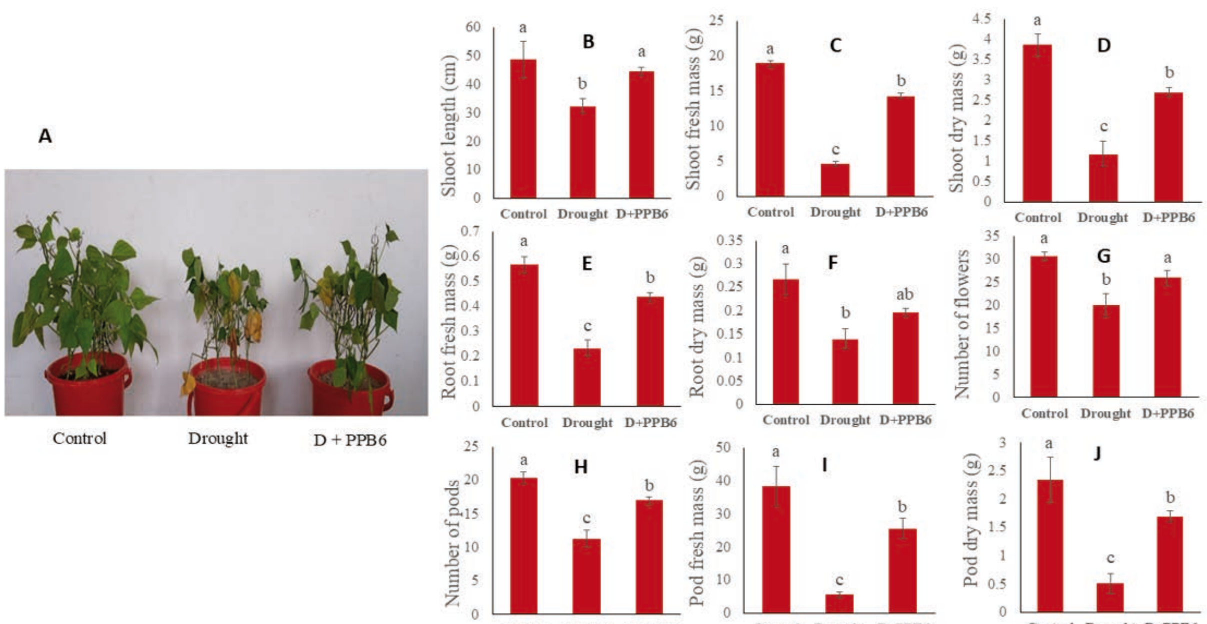

Figure 1: Alteration of morphological characteristics of common bean by *Bacillus amyloliquefaciens* (PPB6) under drought stress. Phenotypic appearance of common bean plants under uninoculated non-stressed condition (Control), uninoculated drought-stressed condition (Drought) and drought-stressed inoculated with PPB6; D+PPB6 (A). Effect of PPB6 on shoot length (B), shoot fresh mass (C), shoot dry mass (D), root fresh mass (E), root dry mass (F), number of flowers (G), number of pods (H), pod fresh mass (I) and pod dry mass (J) under drought stress. Error bars indicate standard error. Different letters on the bars show significant differences at $p < 0.05$.

mg g^{-1} FM) conditions (Fig. 2E). We also recorded data of maximum photochemical efficiency of PSII (Fv/Fm) and found significant reduction of that in plants treated with drought (0.73) as compared with D + PPB6 (0.78) and control (0.79) (Fig. 2F).

The decreased chlorophyll content by drought is considered as the typical symptom of oxidative stress and may be the result of pigment photo-oxidation and chlorophyll degradation (Anjum et al., 2011). Many investigations confirmed that PGPR could increase chlorophyll content by increasing stomatal conductivity, rate of photosynthesis, water and nutrient uptake, and protect chlorophyll degradation under drought stress (Liu et al., 2019). For instance, *Bacillus* strains were reported to increase Chl *a*, Chl *b* under drought stress in *Zea mays* L. and *Triticum aestivum* L. (Ilyas et al., 2020; Saleem et al., 2021). Furthermore, *B. amyloliquefaciens* was reported to increase Chl *a*, Chl *b* in *Medicago sativa* L. under drought stress (Han et al., 2022). Along with those, our results of relatively higher chlorophyll content under D+PPB6 as compared to uninoculated drought stress (Figs. 2A-2C) suggest the conspicuous role of *B. amyloliquefaciens* in the recovery of chlorophyll pigments in drought-stressed common bean. SPAD values was also reported to be enhanced during drought stress condition (He et al., 2019) which is compatible to our findings where higher SPAD value was recorded in drought-stressed plants supplemented with *B. amyloliquefaciens* (Fig. 2D). Alongside, carotenoids, as one of the major classes of secondary metabolites boost up the antioxidant machineries to counter face oxidative stress in plants (Polapally et al., 2022). *Bacillus* strains has been reported to increase carotenoid under drought stress in crop plants (Ilyas et al., 2020; Saleem et al., 2021) which is very compatible to our findings (Fig. 2E). During photosynthesis, abiotic stress quietly alters the maximum quantum yield of PSII having impacts on crop yield. Maximum photochemical efficiency of PSII (*Fv/Fm*) is used as a sensitive indicator of plant photosynthetic performance (Lobos et al., 2012) and found to be greatly reduced by drought stress (Zhuang et al., 2020). The recent efforts indicated that PGPRs including *B. amyloliquefaciens* are able to improve *Fv/Fm* in plants under drought stress (Asghari et al., 2020; Petrillo et al. 2022). Consistently, *B. amyloliquefaciens* (PPB6) could improve *Fv/Fm* under drought stress (Fig. 2F). Thus, *B. amyloliquefaciens* could be the potent mitigator of drought stress effects in common bean by enhancing photosynthetic pigments like chlorophyll and carotenoids, and improving photochemical quantum yield of PSII.

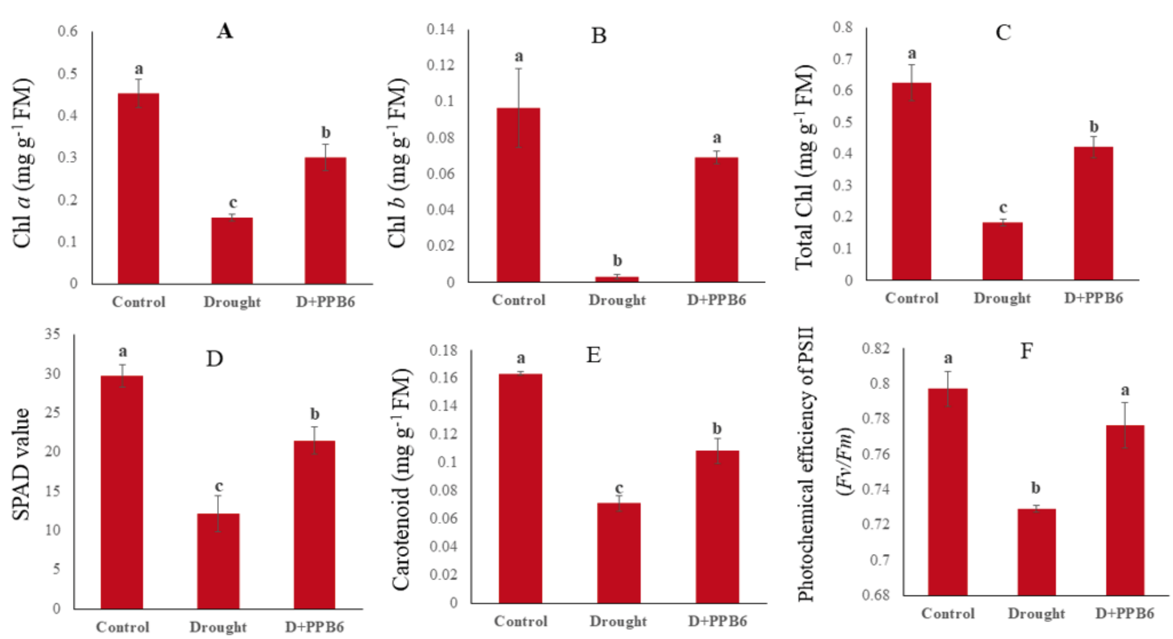

Figure 2: Effects of *Bacillus amyloliquefaciens* (PPB6) on the physiological parameters of common bean under drought stress. Effect of PPB6 on Chl *a* (A), Chl *b* (B), total chlorophyll (total Chl) (C), SPAD value (D), carotenoid contents (E) and photochemical efficiency of PSII (*Fv/Fm*) (F) under drought stress. Error bars indicate standard error. Different letters on the bars show significant differences at $p < 0.05$.

3.4 REGULATION OF OSMOTIC ADJUSTMENT BY PPB6 IN DROUGHT-STRESSED COMMON BEAN PLANTS

Since maintenance of cell turgor, osmotic adjustment and cellular homeostasis are crucial for acclimating drought stress, we measured percentage of leaf relative water content (LRWC) in leaf and found that D + PPB6 maintained more relative water content (59.67 %) as compared to uninoculated drought-stressed plants (43.67 %) (Fig. 3A). However, the highest percentage of LRWC was maintained by control treatment (78 %) (Fig. 3A). Since, cell turgor and osmotic adjustment are highly regulated by the accumulation of osmolytes, we measured proline content in common bean plants under different treatments. Proline content of plants under control, drought, and D + PPB6 was 0.18 μmol g^{-1} FM, 1.42 μmol g^{-1} FM, 0.63 μmol g^{-1} FM respectively (Fig. 3B). The LRWC is vital for the regulation of cell expansion, growth and development while acclimating to drought stress (Ashraf, 2010). PGPR-treated plants maintained relatively higher LRWC compared to non-treated plants under drought stress (Bouremani et al., 2023) and the results of which are compatible to our findings where common bean plants inoculated with PPB6 showed higher LRWC as compared to uninoculated drought-stressed plants (Fig. 3A). Proline accumulation is quite complex for explaining its role during plant-microbs interaction.

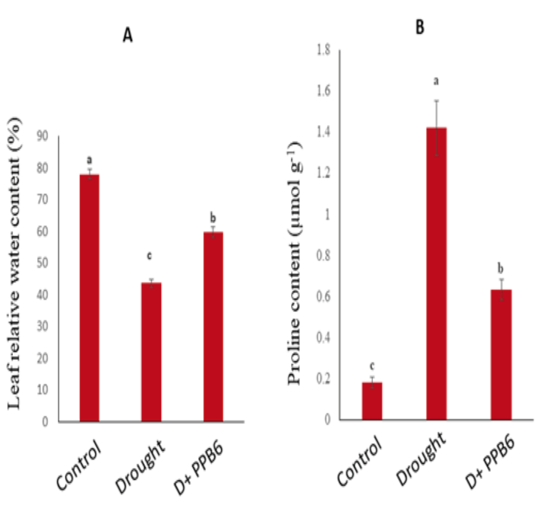

Figure 3: Effects of *Bacillus amyloliquefaciens* (PPB6) on leaf relative water and proline content of common bean subjected to non-stressed uninoculated condition (Control), uninoculated drought-stressed condition (Drought) and drought-stressed condition inoculated with PPB6 (D + PPB6). Error bars indicate standard error. Different letters on the bars show significant differences at $p < 0.05$.

Various aspects of the regulation of proline accumulation by PGPR is due to the differences of mode of action of the bacterial species, intensity of drought and differential ontogenic responses of the plants. For instance, PGPR like *P. fluorescens*, *Burkholderia* sp., *Mitsuaria* sp. and *B. amyloliquefaciens* showed more proline accumulation in wheat, maize and *Arabidopsis* under drought stress (Vardharajula *et al.,* 2011; Huang et al., 2017; Chandra et al., 2018). Contrary, PGPR like *Alcaligenes faecalis* Castellani & Chalmers 1919, *Proteus penneri* Hickman et al. 1982, and *Pseudomonas aeruginosa* were claimed to inhibit proline accumulation in plants under drought stress (Naseem & Bano, 2014), and the findings of which is consistent to our findings where PPB6 reduced proline accumulation under drought stress as compared to uninoculated drought-stressed plants (Fig. 3B). Because PPB6 could help the plants for having osmotic balance by enhancing relative water content, the inoculated plants did not cause substantial proline accumulation under drought stress.

3.5 RECOVERY OF PLANT'S TISSUE DAMAGE BY PPB6 UNDER DROUGHT STRESS

Because drought stress cause oxidative damage leading to tissue death, we measured tissue damage by means of determining electrolyte leakage, ROS; H_2O_2 and lipid peroxidation product; MDA. Electrolyte leakage of the common bean under control, drought, and D+PPB6 was 27.80 %, 55.93 %, 30.20 % respectively (Fig. 4A). H_2O_2 content of plants of control, drought and D+PPB6 was 46.47, 136.25 and 66.60 μmol g^{-1} FM respectively (Fig. 4B) and MDA content of those treatments was 0.03, 0.08 and 0.05 μmol g^{-1} FM respectively (Fig. 4C). Enhanced occurrence of ROS during abiotic stresses causes detrimental effects to cell membrane by increasing lipid peroxidation products leading to higher electrolyte leakage from damaged tissue (Ghosh et al., 2022; Huang et al., 2019). Higher electrolyte leakage in plant tissue indicates the negative consequences to membrane permeability and stability of cell membrane (Abdelaa et al., 2021). Different *Bacillus* strains could maintain cell membrane stability by means of lowering electrolyte leakage in maize (Vardharajula et al., 2011). Similarly, other PGPR were also involved to protect drought-induced membrane damage by means of lowering electrolyte leakage (Bouremani et al., 2023; Chiappero et al., 2019). Consistently, our result with PPB6 in drought-stressed common bean supports the above-mentioned statements and clarified the role of PPB6 in cell membrane stability under drought stress. Enhanced accumulation of H_2O_2, as the crucial member of ROS causes lipid peroxidation and membrane injury in plants (Sachdev et al., 2021). Lipid peroxidation product; MDA is formed by the reaction of free radicals and lipid and alter the structure of cell membrane and its stability (Woźniak et al., 2006). PGPRs like *P. fluorescens* and *Pseudomonas palleroniana* Gardan, et al.2002 decreased H_2O_2 and MDA content in wheat under drought stress (Chandra et al., 2018). Along with those findings, the significant reduction of H_2O_2 and MDA by PPB6 under drought stress as compared to uninoculated drought-stressed plants in this study (Figs. 4B & 4C), suggesting the potential role of this bacterium in maintaining cell membrane stability that could complement of the maintenance of higher LRWC, lower proline content and higher photosynthetic activity by PPB6 under drought stress (Figs. 2 & 3).

3.6 REGULATION OF ANTIOXIDANT ACTIVITIES BY PPB6 UNDER DROUGHT STRESS

Plants boost up non-enzymatic antioxidants to confront drought-induced oxidative damage. Therefore, we determined the activity total phenolic and flavonoid contents. Total phenolics contents of common bean under control, drought and D+PPB6 was 313.59, 508.54 and 325.68 µg g^{-1} FM respectively (Fig. 5A). Flavonoid content of those conditions was 624.15, 2279.66 and 1070.76 µg g^{-1} FM respectively (Fig. 5B). In addition, enzymatic antioxidants also play a crucial role in enhancing plant's tolerance to drought-induced oxidative damage (Hasanuzzaman et al., 2020), Therefore, we measured the activity of enzymatic antioxidant such as APX, CAT, GSTs and POD in common bean under different treatments. APX activity of plants of control, drought and D+PPB6 was 2.06, 3.52 and 2.67 µmol min^{-1} mg^{-1} protein respectively (Fig. 5C). CAT activity of those treatments was 56.62, 6.333 and 20.18 µmol min^{-1} mg^{-1} respectively, and GST activity of those was 169.48, 236.14 and 189.45 nmol min^{-1} mg^{-1} respectively, and POD activity of those was 13.09 and 25.94 and 15.17 nmol min^{-1} mg^{-1} respectively (Figs. 5D-5F).

Modulation of antioxidant activities by PGPR is quite complex and varies depending on the types of abiotic stresses, bacterial strains, plant species and growth conditions. For instance, phenolics and flavonoids content in plant were found to be increased under drought stress upon application of PGPR as compared to untreated plants (Chandra *et al.*, 2019; Azizi et al., 2021). Contrary, *Azospirillium brasilense* Tarrand, Krieg & Döbereiner, 1978 produced no effect on total phenolic contents (Asghari et al., 2020), and *Pseudomonas putida* Trevisan, 1889 showed reduced flavonoid content in *Glycine max* (L.)Merr. under drought stress. Accordingly, our findings showed reduced accumulation of both phe-

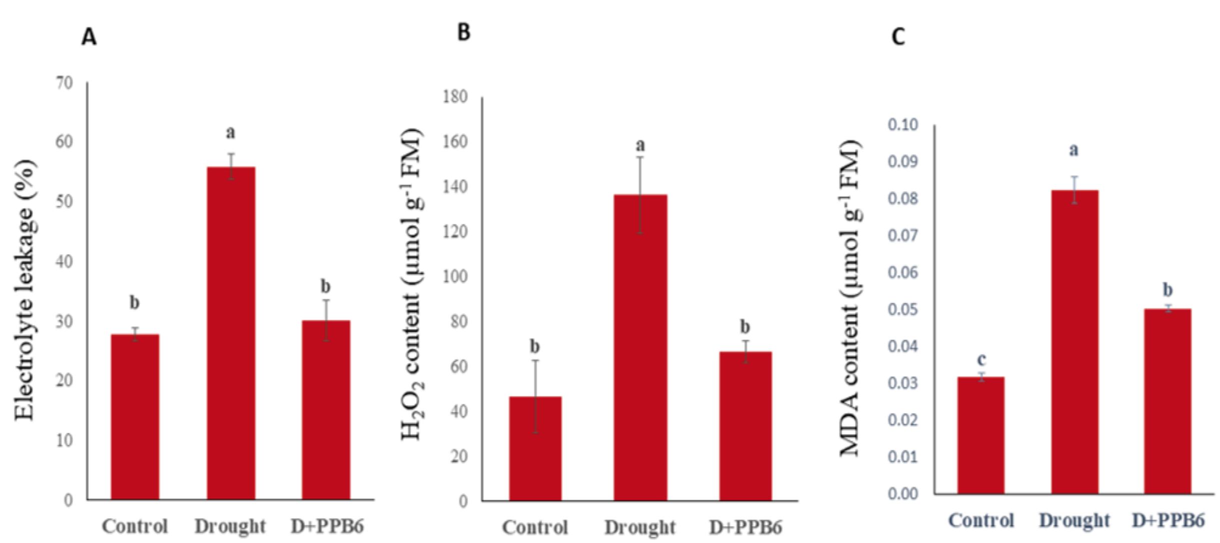

Figure 4: Effects of *Bacillus amyloliquefaciens* (PPB6) on the recovery of tissue damage caused by drought stress in common bean. Percentage (%) of electrolyte leakage (A), H_2O_2 content (B), and lipid peroxidation product; malondialdehyde (MDA) content (C) of common bean subjected to non-stressed uninoculated condition (Control), uninoculated drought-stressed condition (Drought) and drought-stressed condition inoculated with PPB6 (D + PPB6). Error bars indicate standard error. Different letters on the bars show significant differences at p < 0.05.efficiency of PSII (*Fv/Fm*) (F) under drought stress. Error bars indicate standard error. Different letters on the bars show significant differences at $p < 0.05$.

nolics and flavonoids by PPB6 under drought stress as compared to uninoculated drought-stressed plants (Figs. 5A & 5B). The role of enzymatic antioxidants by PGPR is also quite ambiguous while acclimating to drought stress. Although CAT is essential for catalyzing H_2O_2 into water and oxygen (Vitolo, 2021) and having tremendous role in abiotic stresses, but the activity of CAT is found to be reduced sometimes by drought stress (Sofo et al., 2005; Mohi-Ud-Din et al., 2021). The fact is indicating that the activity of CAT is little bit unstable and depending on the intensity of drought and growth phases of plants. Likewise, APX is also crucial and needed to accumulate more in plant for protecting chloroplasts and other cell constituents from oxidative damage (Asada, 1992). Along with those, GSTs and POD were found to be involved in scavenging ROS and reducing oxidative damage in plants (Khan et al., 2014; Kumar & Trivedi, 2018). Although, PGPR have been reported to increase the activity of enzymatic antioxidants under drought stress (Chandra et al., 2021; Akhter et al., 2021), many investigations claimed that PGPR including *Bacillus* species reduce the enzymatic antioxidant activity in drought-stressed plants. For instance, *B. amyloliquefaciens*, *B. licheniformis*, *B. subtilis*, *Bacillus thuringiensis* Berliner 1915, and *Paenibacillus favisporus* decreased APX and CAT activity, and *A. faecalis*, *P. penneri* and *P. aeruginosa* decreased the CAT and POD activity in maize, and *Bacillus cereus* Frankland & Frankland 1887 and *Planomicrobium chinense* Dai et al. 2005 reduced the activity of CAT and POD in wheat under drought stress (Vardharajula et al., 2011; Naseem & Bano, 2014; Khan & Bano 2019). The results of those are consistent to our studies like reduced activities of APX, GST and POD by PPB6 under drought-stress. (Figs. 5C, 5E and 5F). The varied level of both non-enzymatic and enzymatic antioxidant partitivities by PGPR is likely due to the plant species, ontogeny of plants, bacterial strains and intensity of drought stress. The previous findings also demonstrated that PGPR could tend to reduce the

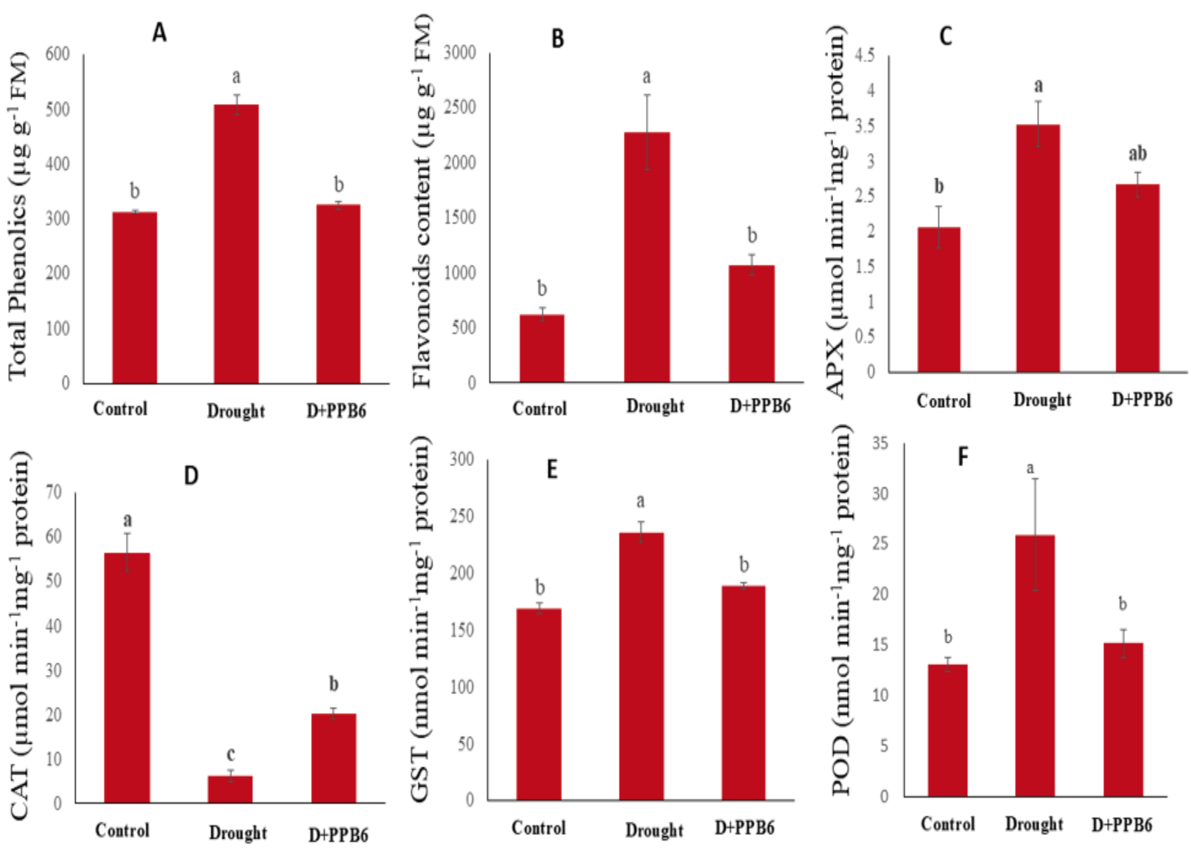

Figure 5: Effects of *Bacillus amyloliquefaciens* (PPB6) on the antioxidant activities of common bean under drought stress. Total phenolics (A) and flavonoids (B) content, and the activities of ascorbate peroxidase; APX (C), catalase; CAT (D), glutathione S-transferase; GST, (E) and peroxidase; POD (F) in common bean subjected to non-stressed uninoculated condition (Control), uninoculated drought-stressed condition (Drought) and drought-stressed condition inoculated with PPB6 (D+PPB6). Error bars indicate standard error. Different letters on the bars show significant differences at $p < 0.05$.

drought stress effects and hence to lessen the activity of antioxidant enzymes (Han & Lee, 2005). Our findings are very compatible to that where PPB6 could produce lower ROS, higher membrane stability and lower level of antioxidant activities as compared to uninoculated drought-stressed plants (Figs. 4 & 5).

4 CONCLUSION

The present study concluded that PPB6 could increase the efficiency of photosynthesis, and maintain cell membrane stability by increasing relative water content and decreasing ROS effects in drought-stressed common bean plant. PPB6 inoculated plants perceived lower drought effects which was evidenced by the reduction of proline and antioxidant enzymes. The comparable occurrence of ROS and antioxidant activities in drought-stressed plants inoculated with PPB6 and non-stressed uninoculated plants suggesting the partial complementation of drought-effects by this bacterium. The findings might help for understanding the implications of *B. amyloliquefaciens* in drought tolerance of common bean. The knowledge of this study will further assist for molecular characterization of common bean plant inoculated with *B. amyloliquefaciens* under drought stress.

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Biochemical and molecular characterization of bread wheat genotypes under drought stress: Implications for antioxidant defense mechanisms and genomic analysis

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Biochemical and molecular characterization of bread wheat genotypes under drought stress: Implications for antioxidant defense mechanisms and genomic analysis

Abstract: Plants face abiotic stresses like drought, salinity, and high temperature, which adversely affect growth and induce physiological and metabolic changes. Drought is a complex stress controlled by many genes, requiring investigation through molecular markers and biochemical characterization in wheat genotypes. This study involved eight bread wheat cultivars and two controls: drought-tolerant 'Gerek-79' and drought-sensitive 'Sultan-95'. These were grown for 40 days and then subjected to 10 days of drought stress. Antioxidants and antioxidant enzyme activities, which neutralize ROS, are key resistance mechanisms against oxidative stress. Levels of polyphenol oxidase (PPO), peroxidase (POD), ascorbate peroxidase (APX), and catalase (CAT) were measured. Plant responses to stress included changes in photosynthetic pigments, total proteins, hydrogen peroxide, lipid peroxidation (MDA), and proline levels. POD showed the highest change in enzyme activity, while PPO was least affected. Chlorophyll b levels increased under stress across all varieties. Notably, proline levels, an abiotic stress marker, significantly rose by the 10th day of drought. Additionally, wheat genotypes were analyzed using drought-related SSR markers (Xwmc 89, Xwmc 118, Xwmc 304, Xgwm 337). This allowed evaluation of the impact of molecular characterization on biochemical changes under drought stress.

Key words: bread wheat, drought stress, antioxidant enzymes, proline, lipid peroxidation, hydrogen peroxide, photosynthetic pigments

Prepoznavanje biokemičnih in molekularnih označevalcev krušne pšenice v razmerah sušnega stresa, ki so vključeni v mehanizme antioksidacijske obrambe in genomske analize

Izvleček: Rastline se izpostavljene abiotskim stresom kot so suša, slanost in visoke temperature, ki negativno vplivajo na rast in vzpodbudijo fiziološke in med njimi presnovne spremembe. Suša je kompleksni stres, ki ga uravnavajo številni geni, kar pri genotipih krušne pšenice zahteva preučevanje molekularnih označevalcev in biokemičnih procesov. V to raziskavo je bilo vključenih osem genotiopov krušne pšenice in dve kontroli: na sušo tolerantna sorta Gerek-79 in občutljiva sorta sorta Sultan-95. Vse rastline so bile gojene 40 dni v standardnih razmerah in nato izpostavljene sušnemu stresu za 10 dni. Antioksidanti in antioksidacijski emcimi, ki nevtralizirajo aktivne zvrsti kisika (ROS) so ključni mehanizmi za odpravo oksidacijskega stresa. Izmerjene so bile vsebnosti polifenol oksidase (PPO), peroksidaze (POD), askorbat peroksidaze (APX) in katalaze (CAT). Analiza odziva rastlin na stres je obsegala še meritve sprememb v vsebnosti fotosinteznih pigmentov, celokupnih beljakovin, vodikovega peroksida, spremembe v peroksidaciji maščob (MDA) in v vsebnosti prolina. POD je pokazala največjo spremembo v aktivnosti med tem, ko je bila PPO najmanj prizadeta. Vsebnost klorofila b se je v stresnih razmerah povečala pri vseh sortah. Značilno se je po desetih dneh suše povečala vsebnost prolina kot označevalca abiotičnega stresa. Dodatno so bili v genotipih krušne pšenice anlizirani na s sušo povezani SSR označevalci (Xwmc 89, Xwmc 118, Xwmc 304, Xgwm 337). To je omogočilo ovrednotenje biokemičnih sprememb v razmerah sušnega stresa na molekularni osnovi.

Ključne besede: krušna pšenica, sušni stres, antioksidacijski encimi, prolin, peroksidacija lipidov, vodikov peroksid, fitosintezna barvila

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1 INTRODUCTION

Global crop production is threatened by the increased frequency of long-term water and heat stress caused by climate change (Arora, 2019). Drought caused by climate change is one of the important natural disasters that significantly impact the agricultural sector. Due to its geographical size and range, it has a complex structure and affects a large population (Javadinejad et al., 2021).

Plants can face very different stress conditions throughout their lives. These stress factors can be biotic (pathogen, competition with other organisms, etc.) or abiotic (drought, salinity, radiation, chemical substances, high temperature or frost etc.). The ability of a plant to grow in a limited area of water is referred to as drought resistance (Kadam et al., 2012). Under drought stress, plants exhibit various metabolic changes such as protein synthesis, ROS accumulation, enzyme activity change, and pigment content (Chen et al., 2016).

The response of plants to drought stress depends on plant growth (development), stress period and plant genetics (Beltrano & Ronco, 2008; Khan et al., 2012). Drought stress induces many common cellular reactions in plants. The stress leads to cellular dehydration, which causes osmotic stresses resulting in reduced cytosolic and vacuolar bulk (Wang et al., 2003). Except for ionic compounds, the early response of this plant to the plant is largely the same. The response of the plant to the stress condition is quite complex in terms of cellularity, and plants respond to stress by changing many biochemical and molecular mechanisms (genes, etc.) at tissue and plant levels (Bohnert & Jensen, 1996; Wang et al., 2003). As a result, there are numerous changes to the plant adaptation, such as reduction in growth, slowing of photosynthesis, closing of stomata, transient increases in ABA levels, induction of gene expressions, accumulation of coherent soluble and protective proteins, increased levels of antioxidants and suppression of energy consumption pathways (Bartels & Sunkar, 2005).

Proteins are essential components of living organism. The most important indicator of wheat quality is the amount of protein and the quality of the existing protein (Finney et al., 1987).

As a result of water loss, the interactions of hydrophobic and hydrophilic amino acids in the structure of proteins with water are disrupted. So, proteins denaturation and enzymes inhibition occur. Proline is a molecular chaperone that protects proteins from degradation and regulates different enzyme activities. It has been suggested that proline accumulation in high plants is an antioxidant because it contributes to osmotic compliance and is effective in preserving the integrity of the plasma membrane by against to drought and salinity, metal toxicity, oxidative stress and biotic stress factors (Mansour, 1998; ÖZDEMİR et al., 2012). Causing cross-linking and polymerization of membrane components, aldehydes inactivate receptors and membrane bound enzymes at

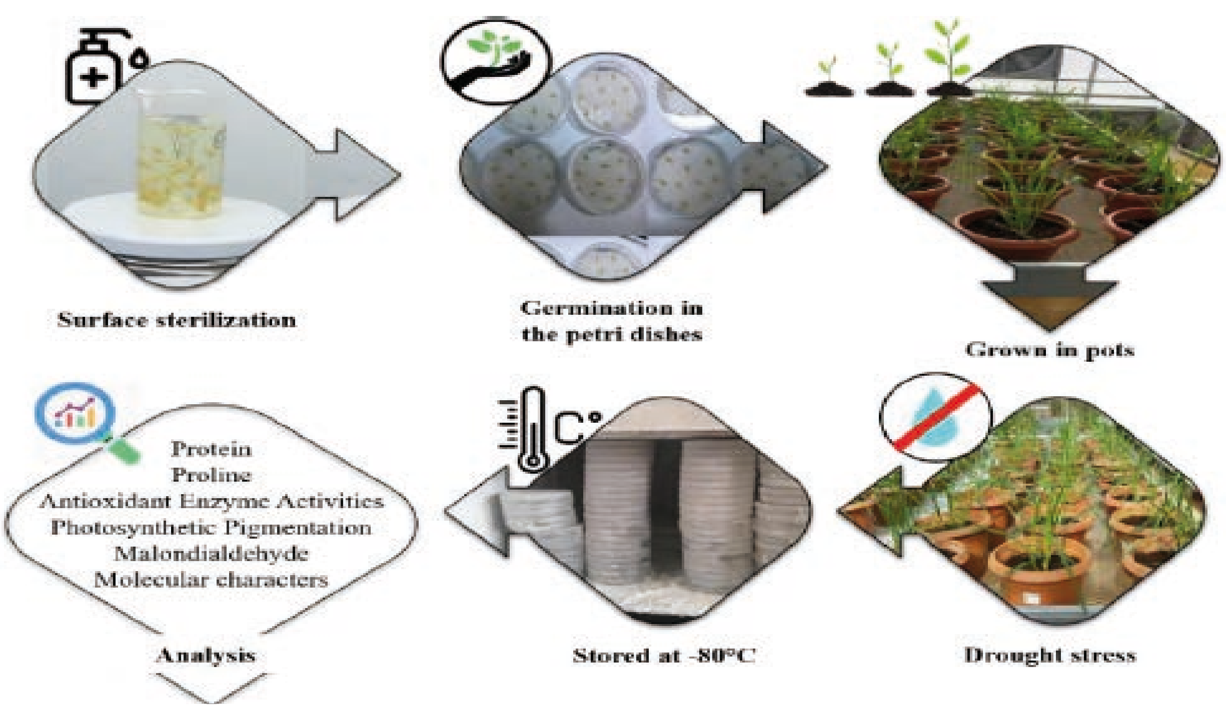

Figure 1: Schematic diagram of the study

membrane. So, serious damage can also occur in membrane proteins. Malondialdehyde (MDA), a reactive aldehyde, is formed as a result of the oxidation of lipids in the biological system. MDA inhibits the production of peroxidized unsaturated fatty acids in plant membranes, triggering transcriptional stress responses (Tenikecier, 2013). In biotic or abiotic stressed plants, the inactivation of reactive oxygen species, which damage plant cells to vital components such as membrane lipids, proteins, enzymes, pigments and nucleic acids, is provided by the increase of antioxidant enzymes (Maheshwari & Dubey, 2009). ROS, such as hydrogen peroxide, superoxide anion, hydroxyl radical and singlet oxygen, are formed by partial reduction of molecular oxygen (Kireçci, 2012). ROSs cause degradation of membrane components, oxidation of protein sulfhydryl groups, formation of cytoplasmic gel phase, and loss of membrane functions. Antioxidants protect cells from oxidative damage by capturing and detoxifying ROS. This system consists of antioxidants such as ascorbate, glutathione, α tocopherol and carotenoids with small molecular weight and enzymatic antioxidants such as SOD, CAT, ASPX and GR (Ahmad et al., 2008; Keleş & Öncel, 2002; Zhang Feng et al., 2004).

Wheat is an important grain product that meets a large portion (22 %) of global food needs (Naderi et al., 2020). Due to population growth worldwide (Faostat, 2017), wheat production needs to be supported to meet food needs around the world. Determining the crop tolerance mechanism in drought is important for wheat breeding programs (Gálvez et al., 2019). Landraces are more tolerant to stressful environments and temporary climatic conditions thanks to their agro-physiological characteristics and genetic structure (Mohammadi et al., 2015). For this reason, determining suitable wheat varieties against drought stress can contribute to creating new varieties and benefit from creating new product plans with improved stress tolerance (Naderi et al., 2020; Zhou et al., 2015). In this study, ten different bread wheat genotypes were subjected drought stress by deficient of water for 10 days. Changes in some antioxidant enzymes and molecular indicator of drought stress such as MDA, prolin, pigments etc. were measured in different wheat genotypes to develop relationship between antioxidant enzymes, molecular indicators and mechanisms of drought tolerance.

2 MATERIAL & METHOD

2.1 PLANT MATERIALS

Ten bread wheat cultivars (Sarıcanak-98, Karahan, Sonmez2001, Kate-1, Altay2000, Bayraktar, Harmankaya99, Izgi2001, Gerek-79, Sultan-95) that differ in drought tolerance were used as plant material. Gerek 79 cultivars for drought tolerance and Sultan 95 cultivars for drought sensitivity were used as control cultivars. The wheat cultivars and lines were kindly provided by the GAP International Agricultural Research Institute, Eskişehir Transition Zone Agricultural Research Institute, Bahri Dağdaş International Agricultural Research Institute, Ankara Field Crops Central Research Institute and Thrace Agricultural Research Institute.

Surface sterilization was applied before the wheat seeds were planted in petri dishes. Briefly, seeds were treated for 1 min with 70 % ethanol and 5 % sodium hypochlorite for 10 min. Finally, the seeds were rinsed three times with distilled water.

The seeds were placed in the petri dishes with 10-12 seeds each. The petri dishes were closed with para-film and were kept at +4 °C for 4-5 days. Then, the germination was observed for 3-4 days at room temperature. Seven- and ten-day seedlings pots were grown in pots for thirty days. Irrigation was left in the application groups after the thirtieth day. On the third, seventh, and tenth days, tissue samples were collected and stored at -80 °C for analysis.

2.2 DETERMINATION OF ANTIOXIDANT ENZYME ACTIVITIES

0.25 g of plant tissue was homogenized with potassium phosphate (pH = 7) and centrifuged at 10000 x G for 15 minutes. The supernatant was used to determine enzyme activities.

Depending on the consumption of the prepared substrates or the product formation, the enzyme activities were examined at the optimum pH and temperature for the enzymes by spectrophotometric method at specific wavelengths. All characterization operations were performed in triplicate.

2.2.1 CATALASE ASSIGNMENT

In the activity measurement, 3 ml of reaction buffer was prepared which including 50 mM phosphate buffer (pH = 7), 30 mM H_2O_2 and 30 µl homogenate. The absorbance was measured during 2 minutes. For the calculations, the amount of enzyme that cleaved 1 µmol of H_2O_2 was determined in one minute at 240 nm from using the A = Є.b.c formula by taking the light path as 10 mm and the extinction coefficient (Є H_2O_2: 0.0394 $mmol^{-1}$x mm^{-1}).

2.2.2 PEROXIDASE ASSIGNMENT

To determine the peroxidase activity 3 ml of the reaction mixture was prepared consisting of 50 mM phosphate buffer (pH = 7), 22.5 mM H_2O_2, 30 mM guaiacol and 20 µl enzyme extract. The activity to the reaction was initiated by the addition of the enzyme solution to the assay medium and optical density was recorded at 470 nm for 2 minutes. An enzyme unit was calculated as the amount of enzyme catalyzing 1 µmol guaiacol per minute.

2.2.3 ASCORBATE PEROXIDASE ASSIGNMENT

To determine the ascorbate peroxidase activity, 3 ml of the reaction mixture was prepared in the activity measurement to consist of 100 mM phosphate buffer (pH = 7), 10 mM H_2O_2, 2.5 mM ascorbic acid and 100 µl enzyme extract. The reaction was initiated by adding hydrogen peroxide to the activity measurement medium last and the absorbance was recorded at 290 nm for 2 minutes. Enzyme activity was calculated using ascorbate extinction coefficient (2.8 $mM^{-1}xcm^{-1}$).

2.2.4 POLYPHENOL OXIDASE ASSIGNMENT

0.3 g of the tissue was taken and 2 ml of buffer (0.2 M Na_2HPO_4 and 5 mM ascorbic acid pH: 6.5) was added to prepare the homogenate. Homogenate was centrifuged at 12000 x G for 10 minutes. Enzyme activity measurement is based on the observation of the increase in absorbance at 420 nm caused by the conversion of catechol to brownish-yellow colored quinone in the presence of oxygen.

2.2.5 THE HYDROGEN PEROXIDE (H_2O_2) ASSIGNMENT

Leaf tissues were homogenized in 5 ml of ice-bath with 0.1 % (w / v) TCA. The homogenate was centrifuged for 15 min at 12000 x G. 0.5 ml of supernatant were added to 0.5 ml of 10 mM potassium phosphate (pH: 7.0) buffer and 1 ml of 1 M KI. The absorbance of the mixture was then determined at 390 nm and the amount of H_2O_2 was determined by using the standard curve.

2.3 PHOTOSYNTHETIC PIGMENTATION ASSIGNMENT

Chlorophyll a, b and carotenoid amounts were determined according to Arnon after extraction with 80 % acetone of plant leaves (Arnon, 1949). The amount of carotenoid was determined according to the Jaspars formula (Ulusu et al., 2017). 0.2 g of leaf tissue was homogenized in 5 ml porcelain mortar with 80 % acetone. The homogenate was transferred to a 15 ml centrifuge tube and centrifuged at 3000 x G for 5 minutes. The absorbance of the supernatant at 645, 663 and 450 nm was measured by spectrophotometer and the amount of pigment/ml extract was calculated from the following formulas with the help of these absorbance values.

Chlorophyll a = 12.7 x A663 – 2.69 x A645

Chlorophyll b = 22.9 x A645 – 4.68 x A663

Total Chlorophyll = 20.2 x A645 + 8.02 x A663

Carotenoid = 4.07 x A450 – (0.0435 x amount of Chlorophyll a + 0.3367 x amount of Chlorophyll b)

2.4 PROTEIN ASSIGNMENT

The amount of protein in the leaves was determined by Bradford method (Bradford, 1976). In order to determine the amount of protein, 0.25 g leaf tissue was homogenized in 2.5 ml of 50 mM KH_2PO_4 (pH = 7) buffer in porcelain mortar. homogenate was transferred to eppendorf tubes and centrifuged at 15000 x G for 20 minutes at +4 °C. 20 µl of supernatant was added in 2.5 ml of Coomassie brilliant blue G-250 and vortexed. After 10 minutes of incubation, the absorbance at 595 nm was measured and the amount of protein in the leaves was determined by means of a standard graphic.

2.5 PROLINE ASSIGNMENT

To determine the amount of proline, 0.4 grams of leaf was homogenized in 4 % sulfosalicylic acid and then filtered through homogenate filter paper. 0.5 ml was taken from the filtrate and diluted 10 times with distilled water. 1 ml of sample, 1 ml of 96 % glacial acetic acid and 1 ml of acid ninhydrin solution were added to the tubes, and all the tubes were allowed to incubate for 60 minutes in a water bath at 100 °C. After incubation, the tubes were held for 10 minutes in ice cubes, and then 2 ml of toluene was added to each tube and vortexed. After this process, five minutes were waited and the absorbance of the pink phase formed at the upper part of each tube was measured at 520 nm and recorded. The results were calculated as the amount of prolin per gram of fresh tissue using standard graphics prepared from pure proline.

2.6 MALONDIALDEHYDE ASSIGNMENT

1 g of plant tissue was homogenized with 0.1 % (w / v) TCA and centrifuged at 10000 x G for 5 minutes.

TCA containing 0.5 % TBA was added to the supernatant and incubated at 95 °C for 30 minutes and then quickly cooled in ice bath. The mixture was centrifuged again at 10000 x G for 15 minutes. The amount of malondialdehyde was determined by measuring the absorbance of the supernatant at 532 nm in the spectrophotometer.

2.7 DNA EXTRACTION AND PCR AMPLIFICATION

Young leaves reaching the two-leaf stage were used for DNA extraction, and it was performed according to the method of Doyle and Doyle (1990) with some modifications. The SSR primers used for this study were selected from the SSR primers that showed polymorphic properties according to the previous drought characterization study (Ateş Sönmezoğlu & Terzi, 2018). PCR conditions used for Xwmc 89, Xwmc 118, Xwmc 304 and Xgwm 337 primers are as specified in the source article.

Agarose gel concentrations were 2 % for PCR products and 1 % for genomic DNAs. Ethidium bromide (10 mg ml^{-1}) was used as an imaging dye in electrophoresis. Electrophoresis was performed in 1 % TBE buffer at 100 V constant for 60 min. PCR reactions were carried out with BIO-RAD C1000 Touch Thermal Cycler. Biorad ChemiDoc MP was used as a gel imaging system, and polymorphic bands were determined.

2.8 STATISTICAL ANALYSIS

Polymorphic bands obtained from PCR were scored 1 (presence) and 0 (no band) for molecular characterization. DendroUPGMA (D-UPGMA) software (//genomes.urv.es/UPGMA) was used for the molecular comparisons of genotypes, and Interactive Tree of Life (iTOL) (https://itol.embl.de) was used to construct the interactive tree.

3 RESULTS AND DISCUSSIONS

3.1 ANTIOXIDANT ENZYME ACTIVITIES

In Bayraktar, Sönmez, Gerek and İzgi cultivars, the amount of H_2O_2 and CAT varied inversely according to the stress days, while in Sultan, Harmankaya, Karahan, Kate and Sarıçanak cultivars, both were parallel. In this case, H_2O_2 produced as a result of stress factors was tried to be kept under control by CAT, but these plants had a

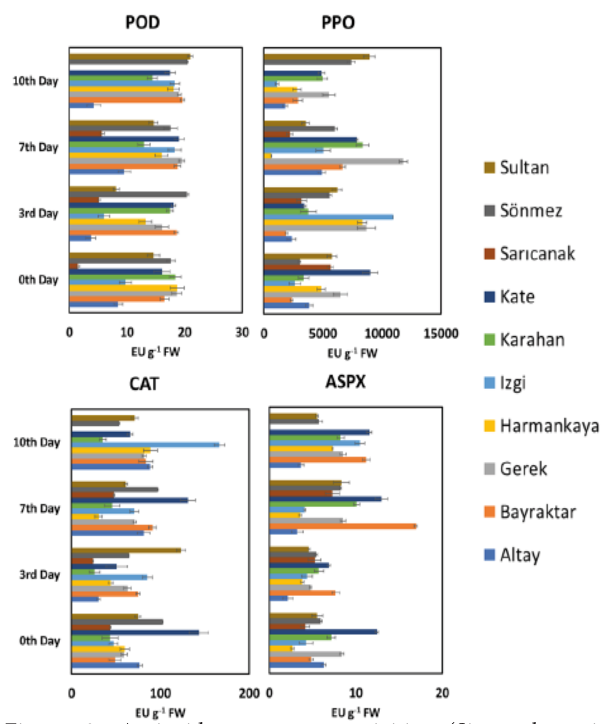

Figure 2: Antioxidant enzyme activities (Since there is not enough vegetative tissue left in the Sarıcanak variety, the analyzes on the 10th day could not be studied)

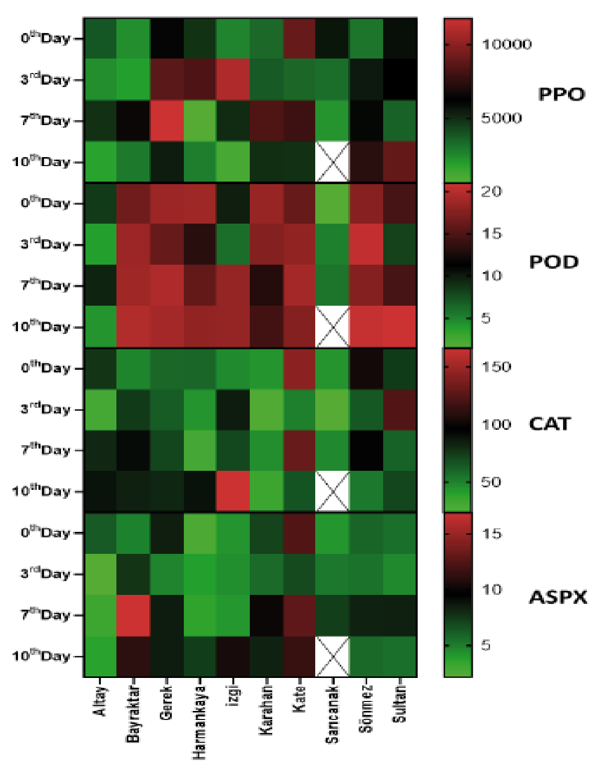

Figure 3: Heatmap analysis of the antioxidant enzyme activities (Since there is not enough vegetative tissue left in the Sarıcanak variety, the analyzes on the 10th day could not be studied).

little more difficulty in maintaining the balance than the others. ASPX activity tended to decrease in Altay variety due to stress, while it increased in other wheat varieties compared to the control group. On the other hand, polyphenol oxidase activity increased until the middle of the stress application in the other varieties except Sarıçanak variety, while it showed a decreasing trend as of the 10^{th} day due to the deterioration of the internal balance (Fig. 2).While an increase in POD activity was observed in Bayraktar, Sultan, Izgi and Sarıçanak varieties, no significant change in POD activity was observed in other treatment groups due to stress (Fig. 3).

3.2 PHOTOSYNTHETIC PIGMENTS

Photosynthesis is one of physiological processes, which is the most sensitively affected by abiotic stress conditions in plants. Drought stress also causes structural and functional effects on chloroplasts. In this study, changes in chlorophyll a, b, total chlorophyll and carotenoid contents were investigated in different wheat varieties under water stress. While no significant pigment change was observed in the samples during the early drought period, a relative decrease occurred in chlorophyll a, b and total chlorophyll amounts in parallel with the increasing stress conditions. (Fig.4, Fig. 5).

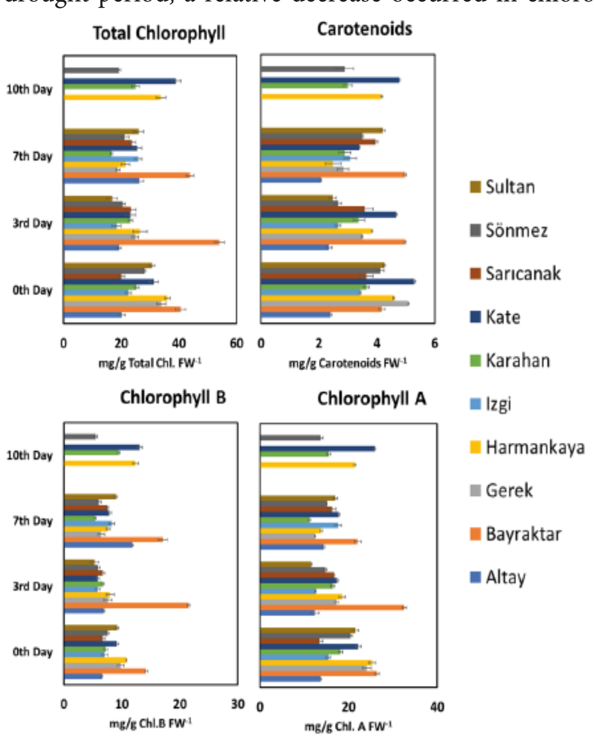

Figure 4: Photosynthetic pigment analysis (Since there is not enough vegetative tissue left in the Altay, Bayraktar, Gerek, Izgı, Sarıcanak, Sultan varieties the analyzes on the 10th day could not be studied)

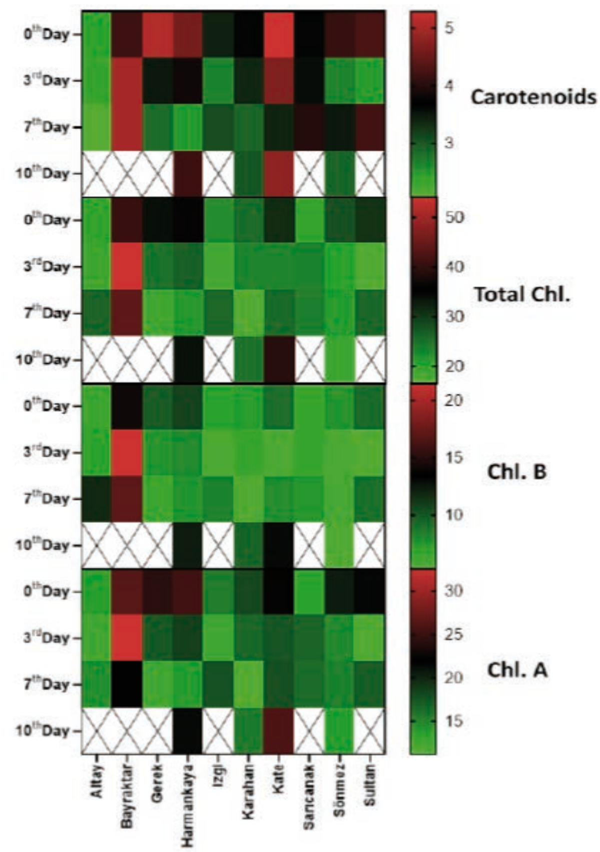

Figure 5: Heatmap analysis of the photosynthetic pigments (Since there is not enough vegetative tissue left in the Altay, Bayraktar, Gerek, Izgı, Sarıcanak, Sultan varieties the analyzes on the 10th day could not be studied)

3.3 PROTEIN

Drought tolerance of plants is usually achieved by changes in antioxidant enzymes and maintenance of H_2O_2 levels within certain limits. Water stress is associated with increased oxidative stress due to increased accumulation of ROS, especially O^{2-} and H_2O_2 in chloroplasts, mitochondria and peroxisomes. Induction of antioxidant enzyme activities is a general adaptation strategy used by plants to overcome oxidative stresses (Fig. 6) (Foyer & Noctor, 2003).

In plants subjected to drought stress, changes in the amount of hydrophilic total protein within the cells were observed as a response to drought. A statistically significant increase in the amount of protein was observed in 7 out of 10 different varieties (Altay, Bayraktar, Gerek, Sultan, İzgi, Karahan and Kate) analyzed. This increase is thought to have been provided by the activities in the

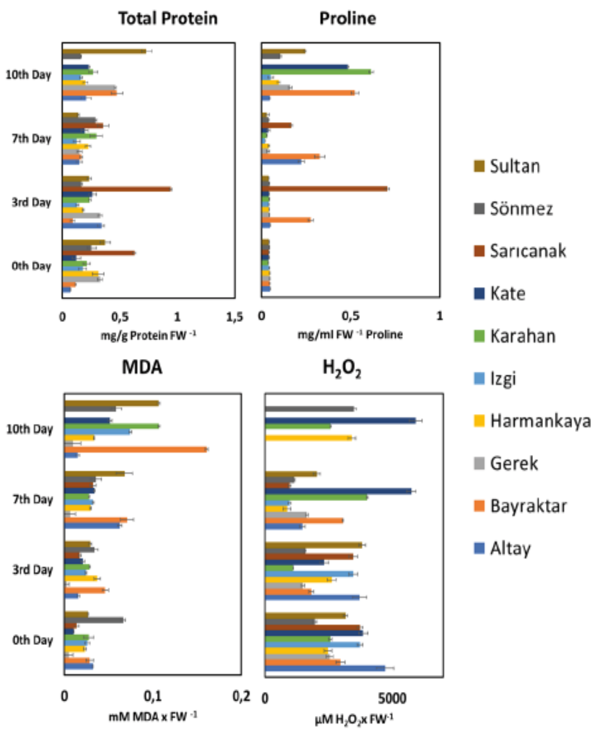

Figure 6:Total protein, proline, MDA and H_2O_2 levels of treatment groups (Since there is not enough vegetative tissue left in the Altay, Bayraktar, Gerek, Izgı, Sarıcanak, Sultan varieties the H_2O_2 analyzes on the 10th day could not be studied)

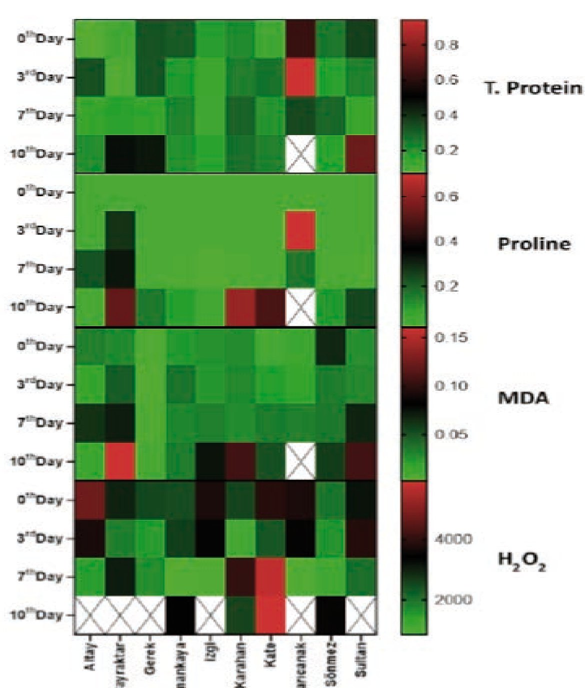

Figure 7: Heatmap analysis of al protein, Proline, MDA and H^2O^2 levels (Since there is not enough vegetative tissue left in the Altay, Bayraktar, Gerek, Izgı, Sarıcanak, Sultan varieties the H^2O^2 analyzes on the 10th day could not be studied)

synthesis of antioxidant enzymes, which are one of the intracellular defense systems. On the other hand, while no significant change was observed in Harmankaya variety, in Sönmez and Sarıçanak varieties, the plant was out of the hemostasis state and in parallel with this, a decrease in total protein amount was observed.

3.4 PROLINE

In the present study, proline levels increased significantly in all treatment groups except Altay and Sarıçanak, especially on the 10^{th} drought day. This indicates that the plants were stressed after drought and this provided a sufficient signal for the activation of many biochemical and physiological antioxidant defense mechanisms within the organism. In Altay, the amount of free proline increased about five times after the 7^{th} treatment day compared to the control group; however, it decreased back to its previous level by the 10^{th} day. A similar situation was observed in Sarıçanak varietal as of the 3^{rd} day (Fig. 7).

3.5 MALONDIALDEHYDE

In Altay varietal, the amount of MDA, which reached its highest level on the 7th day, decreased on the 10th day. In this case, it is thought to be an indication that especially membrane lipids started to abound due to stress and the hemostasis state of the plant gradually started to disappear.

3.6 ANALYSES OF MOLECULAR CHARACTERS

In this study, the dendrogram of the genotypes was conducted by using 4 SSR primers (Xwmc 89, Xwmc 118, Xwmc 304, and Xgwm 337), which show polymorphic features in drought-related gene regions (Ateş Sönmezoğlu & Terzi, 2018; Kirigwi et al., 2007; Kumar et al., 2012; Mdluli et al., 2020). According to *Figure 8*, bread-wheat genotypes were divided into two main groups. While the first of these main groups were the Sultan-95 genotype, which is sensitive to drought, the second main group included the Gerek-79 genotype, which is resistant to drought. Group 1 contained the Sultan-95 and Karahan genotypes, while the other group contained all remaining genotypes. The second group was also divided into subgroups within itself. According to the dendrogram values, the closest genotypes to each other were Kate-1 and Sönmez 2001. The results of this study showed that the genotypes used have a high genetic diversity.

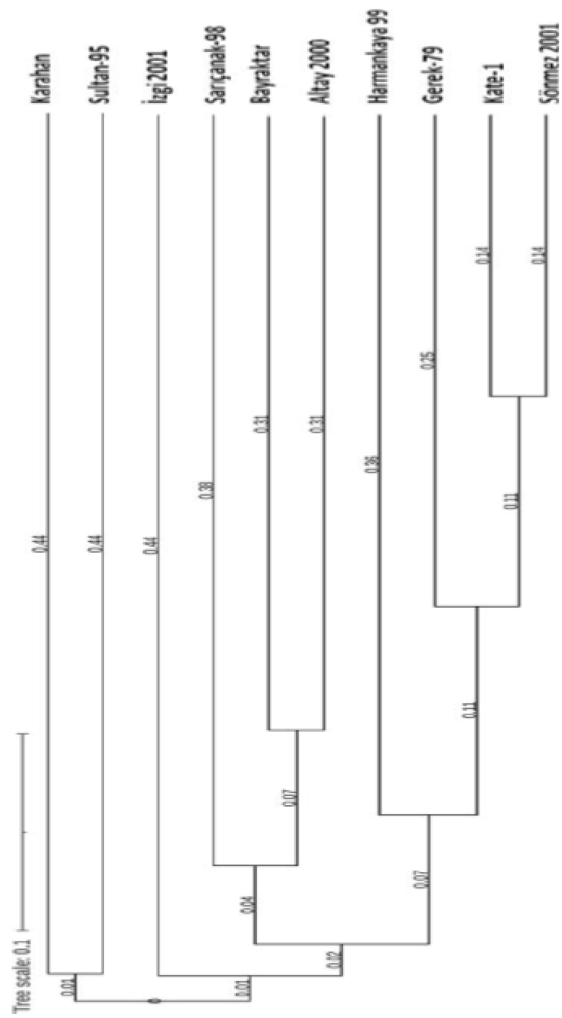

Figure 8: Dendrogram based on molecular data.

The antioxidant response is often enhanced in relation to plant tolerance when abiotic stress conditions occur because enhanced antioxidant enzyme activities contribute to drought tolerance to reduce oxidative damage (Gill & Tuteja, 2010). It has been stated in the literature that high antioxidant enzyme activities contribute to stress tolerance (Mehrabad Pour-Benab et al., 2019), but different studies may show different results (Cruz de Carvalho, 2008).

In this study, catalase enzyme activity increased gradually from day 0 in the tolerant genotype (Gerek-79), while it decreased significantly after the 3rd day for the sensitive genotype (Sultan-95). Catalase activities increased on the 10th day for Altay 2000, Harmankaya 99, and Izgi 2001 varieties. While the peroxidase enzyme activity did not show a big difference for Gerek-70, it decreased and then increased for the Sultan on the 3rd day. Izgi 2001 and Altay 2000 cultivars were also reduced on the 3rd day the other days, like the Sultan-95. Ascorbate peroxidase activity decreased on the 3rd day for Gerek-79 but increased for the following days, it decreased on the 10th day in the Sultan-95 cultivar. Bayraktar, Altay 2000, Sönmez 2001 and Karahan also decreased on the 10th day.

When evaluated biochemically, some of the wheat genotypes selected in different genotypes under drought stress are more resistant, and some are more sensitive. However, this varies for different biochemical analyzes. Therefore, no significant correlation was observed between genotypes when the biochemical results were examined in our study.

Vuković et al. (2022) did not find a linear correlation between the results on the biochemical and molecular responses of winter wheat varieties under drought stress. They stated that the mechanism of drought tolerance is quite complex. Accordingly, factors such as the period of the plant and the intensity or duration of the stress to which the plant is exposed should be considered (Vuković et al., 2022).

Since genotypes under drought stress exhibit various biochemical properties, it is not enough to generalize the molecular data according to biochemical results. The reason for this can be explained as follows: 1) wheat has a large genome (16,000 Mb for bread wheat), and drought is a quantitative trait controlled by many genes. In this regard, the molecular markers may not be specific to the gene regions controlling the relevant biochemical processes; 2) molecular markers can also cover non-coding regions of the genome.

4 CONCLUSIONS

In this study, the characterization of different wheat genotypes for drought tolerance as well as detailed biochemical analyzes, was evaluated. The tolerance mechanism of drought, which has a complex mechanism, was examined in terms of different genotypes. This study is expected to provide molecular and biochemical preliminary information for local wheat varieties in our country. In addition, we propose a detailed molecular, biochemical, and morphological examination of the drought mechanism for essential crop plants with subsequent studies.

5 ACKNOWLEDGEMENT

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Prve najdbe štirih vrst jajčnih parazitoidov ščitastih stenic (Pentatomidae) v Sloveniji: *Trissolcus scutellaris* (Thomson, 1861), *Trissolcus viktorovi* Kozlov, 1968, *Trissolcus festivae* (Viktorov, 1964) in *Telenomus chloropus* (Thomson, 1861) (Insecta, Hymenoptera, Scelionidae)

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Prve najdbe štirih vrst jajčnih parazitoidov ščitastih stenic (Pentatomidae) v Sloveniji: *Trissolcus scutellaris* **(Thomson, 1861),** *Trissolcus viktorovi* **Kozlov, 1968,** *Trissolcus festivae* **(Viktorov, 1964) in** *Telenomus chloropus* **(Thomson, 1861) (Insecta, Hymenoptera, Scelionidae)**

Izvleček: Jajčni parazitoidi so skupina naravnih sovražnikov, ki v naravi vplivajo na zmanjševanje števila škodljivih žuželk, uporabljajo pa se tudi v okoljsko sprejemljivih sistemih zatiranja škodljivcev v rastlinski pridelavi. V članku predstavljamo štiri vrste parazitoidnih os iz družine Scelionidae, in sicer vrste *Trissolcus scutellaris*, *Trissolcus viktorovi*, *Trissolcus festivae* in *Telenomus chloropus*, ki spadajo med jajčne parazitoide ščitastih stenic (Pentatomidae). Prve tri vrste smo v Sloveniji potrdili kot naravne sovražnike pisane stenice (*Eurydema ventralis* Kolenati, 1846), zadnjo vrsto pa kot jajčnega parazitoida marmorirane smrdljivke (*Halyomorpha halys* Stål, 1855). Nobena od navedenih vrst naravnih sovražnikov še ni navedena v Pozitivnem seznamu Evropske in sredozemske organizacije za varstvo rastlin (EPPO), zato jih ne moremo uvrstiti na Seznam domorodnih vrst organizmov za biotično varstvo rastlin, s čimer bi bila v Sloveniji dovoljena njihova uporaba v inokulativnem biotičnem zatiranju škodljivcev v kmetijski pridelavi na prostem. Na večji pomen navedenih jajčnih parazitoidov pri zatiranju gospodarsko škodljivih stenic v okviru varovalnega biotičnega varstva pa lahko vplivamo z uporabo drugih okoljsko sprejemljivih načinov zatiranja škodljivih organizmov.

Ključne besede: parazitoidne ose, *Trissolcus scutellaris*, *Trissolcus* festivae, *Telenomus chloropus*, *Trissolcus viktorovi*, Slovenija, biotično varstvo rastlin, Pentatomidae

First records of four species of egg parasitoids of stink bugs (Pentatomidae) in Slovenia: *Trissolcus scutellaris* **(Thomson),** *Trissolcus viktorovi* **Kozlov,** *Trissolcus festivae* **(Viktorov) and** *Telenomus chloropus* **(Thomson) (Insecta, Hymenoptera, Scelionidae)**

Abstract: Egg parasitoids are a group of natural enemies that reduce the number of harmful insects in nature, and are also used in environmentally acceptable pest control systems in crop production. In the article, we present four species of parasitoid wasps from the family Scelionidae, namely the species *Trissolcus scutellaris*, *Trissolcus viktorovi*, *Trissolcus festivae* and *Telenomus chloropus*, which belong to egg parasitoids of stink bugs (Pentatomidae). In Slovenia, we confirmed the first three species as natural enemies of the cabbage bug (*Eurydema ventralis* Kolenati, 1846), and the last species as an egg parasitoid of the brown marmorated stink bug (*Halyomorpha halys* Stål, 1855). None of the mentioned species of natural enemies is yet listed in the Positive List of the European and Mediterranean Plant Protection Organization (EPPO), so in Slovenia we cannot include them in the List of native species of organisms for biological control, which would allow their use in inoculative biological control in outdoor agricultural production. The greater importance of the mentioned egg parasitoids in the suppression of economically harmful stink bugs in the context of conservation biological control can be influenced by using other environmentally acceptable methods of controlling harmful organisms.

Key words: parasitoid wasps, *Trissolcus scutellaris*, *Trissolcus* festivae, *Telenomus chloropus*, *Trissolcus viktorovi*, Slovenia, biological control, Pentatomidae

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1 UVOD S POUDARKOM NA JAJČNIH PARAZITOIDIH

Biotično varstvo rastlin spada med pomembnejše sestavne dele integriranega varstva rastlin, pri katerem uporabljamo nekemične in ciljno specifične metode zatiranja škodljivcev gojenih in samoniklih rastlin. Zaradi stalnega zmanjševanja števila registriranih fitofarmacevtskih sredstev je v zadnjih 30 letih v Evropi in s tem tudi v Sloveniji vse več poudarka na biotičnem zatiranju rastlinskih škodljivcev (Trdan et al., 2020, 2023). V Sloveniji je za potrebe biotičnega varstva rastlin trenutno na voljo 38 koristnih organizmov. Gre za vrste, ki so v Sloveniji domorodne in so uvrščene na Seznam domorodnih vrst organizmov za biotično varstvo rastlin (Seznam,…2024).

Jajčni parazitoidi so pomembna skupina naravnih sovražnikov. Zaradi njihovega posebnega življenjskega kroga jih lahko uporabljamo za biotično zatiranje nekaterih gospodarsko pomembnih vrst rastlinskih škodljivcev (Bohinc in sod., 2015). Parazitoidne ose, ki smo jih v Sloveniji prvič našli v letih 2021 in 2022, pripadajo rodovoma *Trissolcus* in *Telenomus*, ki sta sestavni del družine Scelionidae. V slednji najdemo veliko vrst jajčnih parazitoidov. Rod *Trissolcus* je eden večjih v družini Scelionidae, saj vanj uvrščamo prek 160 različnih vrst. Samice odlagajo svoja jajčeca v jajčeca njihovih gostiteljev, z vsebino katerih se prehranjujejo ličinke parazitoidov. Na ta način vplivajo na zmanjšanje števila odraslih osebkov rastlinskih škodljivcev. Gospodarski pomen jajčnih parazitoidov je velik, žal pa se v svetu v sistemih biotičnega zatiranja rastlinskih škodljivcev še vedno premalo uporabljajo. Zato so potrebne še nadaljnje raziskave na tem področju (Petrov, 2013).

V prispevku predstavljamo štiri vrste jajčnih parazitoidov, in sicer *Trissolcus scutellaris* (Thomson, 1861), *Trissolcus viktorovi* Kozlov, 1968, *Trissolcus festivae* (Viktorov, 1964) in *Telenomus chloropus* (Thomson, 1861), ki so v Evropi domorodne. Nekatere vrste jajčnih parazitoidov iz obeh rodov so bili doslej že uspešno uporabljene v klasičnem biotičnem varstvu različnih škodljivcev. Tako se na primer vrsta *Trissolcus basalis* (Wollaston, 1858) uporablja za zatiranje stenice *Nezara viridula* (Linnaeus, 1758). S tem namenom so iz Japonske v Združene države Amerike in v Avstralijo vnesli tudi vrsto *T. chloropus*, ki jo v nadaljevanju predstavljamo v tem prispevku, vendar se ta parazitoidna osica na celinah ni obdržala (Jones, 1988; Haye in sod., 2015).

Jajčni parazitoidi iz rodov *Trissolcus* in *Telenomus* so generalisti, ki so razvili učinkovito strategijo, ki jim omogoča preseči odziv jajčec različnih vrst gostiteljev, s čimer jih lahko uspešno parazitirajo. Tako ima osica *T. chloropus* 21 različnih vrst gostiteljev iz 13 različnih rodov. Posledično strokovnjaki sklepajo, da bi lahko bili jajčni parazitoidi, ki napadajo evropske ščitaste stenice (Pentatomidae), potencialno učinkoviti tudi pri zatiranju tujerodne marmorirane smrdljivke (*Halyomorpha halys* [Stål, 1855]) (Haye in sod., 2015).

Eden od najbolj obetavnih načinov zatiranja stenice *Halyomorpha halys* je prav biotično varstvo, zlasti uporaba jajčnih parazitoidov (Leskey in sod., 2012). Navedena invazivna tujerodna stenica je bila v Sloveniji prvič najdena spomladi 2017 v Šempetru pri Gorici (Rot in sod., 2018). Od takrat se na ozemlju Slovenije intenzivno preučuje domorodna favna naravnih sovražnikov ščitastih stenic in rezultate teh raziskav so tudi najdbe štirih jajčnih parazitoidov, ki jih predstavljamo v nadaljevanju. Poznavanje domorodnih jajčnih parazitoidov v Sloveniji je namreč pri nekaterih vrstah (če so namreč tudi sestavni del pozitivnega seznama Evropske in sredozemske organizacije za varstvo rastlin) začetek njihove implementacije v sisteme biotičnega zatiranja rastlinskih škodljivcev (Trdan et al., 2020, 2023; Rot in sod., 2022).

2 MATERIALI IN METODE

Zastopanost jajčnih parazitoidov ščitastih stenic (Pentatomidae) smo v obdobju 2014-2022 spremljali na območju celotne Slovenije. V rastni dobi smo vzorčili potencialno parazitirana jajčna legla različnih vrst ščitastih stenic na različnih vrstah gojenih rastlin (zelenjadnice, poljščine, sadno drevje). Nabrana jajčna legla smo prenesli v insektarije Laboratorija za entomologijo na Oddelku za agronomijo Biotehniške fakultete v Ljubljani. Izletele parazitoidne osice smo shranili v 70 % etanol. Determinacijo parazitoidov je opravil samostojni raziskovalec Peter Neerup Buhl na Danskem, z uporabo določevalnega ključa Talamas in sod. (2017).

3 REZULTATI S PREDSTAVITVIJO ŠTIRIH VRST JAJČNIH PARAZITOIDOV ŠČITASTIH STENIC

3.1 *Trissolcus scutellaris* (Thomson, 1861)

To parazitoidno osico smo v Sloveniji prvič našli na njivi z zeljem (*Brassica oleracea* L. ssp. *oleracea* convar. *capitata* [L.] Alef.) v Škocjanu pri Kopru (45.539396, 13.764718). Vzorčenje je bilo izvedeno 22. julija 2021 na njivi s številčno populacijo pisane stenice (*Eurydema ventralis* Kolenati, 1846).

Rod *Trissolcus* Ashmead predstavlja enega od večjih taksonov družine Scelionidae. Njegovi predstavniki se prehranjujejo z jajčeci stenic, dvokrilcev, metuljev in nekaterih drugih redov žuželk (Bohinc in sod., 2015). V svetu je bilo do leta 2013 opisanih več kot 160 vrst, od tega 50 v Evropi. Na Balkanu je bilo najdenih in opisanih 19 vrst (Petrov, 2013; Fauna Europaea, 2023). Sem spada tudi parazitoidna osa *Trissolcus scutellaris* (slika 1). Ta parazitoidna osa se pojavlja po celotnem območju Palearktika (Fauna Europaea, 2023) in spada med bolj razširjene predstavnike svojega rodu (Vastilia in sod., 2020).

O tej vrsti poročajo iz Avstrije, Bolgarije, Hrvaške, Francije, Italije, Grčije, Rusije, Nemčije, Turčije, Švedske, Sirije in Španije (Talamas in sod., 2017). Ker se pojavlja na širokem geografskem območju, lahko njene telesne značilnosti variirajo (Vasilita in sod., 2020). Med vrstami iz rodu *Trissolcus* sta na območju Palearktika osici *T. scutellaris* najbolj podobni vrsti *T. vesta* Kozlov & Lê, 1893 in *T. viktorovi* Kozlov, 1968 (GBIF, 2023; Vasilita in sod., 2020). Laznik in sod. (2021) navajajo parazitoida *T. scutellaris* kot naravnega sovražnika marmorirane smrdljivke.

Leta 2013 so v dolini Delemont v Švici nastavili več kot 10 000 kontrolnih jajčnih legel stenice *Halyomorpha halys*, z namenom, da privabijo jajčne parazitoide. Na ta način so potrdili 4 vrste jajčnih parazitoidov, med drugim tudi vrsti *T. scutellaris* in *T. chloropus*. Ugotovili so, da se lahko osica *T. scutellaris* popolnoma razvije v svežih jajčecih stenice *H. halys* (Haye in sod., 2015). V Švici so odkrili vrsto *T. scutellaris* tudi v jajčecih uhate rusonožke (*Pentatoma rufipes* [Linnaeus, 1758]), potencialnega škodljivca gozdnega in sadnega drevja (Powell, 2020). Prav tako so osico *T. scutellaris* našli v jajčecih azijske žitne stenice (*Eurygaster integriceps* Puton, 1881) (Talamas in sod., 2017).

Slika 1: *Trissolcus scutellaris* (Thompson, 1861) (foto: Peter Neerup Buhl)
Figure 1: *Trissolcus scutellaris* (Thompson, 1861) (photo: Peter Neerup Buhl)

3.2 *Trissolcus viktorovi* Kozlov, 1968

Tega jajčnega parazitoida smo našli v parazitiranih jajčecih pisane stenice, nabranih 7. julija 2022 na njivah z zeljem v Orehovljah (45.89264, 13.61065) in Vrtojbi (45.9072040, 13.6172590).

Trissolcus viktorovi je vrsta jajčnega parazitoida, ki se pojavlja v Evropi. Znana je tudi pod sinonimom *Tissoleus victorovi* Kozlov, 1968. V Evropi je vrsta najbolj podobna parazitoidu *T. scutellaris* in strokovnjaki predvidevajo, da se je iz njega tudi razvila. To sklepajo zaradi podobnih telesnih značilnosti pri obeh vrstah (Vasilita in sod., 2020).

O najdbah vrste *T. viktorovi* poročajo iz Rusije, Turčije, Armenije in Portugalske. Vrsto je prvi opisal Kozlov leta 1968 (Talamas in sod., 2017), še prej pa je bila najdena in vrstno določena leta 1958 v Turčiji, kjer so jo našli v jajčecih stenice Eurydema festiva (Linnaeus, 1767) (The Ohio State University, 2023). Osica *T. viktorovi* (Kozlov, 1968) lahko parazitira več vrst stenic, med drugim tudi ščitasti stenici *Dolycoris baccarum* (Linnaeus, 1758) in *Eurydema ventralis* Kolenati, 1846 (Talamas in sod., 2017).

3.3 *Trissolcus festivae* (Viktorov, 1964)

To vrsto smo v Sloveniji prvič našli 1. junija 2014 na njivi z zeljem na Zgornji Lipnici (46.322055, 14.18.5942), kjer se je pojavljala pisana stenica.

Tako kot ostale v tem prispevku opisane vrste tudi *Trissolcus festivae* (Viktorov) spada v družino Scelionidae in ima velik pomen v biotičnem varstvu rastlin, predvsem pri zatiranju ščitastih stenic. Ličinke te parazitoidne ose se prehranjujejo z jajčeci stenic, poleg tega pa tudi z jajčeci dvokrilcev, metuljev in hroščev. Spada med solitarne idiobionte (Bohinc in Trdan, 2015). Njen sinonim je tudi *Asolcus festivae* Viktorov, 1964. O pojavu te vrste poročajo iz Irana, kjer je parazitirala kapusovo stenico (*Eurydema ornata* [Linnaeus, 1758]) (Samin in Asgari, 2012), iz Bolgarije (Petrov, 2013), Danske, Turčije in Madžarske (GBIF, 2024).

Iz Turčije poročajo o vrsti *T. festivae* kot o jajčnem parazitoidu azijske žitne stenice (*Eurygaster integriceps* Puton, 1881). Tarla in Kornoşor (2009) ugotavljata, da sta vrsti *T. festivae* in T. semistriatus (Nees, 1834) odgovorna za 87 % naravne parazitiranosti predhodno navedene vrste stenice na jugu Turčije. Obe vrsti pa se uporabljata v biotičnem varstvu azijske žitne stenice v Turčiji.

3.4 *Telenomus chloropus* (Thomson, 1861)

To vrsto parazitoidne ose smo potrdili v jajčnih leglih marmorirane smrdljivke (*Halyomorpha halys*), nabranih 21. julija 2021 v sadovnjaku na Lesnem Brdu (46.003205, 14.327141).

Jajčni parazitoid *Telenomus chloropus* (slika 2) je bil prvič opisan leta 1860, ko je bil na Švedskem najden na vrstno nedoločenem gostitelju. Takrat ga je Thompson poimenoval s *Phanurus chloropus* Thomson, 1861. Njegova sinonima sta še *Telenomus turesis* Walker, 1836 (Mineo in sod., 2010) in *Telenomus sokolovi* Mayr, 1897. Je domorodna evropska vrsta parazitoidnih osic (Samin in sod., 2010), ki jo najdemo na različnih geografskih območjih. Samin in sod. (2010) poročajo, da je bila doslej najdena v Veliki Britaniji, Franciji, Španiji, na Madžarskem in Švedskem, Ukrajini, Moldaviji, Azerbajdžanu, Armeniji, Gruziji, Kazahstanu, Rusiji, Turčiji, na Irskem, Japonskem in v ZDA. V Severno Ameriko in na Filipine so osico *T. chloropus* vnesli v okviru klasičnega biotičnega varstva (O´Connor in sod., 2013).

Viktorov (1966) poroča, da naj bi bila osica *T. chloropus* (Thomson, 1861) hiperparazitoid vrste *Trissolcus semistriatus*, našli pa so jo tudi v jajčecih zelene smrdljivke na različnih sortah soje (*Glycine max* [L.] Merr.). Uspešnost razvoja osic v jajčecih zelene smrdljivke je bila odvisna tudi od sorte soje, torej od kakovosti hrane, ki jo je zaužil škodljivec (Jones, 1985). Znano je, da ista osica parazitira tudi jajčeca drugih stenic, npr. zeleno listno stenico (*Palomena prasina* [Linnaeus, 1761] (O´Connor in sod., 2013).

Slika 2: *Telenomus chloropus* (Thomson, 1861) (foto: Peter Neerup Buhl)
Figure 2: *Telenomus chloropus* (Thomson, 1861) (photo: Peter Neerup Buhl)

4 ZAKLJUČEK

V prispevku predstavljamo štiri vrste jajčnih parazitoidov ščitastih stenic, katerim smo z najdbami na območju Slovenije potrdili domorodnost in s tem tudi njihov pomen v varovalnem biotičnem varstvu rastlin. Njihov načrtni vnos v agroekosisteme v Sloveniji namreč še ni mogoč, saj nobena od v tem prispevku predstavljenih vrst naravnih sovražnikov še ni uvrščena na Pozitivni seznam Evropske in sredozemske organizacije za varstvo rastlin (EPPO) (Trdan et al., 2020, 2023).

Na Seznamu domorodnih vrst organizmov za biotično varstvo rastlin sicer najdemo vrsto *Trissolcus* basalis (Wollaston, 1858), katere načrtni vnos v rastlinjake in na prosto je namenjen biotičnemu zatiranju zelene smrdljivke in marmorirane smrdljivke (Seznam,…2024). Ker je učinkovitost naravnih sovražnikov navadno precej manjša kot učinkovitost insekticidov, se za povečanje učinkovitosti zatiranja rastlinskih škodljivcev v zadnjem obdobju vse pogosteje predlaga sinergistični pristop, to je hkratna uporaba dveh ali več okoljsko sprejemljivih načinov zatiranja (Curk in Trdan, 2024). Med te uvrščamo tudi hkratno uporabo dveh ali več vrst naravnih sovražnikov. Potrditev domorodnosti štirih vrst jajčnih parazitoidov, predstavljenih v tem prispevku, ima zato v Sloveniji precejšen pomen v varovalnem biotičnem varstvu rastlin pred ščitastimi stenicami in potencialni prihodnji pomen v inokulativnem biotičnem varstvu rastlin.

5 ZAHVALA

Rezultati, predstavljeni v tem prispevku, so bili pridobljeni v okviru Programa strokovnih nalog s področja zdravstvenega varstva rastlin, ki ga financira Ministrstvo za kmetijstvo, gozdarstvo in prehrano RS – Uprava za varno hrano, veterinarstvo in varstvo rastlin.

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Ogorčica *Xiphinema index* Thorne & Allen, 1950, prenašalec virusa pahljačavosti listov vinske trte

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Ogorčica *Xiphinema index* Thorne & Allen, 1950, prenašalec virusa pahljačavosti listov vinske trte v vinogradih

Izvleček: Virusi na rastlinah povzročajo veliko gospodarsko škodo, saj je zdravljenje okuženih trt praktično nemogoče v poznejši fazi rasti, ali pa so uveljavljeni postopki precej dolgotrajni, prav tako proti virusom trenutno ni učinkovitega pripravka ali ukrepa. Med očem skrite prenašalce virusov spadajo tudi ogorčice – majhni organizmi , ki lahko skupaj z virusi povzročajo velike izgube pridelka. Med takšne ogorčice spada tudi vrsta *Xiphinema index*. Je glavni prenašalec virusa pahljačavosti listov vinske trte (Grapevine fanleaf virus, GFLV). Vinska trta je glavni gostitelj te rastlinsko-parazitske ogorčice. S svojim značilnim dolgim cevastim bodalom – stiletom prodre in se prehranjuje na mladih koreninicah. Okužba vinske trte z GFLV iz rodu *Nepovirus* vodi v postopno izrojevanje vinske trte in pozneje v gospodarsko nekonkurenčnost vinograda. Obvladovanje okužb z GFLV v vinogradih je večinoma omejeno na obvladovanje ogorčice *X. index*, ki pa je izjemno težavno, predvsem zaradi njihove relativno dolge življenjske dobe in prostorske razporeditve v tleh. Kot najbolj učinkovita ukrepa sta se izkazala kolobarjenje na zemljišču, kjer se pojavlja ta ogorčica in praha, a sta ta pristopa ekonomsko neprivlačna, saj je za ta ukrep potrebno pustiti zemljišče pred ponovno zasaditvijo novega vinograda brez vinske trte za več let. V prihodnosti je potrebno pozornost posvetiti področju razvoja novih, okolju prijaznejših in učinkovitejših pristopov obvladovanja virusonosnih ogorčic *X. index* in nadomestiti uporabo nematocidov.

Ključne besede: rastlinsko-parazitske ogorčice, *Xiphinema index*, nepovirusi, *Vitis* spp., GFLV, biotično zatiranje

Dagger nematode *Xiphinema index* Thorne & Allen, 1950, as virus vector of grapevine fanleaf virus

Abstract: Viruses on plants cause significant economic damage, as treating infected seedlings during later stages of growth is practically impossible. Established procedures for eliminating plant viruses from plants can be extremely challenging and time-consuming, and currently, there is no effective treatment or measure against these viruses. Among the hidden vectors of viruses are also nematodes – small organisms that can, along with viruses, cause significant yield losses. One such soil-borne nematode is *Xiphinema index*. It is the main vector of grapevine fanleaf virus (GFLV). Grapevines (*Vitis* spp.) are the main host of this plant-parasitic nematode. It penetrates and feeds on young roots with its characteristic long tubular stylet. Infected vines with GFLV from the genus *Nepovirus* lead to the gradual degeneration of the vine and, subsequently, to the economic uncompetitiveness of the vineyard. The control of GFLV infections in vineyards is largely limited to the control of the vector nematodes. However, control of *X. index* nematode is extremely difficult, mainly due to its relatively long life span and spatial distribution in the soil. Crop rotation on land where this nematode is present and set aside have proven to be the most effective measures, but these approaches are economically unattractive as they require the land to be left free of vines for several years before replanting a new vineyard. In the future, attention should be given to the development of new, more environmentally friendly and more effective approaches to control virulent *X. index* nematodes and to replacing the use of nematocides.

Key words: plant-parasitic nematodes, *Xiphinema index*, nepoviruses, *Vitis* spp., GFLV, biological control

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1 UVOD

Bolezni in škodljivci rastlin v kmetijstvu povzročajo nemalo težav. Na podlagi soočanja z manjšimi količinami oziroma izpadi pridelka ter slabšo kakovostjo plodov, prihaja do manjše konkurenčnosti na trgu. Bolezni in škodljivci rastlin lahko v najslabšem primeru privedejo tudi do popolnega uničenja rastlin. Med povzročitelji bolezni predstavljajo pomembno skupino virusi. Največ virusov med vsemi gojenimi rastlinskimi vrstami okužuje vinsko trto (*Vitis* spp.), do danes so potrdili 101 različnih vrst virusov iz 21 različnih družin (Fuchs, 2023).

Med gospodarsko pomembne viruse, ki okužujejo vinsko trto, sodi tudi virus pahljačavosti listov vinske trte (Grapevine fanleaf virus, GFLV). Virus povzroča kompleks kužne izrojenosti vinske trte (Pompe-Novak in sod., 2005; Martelli in Boudon-Padieu, 2006). Ta bolezen je razširjena v vinogradih po celem svetu in je ena od najstarejših znanih ter gospodarsko pomembnih virusnih bolezni v vinogradih. Bolezen se kaže v zmanjšanem pridelku, pridelek je slabše kakovosti, okužene trte imajo krajšo življenjsko dobo, prav tako pa so dovzetnejše na okoljske spremembe (Martelli in sod., 2008; Bashir in sod., 2015). V preteklosti pa se je že izkazalo, da je prišlo do manjše stopnje okužb trt s pepelasto plesnijo in peronosporo na trtah okuženih z GFLV v primerjavi z neokuženimi trtami (Gilardi in sod., 2020). V vinogradih bolezen prepoznamo po rumenenju in deformaciji listov, ter nepravilnem izraščanju rozg (Martelli in sod., 2008; Bashir in sod., 2015). Širjenje GFLV povzročajo ogorčice vrste *Xiphinema index* Thorne & Allen, 1950 (Taylor in Brown, 1997).Ocenjujejo, da je globalna škoda, povzročena s strani rastlinskih ogorčic od 80 do 157 milijard ameriških dolarjev letno (Abad in sod., 2008; Nicol in sod., 2011). Verjetno je številka še precej večja z ozirom na mnoge pridelovalce, zlasti v državah v razvoju, ki ne poznajo škodljivosti ogorčic. Zaradi majhnosti so ogorčice pogosto spregledane, hitro se lahko prenašajo s sadilnim materialom, mehansko ali s prenašalci, prav tako pa so lahko bolezenska znamenja, ki se ob pojavu ogorčic izražajo na rastlinah, precej nespecifični (Jones in sod., 2013).

Zastopanost rastlinsko-parazitskih ogorčic v vinogradu predstavlja eno pomembnejših težav, saj vpliva na koreninski sistem vinske trte, kar se navadno odraža v manjši masi pridelka, v nekaterih primerih prihaja tudi do popolne izgube pridelka. V več študijah so ocenili, da rastlinsko-parazitske ogorčice v vinogradništvu povzročajo letne globalne izgube pridelka tudi do 12,5 % samo pri namiznem grozdju (Sasser in Freckman, 1987; Smiley, 2005). Škoda, ki jo povzročajo ogorčice se razlikuje glede na dejavnike, kot so tip tal, sorta, podnebje in tehnologija pridelave rastlinske vrste (Ferris in McKenry, 1974; Baginsky in sod., 2013).

Po podatkih EPPO sta tako GFLV kot njegov prenašalec ogorčica *X. index,* v Evropski uniji uvrščena na seznam nadzorovanih nekarantenskih škodljivih organizmov na vinski trti.

2 VIRUSI VINSKE TRTE

Vinska trta je ena od najbolj dovzetnih rastlinskih vrst za virusne okužbe. Več kot 101 virusov, ki pripadajo različnim družinam in rodovom, lahko okužujejo vinsko trto (Fuchs, 2023). Število virusov, ki okužujejo vinsko trto, pa se neprestano povečuje (Miljanić in sod., 2022). Okužbe vinske trte z virusi veljajo za gospodarsko najbolj uničujoče (Šutić in sod., 1999). Med virusi, ki okužujejo vinsko trto, največ škode povzročijo virusi iz rodov *Nepovirus*, *Closterovirus*, *Ampelovirus*, *Maculavirus*, *Vitivirus* in *Foveavirus* (Fauquet in sod., 2005). Virusna okužba navadno sistemsko prizadene vsa tkiva, z občasno izjemo apikalnega meristema, cvetnega prahu in semena. Pri rastlinah, kjer apikalni meristem ni okužen, je eliminacija virusa možna z mikropropagacijo (izrez meristema in gojenje v tkivni kulturi). Prav tako lahko zdrav sadilni material pridobimo s tehniko termoterapije, krioterapijo in mikrograftingom (Pompe-Novak in sod., 2005; Marković in sod., 2015; Miljanić in sod., 2022). To je še posebno pomembno pri pojavu sistemskih okužb pri tradicionalnem vegetativnem razmnoževanju. Sistemsko širjenje virusnih okužb gre pripisati cepljenju, saj se z okuženim sadilnim materialom virusi in viroidi vinske trte hitro prenašajo in širijo povsod po svetu (Jackson, 2020). Problem nastane predvsem v matičnih vinogradih, ki jih vinogradniki uporabljajo za namen pridobivanja cepičev. V kolikor je tak vinograd okužen z virusi, se lahko le ti hitro širijo s sadilnim materialom (Miljanić in sod., 2022). Tako se na večje razdalje virusi prenašajo z okuženim sadilnim materialom, znotraj vinograda ali nekega območja, pa se virusi prenašajo mehansko ali z vektorji – žuželkami, pršicami, kaparji in ogorčicami (Martelli in sod., 2017; Miljanić in sod., 2022). Do mehanskega prenosa virusov prihaja z opremo za rez, traktorji in raznimi stroji, zato je pomembno pogosto površinsko razkuževanje opreme (Jackson, 2020). Rast vinske trte in končni pridelek sta odvisna od zdravega koreninskega sistema in njegove sposobnosti črpanja ter absorpcije vode in hranil iz tal (Meza in sod., 2012).

Bolezenska znamenja okužbe virusov na vinski trti se odražajo zelo različno. Na to vplivajo različne podnebne razmere, letni čas, starost vinograda in njegova lega, lastnost oziroma tip tal, sorta vinske trte in gojitvena oblika ter vrsta rezi. Vsekakor pa imajo velik vpliv virusi, njihovi različki ali kombinacije različnih virusov, ter kako dolgo je vinska trta z virusom okužena (Mannini in sod., 2012). Virusne okužbe se med sabo razlikujejo z bolezenskimi znamenji in glede na vrsto povzročiteljev in prenašalcev (Martelli in sod., 2008).

Poznamo štiri glavne virusne okužbe vinske trte (Martelli in Boudon-Padieu, 2006; Martelli in sod., 2008; Fuchs, 2020):

- kompleks bolezni razbrazdanosti lesa vinske trte (rugose wood complex),
- kompleks kužne izrojenosti vinske trte (grapevine fanleaf degeneration-decline complex),
- kužna marmoriranost vinske trte (grapevine fleck complex) in
- kompleks predčasnega rdečenja in zvijanja listov vinske trte (grapevine leafroll disease).

Beseda nepovirus je izpeljana iz angleškega izraza "NEmatode-transmitted virus with POlyhedral particles", kar pomeni da so prav ogorčice prenašalci teh virusov, ki parazitirajo višje organizme. Za viruse je značilno, da jim gostiteljske celice zagotavljajo gradnike, energijo in encime ter posledično omogočajo njihovo razmnoževanje (Taylor in Brown, 1997). Prenašalci virusov iz rodu *Nepovirus* so talne ogorčice (Bovey in sod., 1980) iz rodov *Xiphinema* in *Longidorus* (Urek in Hržič, 1998). Med najpomembnejša predstavnika povzročiteljev bolezni kompleksa kužne izrojenosti vinske trte uvrščamo virus pahljačavosti listov vinske trte (GFLV) in virus mozaika repnjaka (ArMV) (Martelli in Boudon-Padieu, 2006).

2.1 VIRUS PAHLJAČAVOSTI LISTOV VINSKE TRTE (GFLV)

GFLV uvrščamo v rod *Nepovirus*, ki spada v družino Secoviridae in poddružino Comovirinae (Hao in sod., 2018; Fuchs, 2020). Okužba vinske trte z GFLV lahko povzroči zmanjšanje pridelka grozdja tudi do 80 %, vpliva pa tudi na kakovost plodov in skrajša življenjsko dobo vinske trte s hitrim odmiranjem mladih rastlin ali postopnim propadanjem – kužno izrojevanje (Andret-Link in sod., 2004; Hao in sod., 2018).

Med najpogosteje izražena in značilna bolezenska znamenja na listih vinske trte, okužene z GFLV, štejemo pahljačavost listov, njihovo nepravilno nazobčanost (izgled petršiljavosti), široko odprtost sinusov ob listnem peclju, klorozo žil in tudi medžilnih prostorov, nagubanost listne ploskve ter rumenenje in zvijanje listov. Rozge so lahko deformirane, pogosto se razdvojijo (bifurkacije), členki so precej krajši kot pri neokuženih trtah, stebla mladik so lahko sploščena in se prav tako rumeno obarvajo. Pri okuženih trtah z GFLV lahko prihaja do motenj cvetenja, cvetni nastavki so lahko krajši in razbarvani. Bolezenska znamenja ob okužbah se lahko izrazijo tudi pozneje pri grozdih, saj so na okuženih trtah grozdi in prav tako jagode majhni, jagode lahko odpadejo že takoj po cvetenju, grozdi se osipajo, jagode nepravilno zorijo, slabša je njihova kakovost (Pearson in Goheen, 1998; Pompe-Novak, 2005; Bashir in sod., 2015; Jackson, 2020). Značilen je grmičast izgled trt, te hitreje propadajo, njihova življenjska doba je krajša (Pearson in Goheen, 1998; Pompe-Novak in sod., 2005). Okužene trte imajo zmanjšan potencial cepljenja in ukoreninjenja. Kako virusna okužba vpliva na rastlino je odvisno od tolerantnosti sorte in okoljskih razmer. Tolerantne sorte so manj prizadete v primeru okužbe, medtem ko občutljive sorte kažejo postopno izrojevanje. Kljub temu velja GFLV za enega od škodljivejših povzročiteljev virusnih bolezni vinske trte (Jackson, 2020; Bertazzon in sod., 2023).

Thorne in Allen sta leta 1958 prvič dokazala povezavo med GFLV in ogorčico *Xiphinema index* (Taylor in Brown, 1997). Hewitt in sod. (1958) so s prenosom okuženih ogorčic vrste *X. index* v predhodno steriliziran substrat potrdili, da je prav ogorčica *X. index* prenašalec GFLV, saj se jevinska trta, posajena v ta substrat okužila z GFLV (Martelli, 1978). Zaradi dolgotrajnega pojavljanja virusa v Evropi in tolerantnosti prostoživečih severnoameriških vrst vinske trte na okužbo z virusom, se domneva, da GFLV izvira iz Evropskega prostora ali Bližnjega vzhoda. Njegova trenutna razširjenost je posledica cepljenja in razpršenosti evropskih sort po vsem svetu (Jackson, 2020). Poleg ogorčice *X. index* je kot znani prenašalec GFLV tudi ogorčica *X. italie* Meyl, 1953 (Cohn in sod., 1970), ki pa se v vinogradih v Evropi zaenkrat pojavlja precej redkeje.

3 VIRUSONOSNE OGORČICE *Xiphinema index* THORNE & ALLEN, 1950

Virusonosna ogorčica *Xiphinema index* ima ozek krog gostiteljev. Najpogosteje se pojavljajo na vinski trti in njenih podlagah. Gostiteljice so lahko še figa (*Ficus carica* L.), vrtnice (*Rosa* spp. L.) in citrusi (*Citrus* spp. L.) (Nicholas in sod., 2007), njeno prisotnost pa so v Italiji potrdili tudi na murvi (*Morus* spp. L.) (Siddiqi, 1974). Taylor in Brown (1997) poročata, da so gostitelji te ogorčice lahko tudi pistacija (*Pistacia vera* L.) in nekatere druge lesnate rastline. V Sloveniji je bila ogorčica *X. index* prvič potrjena leta 1978 (Hržič, 1978), kasneje so bile potrjene vsakoletne najdbe v sklopu zdravstvenih pregledov vinogradov (Urek in Širca, 2005). Tako ogorčica *X. index* in GFLV sta po doslej znanih in pridobljenih podatkih omejena le na vinorodno deželo Primorsko (Širca in Theuerschuh, 2020; Mavrič Pleško in sod., 2023).

Za ogorčice iz rodu *Xiphinema* je značilno dolgo cevasto bodalo – stilet, ki služi za vbadanje in izsesavanje tkiva mladih koreninic. S tem, ko številne vrste iz tega rodu prebadajo celične stene, da prodrejo do rastlinskega soka, lahko na koreninicah opazimo nekroze in nabrekline. Z vbodom bodala v rastlinsko celico začnejo ogorčice črpati rastlinski sok. Črpanje poteka s stikanjem oziroma utripanjem požiralniške razširitve (ezofagalni bulbus). Ko ogorčice črpajo rastlinski sok se iz njih izločajo snovi skozi požiralnikove žleze. V rastlinsko tkivo te snovi prehajajo v stanju mirovanja, ko ogorčice prenehajo s stiskanjem bulbusa in vbadanjem bodala v tkivo. Prav v tej fazi pride do vnosa virusa iz ogorčic v rastlino (Urek in Hržič, 1998). V nasprotju z ogorčicami koreninskih šišk so ogorčice z bodalom migratorne in se prehranjujejo s celicami povrhnjice v bližini vrha korenine (Van Zyl in sod., 2011). Intenzivno hranjenje lahko povzroči deformacijo korenin (tvorbo šišk), čemur sledi potemnitev. Obsežne poškodbe vodijo v odmiranje korenin in povzročijo nastanek šopov stranskih korenin. Poleg uničevanja korenin je vrsta *X. index* najpomembnejši prenašalec virusa pahljačavosti listov vinske trte (GFLV) (Jackson, 2020). Prav povezava med ogorčico *X. index* in GFLV je bila dokazana kot prvi prenos nepovirusa med ogorčico in vinsko trto (Hewitt in sod., 1958, v Urek in Širca, 2005). Skupno delovanje obeh, tako ogorčic kot virusnih okužb lahko hitro povzroči tržno nekonkurenčnost vinograda. Druge vrste ogorčic iz rodu *Xiphinema* lahko prenašajo vrsto drugih, a manj nevarnih virusov vinske trte (Jackson, 2020). Tako ličinke kot odrasli osebki ogorčic *X. index* lahko pridobijo in prenesejo GFLV na zdrave trte ali na trte, ki so že okužene z drugimi virusi (Taylor in Raski, 1964). GFLV se ohranja v prenašalcih še vsaj devet mesecev (Taylor in Robertson, 1970; Demangeat in sod., 2004).

V raziskavi Ozturka in sod. (2018) je bila populacija ogorčice *X. index* večja v vinogradih z bolj peščenimi tlemi. Prav tako se je število ogorčic povečalo jeseni in spomladi po padavinah. Bolezenska znamenja so manj vidna pri okuženih starejših trtah, saj bolezenska znamenja prikrijejo in si lažje opomorejo. Predvsem vinogradi z mlajšimi trtami in v začetku rastne dobe so dovzetnejše za okužbe. Do hujših izbruhov bolezenskih znamenj virusne okužbe lahko pride v prestrukturiranih vinogradih, torej kjer je bila vinska trta že predhodno posajena in so jo nadomestili z novimi cepljenkami (Širca in Theuerschuh, 2020).

Virusi se v ogorčicah zadržujejo na različnih mestih. GFLV, ki ga prenašajo ogorčice iz rodu *Xiphinema*, povezujejo s povrhnjico, ki obdaja lumen osnovnega dela bodala (odontofor) in požiralnik (Sanfaçon, 2020). Nepovirusi se namreč zadržujejo v sprednjem delu prebavil virusonosnih ogorčic (lumen požiralnika in bodala) (Taylor in Robertson, 1970; Brown in sod., 1995; Wang in sod., 2002; Širca in Urek, 2016).

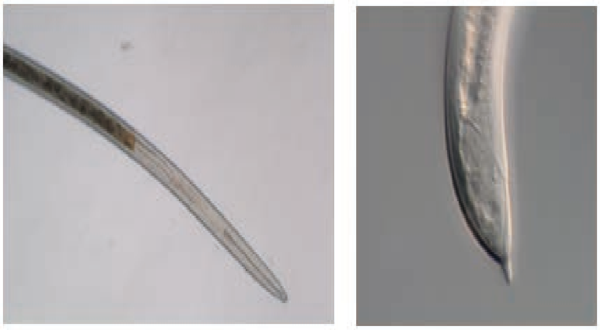
3.1 MORFOLOGIJA

Ogorčica *X. index* je bistveno daljša kot večina drugih rastlinsko-parazitskih ogorčic (doseže namreč tudi 3 mm v dolžino) (Jones in sod., 2013). Ogorčice iz rodu *Xiphinema* so podolgovate, valjaste, bilateralno simetrične, lahko tudi zaobljene oblike, vrste pa se med sabo razlikujejo (Urek in Hržič, 1998). Velik vpliv na raznolikost med posameznimi ogorčicami imajo talne in podnebne razmere ter prehranske razmere ogorčic (Taylor in Brown, 1997).

Določevalni ključi, ki se uporabljajo za identifikacijo ogorčic vrste *X. index*, pri tem pa se upoštevajo značilnosti odrasle samice (Hunt, 1993; Taylor in Brown, 1997; Lamberti in sod., 2000; Lamberti in sod., 2004; Širca, 2007) so dolžina telesa, oblika glave, dolžina bodala, položaj vodilnega obroča, oblika in dolžina repa ter tip genitalnih organov samice. Prav tako se upoštevajo De Man-ova razmerja:

- a: razmerje med dolžino in širino telesa ogorčice,
- b: razmerje med dolžino telesa in dolžino požiralniškega dela (od glave do konca požiralnika),
- c: razmerje med dolžino telesa in dolžino repa,
- c': razmerje med dolžino in širino repa in
- V (%): razmerje med dolžino telesa in položajem spolne odprtine samic (dolžina od glave do spolne odprtine).

Vsi parametri so navadno podani v mikrometrih (µm), razen pozicija spolne odprtine (V) se preračuna na odstotke (%).



Slika 1: Virusonosna ogorčica *Xiphinema index* pod svetlobnim mikroskopom (foto: Polanšek J., 2023).
Figure 1: Virus-vector nematode *Xiphinema index* under a light microscope (photo: Polanšek J., 2023).

3.2 RAZVOJNI KROG

Razmnoževanje ogorčice vrste *X. index* poteka večinoma partenogenetsko oziroma nespolno (Dalmasso in Younes, 1969; Villate in sod., 2010). Razvojni krog ogorčic te vrste pa traja razmeroma dolgo, od 2 do 14 mesecev, pri čemer je hitrost razvoja odvisna predvsem od temperature tal (Taylor in Brown, 1997). Pri vrsti *X. index* poteka razvoj skozi tri razvojne stadije. Prvi stadij so jajčeca, ki so odložena spomladi in se skozi štiri razvojne stopnje ličink (juvenilna stopnja) razvijejo v tretji stadij – odrasle osebke (Dalmasso in Younes, 1969; na CABI, 2022). Na to, kako hitro se bodo jajčeca izlegla, vplivata vlaga v tleh in temperatura tal (Širca in Urek, 2016). Med razvojem se ličinke večkrat levijo. Takrat pride do menjave kutikule, bodala in povrhnjice požiralnika. Zaradi zadrževanja virusa prav v tem delu ličinke (območje bodala in notranje plasti požiralniške cevi) se virusi med zaporednimi razvojnimi stopnjami ličink ne prenašajo (Bitterlin in Gonsalves, 1986; Hunt, 1993; Brown in sod., 1994; Taylor in Brown, 1997; Širca in Urek, 2016). Zaporedne razvojne stopnje posamezne ličinke se med sabo težje ločijo. Za lažjo določitev razvojne stopnje se uporabljajo značilnosti, kot so dolžina telesa, dolžina funkcionalnega votlega sprednjega dela bodala (odontostilet) in dolžina nadomestnega odontostileta (Garau in Prota, 1977; Van Zyl in sod., 2011).

4 ŠIRJENJE IN POSLEDICE ZASTOPANOSTI OGORČIC *Xiphinema index* IN GFLV V VINOGRADIH

Aktivno širjenje ogorčic v tleh je počasno in omejeno. Prav tako se lahko pasivno ogorčice prenašajo z razmnoževalnim in sadilnim materialom. Do prenosa lahko pride z orodjem in opremo, ki se uporablja v vinogradih in trsnicah. Drug vidik širjenja pa je širjenje s prenosom zemlje z mehanizacijo, prenašalci so lahko tudi človek in živali, prenos zemlje in okuženega odpadnega materiala. Do prenosa na dolge razdalje lahko prihaja tudi z vodo (reke, potoki,…) in vetrom (Jackson, 2020; Širca in Theuerschuh, 2020). Širjenje virusa v vinogradih je počasno zaradi omejenega gibanja v tleh glavnega prenašalca virusa, ogorčice *X. index* (približno 1,5 m/letno). Čeprav se virus prenaša na druge rastline, je v poljskih razmerah omejen na vinsko trto (Jackson, 2020).

Izgube pridelka, ki jih povzroča GFLV so odvisne od virulence virusa, občutljivosti sorte vinske trte in okoljskih dejavnikov (Bovey in sod., 1980; Martelli in Savino, 1990). Okužba vinske trte z GFLV spremeni tudi kakovost grozdja, znatno se zmanjša vsebnost sladkorjev in vsebnost kislin v grozdnih jagodah (Andret-Link in sod., 2004). Prav tako se letna ocena izgube pridelka zaradi ogorčic po vsem svetu giblje okrog 14,5 % (Abd-Elgawad in Askary, 2015). Ocenjujejo, da je le na območju pokrajine Champagne v Franciji GFLV prizadel okrog 2.000 hektarjev vinogradov, kar je 6 % celotne vinogradniške površine na tem območju (Andret-Link in sod., 2004). Aballay in sod. (2009) poročajo, da je v Čilu na kar 48 % vinogradniških tal zastopana ogorčica *X. index* in velja za eno bolj škodljivih ogorčic. Večja kot je populacija ogorčic *X. index* v tleh (več kot 50 osebkov na liter tal), manjši je pridelek grozdja (Lamberti in Melillo, 1991). V Kaliforniji je vrsta *X. index* v vinogradniških tleh občutno zmanjšala rast korenin in mladik trte sorte ‚Colombard'. Razcvet brstov je bil zakasnjen, prav tako je bila njihova bujnost manjša kot pri rastlinah, kjer ogorčica ni bila zastopana (Anwar in Van Gundy, 1989). Rastlinam vinske trte, okulirane s 500 osebki *X. index*, je v prvem letu odpadlo 23 % listov več kot navadno, v drugem letu pa se je masa nadzemnega dela in korenin zmanjšala za 65 % oziroma 38 %, pojavnost socvetij je bila manjša za 60 %, prav tako so se grozdne jagode zmanjšale za 89 % (Kirkpatrick in sod., 1965; Boubals in sod., 1971). Auger in sod. (1992) so poročali, da je bila povprečna populacija v vinogradu 250 osebkov *X. index*/liter tal. Prav tako so potrdili okužbo z GFLV v 11-letnem nasadu namizne sorte grozdja ‚Thompson Seedless' v osrednjem delu Čila. V primerjavi z zdravimi rastlinami je prišlo do večjih odstopanj v aktivnosti fotosinteze, premeru debla in jagod, ter v pridelku. Napredek v rasti in razvoju okuženih trt sta se slabše izkazala v primerjavi z neokuženimi trtami.

4.1 OMEJEVANJE ŠIRJENJA OKUŽBE

Z uporabo zdravega sadilnega materiala, torej, da so trsi predhodno testirani v okviru zdravstvene selekcije klonov, lahko nadzorovano sadimo neokužen material (Pompe-Novak in sod., 2005). Potreben je redni nadzor trsnic in matičnih vinogradov, ki vključuje preglede in vzorčenje tako nadzemnih delov rastlin kot tudi tal. Namen nadzora je ugotoviti prisotnost virusov v cepičih oziroma trtah ter ogorčic v tleh. Na ta način se uradno potrjuje in certificira sadilni material in cepiče. Pri omejevanju širjenja okužbe z GFLV je zatiranje oziroma zmanjšanje populacije prenašalcev eden pomembnejših dejavnikov (Demangeat in sod., 2004). Preverjena je bila tudi metoda navzkrižnega varstva z drugimi virusi in pa gojenje na GFLV in *X. index* odpornih podlag. Več raziskav pa se nagiba k uporabi gensko spremenjenih rastlin vinske trte, ki so lahko na GFLV tolerantne ali celo odporne (Pompe-Novak in sod., 2005). Zaplinjevanje tal z nematocidi lahko v različni meri zmanjša številčnost prenašalcev – ogorčic, jih pa ne iztrebi (McKenry in Buzo, 1996; Jackson, 2020). Kot učinkovita strategija za zmanjšanje oz. izkoreninjenje populacije *X. index* iz tal se je obnesla najmanj 10-letna praha na zemljišču, s potrjeno zastopanostjo ogorčice (Vuittenez in sod., 1969; v Demangeat in sod., 2004), a je to v vinogradih ekonomsko neprivlačno (Demangeat in sod., 2004). Dolgo obdobje prahe je verjetno posledica preživetja ogorčic na neodstranjenih koreninah. Korenine namreč lahko v tleh ostanejo in preživijo do 8 ali več let po izruvanju trte (McKenry in Buzo, 1996; Jackson, 2020). Nematocidi so navadno manj učinkoviti zlasti v težkih tleh in na večjih globinah, kljub temu da lahko delujejo tudi kot fumiganti. Poleg tega so precej toksični in je zato njihova uporaba prepovedana v več državah zaradi možnih škodljivih učinkov in vplivov na okolje (Burrows in sod., 1998; Abawi in Widmer, 2000).

Baginsky in sod. (2013) poročajo o zatiranju rastlinsko-parazitskih ogorčic v Čilu, ki temelji na uporabi kemičnih sredstev, predvsem karbamatov in organskih fosforjevih estrov, ki jih uporabljajo enkrat ali dvakrat letno. Vendar ta način zatiranja ne vodi do želenih učinkov; populacija ogorčic se namreč v tleh ne zmanjšuje, kar lahko pripišemo ostankom nematocidov v tleh, izgubi njihove učinkovitosti zaradi pogostega namakanja ali uporabe organskih dodatkov pri različnih tipih tal in načinu vnosa (Baginsky in sod., 2013). Zatiranje ogorčic v tleh je lahko problematično predvsem z vidika večletnega preživetja jajčec v tleh v mirujočem stanju. Z uporabo fumigantov naj bi preprečili širjenje in pojavnost ogorčic. Večji učinek so imeli predvsem v kombinaciji s praho, na plitvih in peščenih tleh. Fumiganti slabše učinkujejo globlje in v ilovnatih tleh. Za uspešno zatiranje pa je zaželeno, da prodrejo vsaj 40 do 120 cm globoko, kjer se nahaja glavnina koreninskega sistema (Jackson, 2020). Kot navajajo Aballay s sod. (2004) pa lahko kot dobra alternativa kemičnemu zatiranju služi kolobarjenje in strniščni dosevki. V nekaterih vinogradih se poslužujejo uporabe antagonističnih rastlin. Te rastline lahko s svojim nematotoksičnim eskudatom korenin vplivajo na rastlinsko-parazitske ogorčice (Bello in sod., 1998) ali stimulirajo rast korenin (Birch in sod., 1993; Baginsky in sod., 2013).

Več raziskav je potekalo na podlagi preučevanja različnih vmesnih posevkov, ki pozitivno vplivajo na zmanjšanje pojavnosti in aktivnost *X. index* v tleh. Na podlagi obetavnih lončnih poskusov je bila njihova učinkovitost preverjena tudi v vinogradih. Najpogosteje so se v vinogradih izkazali posevki iz rodov *Brassica* spp., *Tagetes* spp. in *Vicia* spp. (Halbrendt, 1996; Insunza in sod., 2001; Aballay in Insunza, 2002; Aballay in sod., 2004; Tsay in sod., 2004; Pensec in sod., 2013). Alternativo kemičnemu pristopu lahko predstavljajo tudi koristni talni mikroorganizmi, ki omilijo škodo, povzročeno s strani rastlinsko parazitskih ogorčic (Pozo and Azcon-Aguilar, 2007; Schouteden in sod., 2015, v Hao in sod., 2018). Raziskave so potekale na podlagi izolatov različnih gliv in se proti *X. index* najbolj izkazale glive iz rodov *Rhizophagus* spp., *Arthrobotrys* spp. in *Trichoderma* spp. (Boosalis in Mankau, 1965; Voss in Yyss, 1990; Galper in sod., 1991; Hao in sod., 2012; Daragó in sod., 2013; Hao in sod., 2018; Wernet in Fischer, 2022). Eno od možnosti v boju proti ogorčicam *X. index* pa so raziskovali v različnih študijah z izolati bakterij. V preteklosti so se že izkazali nekateri sevi iz rodov *Bacillus* spp., *Pasteuria* spp., *Paenibacillus* spp. in *Pseudomonas* spp.. S sprva zasnovanimi *in vitro* poskusi so nato učinkovitost delovanja bakterij na *X. index* nato preverjali še z lončnimi poskusi (Sturhan, 1985; Aballay in sod., 2011; Aballay, 2012; Castaneda-Alvarez in sod., 2016; Hao in sod., 2017; Aballay in sod., 2020).

5 ZAKLJUČEK

Okužba vinske trte z virusom pahljačavosti listov vinske trte (GFLV) v vinogradih predstavlja veliko tveganje. Virus v vinogradih povzroča bolezen, imenovano kužna izrojenost vinske trte, kar vodi do velikih izgub pridelka in slabše kvalitete grozdja. Obvladovanje GFLV okužb v vinogradu je večinoma omejeno na obvladovanje ogorčic, prenašalcev tega virusa. Obvladovanje ogorčic vrste *Xiphinema index* je omejeno, predvsem zaradi njihove relativno dolge življenjske dobe in prostorske razporeditve ogorčic v tleh. Najdemo jih lahko tudi na globini več metrov. Kolobarjenje na okuženem zemljišču in zemljišče v prahi so sicer učinkoviti ukrepi, vendar so ekonomsko neprivlačni, saj je potrebno pustiti zemljišče brez vinske trte več let, preden lahko opravimo zasaditev novega vinograda.

Najučinkovitejša v boju proti ogorčicam je zagotovo preventiva. Ker se prenašajo s trtami in cepljenkami, je vsekakor potrebno zagotoviti zdrav sadilni material z rednim nadzorom in pregledi. Prav tako je možen mehanski prenos z orodjem in opremo, ki se uporablja v vinogradih, zato je potrebna pazljivost pri prehodu iz vinograda v vinograd.

V prihodnosti je potrebno pozornost posvetiti področju razvoja novih, okolju prijaznejših pristopov obvladovanja ogorčice *X. index* in nadomestiti uporabo nematocidov, ki se ravno zaradi obstojnosti in pojavnosti teh ogorčic globoko v tleh niso izkazali za učinkovite. Uporaba rastlinske biomase z biocidnim učinkom in bionematocidov na podlagi patogenih gliv ali bakterij se je že večkrat izkazala za učinkovit in okoljsko sprejemljiv pristop zatiranja. Učinkovito obvladovanje ogorčic *X. index* v vinogradih bi bilo mogoče doseči s pristopi za zmanjševanje populacije ogorčic v tleh ter hkratno uporabo pristopov za varstvo na novo zasajenih trt s pripravki na podlagi mikroorganizmov ali drugimi pripravki. Stremeti je potrebno k skrajšanju večletnega obdobja, namenjenega kolobarju/prahi pri obnovi vinograda, oz. čim bolj zmanjšati populacijo ogorčic v vinogradih in jih na ta način obvladovati.

6 ZAHVALA

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Logistična regresija in problem ločenosti

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Logistična regresija in problem ločenosti

Izvleček: Logistična regresija se uporablja za preučevanje povezanosti med binarno odzivno spremenljivko (nek dogodek se zgodi ali pa ne) in množico neodvisnih spremenljivk. Z modelom lahko napovemo verjetnosti dogodka za nove enote, pogojno na vrednosti neodvisnih spremenljivk. Poleg tega ocene parametrov modela, ki jih dobimo z metodo največjega verjetja, lahko interpretiramo kot logaritem razmerja obetov. Kadar so vzorci majhni ali dogodki, v kateri od skupin, ki določajo neodvisno spremenljivko, redki, se lahko zgodi, da algoritem po metodi največjega verjetja ne konvergira, ocene parametrov modela pa so posledično nesmiselne. Pojav se v statistiki imenuje »ločenost«. Ker programska oprema problema pogosto ne identificira, raziskovalci ločenost v praksi lahko prezrejo. Dobljeni rezultati raziskovalce lahko begajo ali pa jih napačno interpretirajo. S člankom zato želimo: motivirati uporabo logistične regresije za preučevanje povezanosti binarne odzivne spremenljivke z množico neodvisnih spremenljivk; bralcem predstaviti problem ločenosti na konkretnem primeru; pokazati, kako problem ločenosti premostiti.

Ključne besede: logistična regresija, metoda največjega verjetja, majhni vzorci, redki dogodki, ločenost, Firthov tip penalizacije

Logistic regression and the problem of separation

Abstract: Logistic regression is used to study the relationship between a binary outcome variable (one event may occur or not) and a set of covariates. Individualized prognosis can be obtained by estimating the probability of an event given the covariates. Moreover, regression coefficients, usually estimated by the method of maximum likelihood, can be interpreted as the log odds ratios. In situations where the data are small or sparse, the likelihood maximization algorithm may fail to converge, leading to implausible parameter estimates. In statistics, this situation is known as 'separation'. In practice, separation may go unnoticed due to software limitations in identifying the problem. The results obtained from such analyses can be puzzling and may be misinterpreted. Therefore, in this manuscript, we aim to: motivate the use of logistic regression to study the relationship between a binary outcome and a set of covariates; demonstrate the problem of separation with a real-data example; and show how to overcome separation.

Key words: logistic regression, maximum likelihood, small datasets, sparse datasets, separation, Firth's penalized likelihood

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1 UVOD

Raziskovalci se v svojih raziskavah pogosto osredotočajo na preučevanje binarnih odzivnih spremenljivk, ki lahko zavzamejo le dve možni vrednosti – nek dogodek se zgodi ali pa ne. Tipični primeri iz biotehnike so: okuženost rastline z neko boleznijo (rastlina je okužena ali ne); zdravstveno stanje živine (prisotnost ali odsotnost bolezni); kalivost semen na določenih tleh (seme kali ali ne); učinkovitost posega, npr. gnojenja, pesticidov, s katerim dosežemo nek rezultat ali ne; prisotnost ali odsotnost določene fenotipske značilnosti; dovzetnost za zdravljenje (se odziva na zdravljenje ali ne); status preživetja (preživetje ali smrt).

V tovrstnih raziskavah nas pogosto zanima povezanost med binarno odzivno spremenljivko ter eno ali več neodvisnimi spremenljivkami. V primeru, ko je neodvisna spremenljivka opisna, podatke lahko preprosto analiziramo z računanjem deležev dogodka v vsaki od dveh ali več skupin, ki določajo neodvisno spremenljivko. Tak pristop postane problematičen, ko je neodvisna spremenljivka številska ali ko želimo opisati povezanost med odzivno spremenljivko ter več neodvisnimi spremenljivkami hkrati (Harrell, 2015), kar je tipično na primer v genetiki, kjer so neodvisne spremenljivke lahko številne genetske različice (npr. polimorfizem posameznega nukleotida – SNP). Raziskovalci tako pogosto številske spremenljivke kategorizirajo, kar privede do nepotrebne izgube informacije in pristranskosti, povezanost med odzivno in neodvisnimi spremenljivkami pa ovrednotijo za vsako neodvisno spremenljivko posebej, kar ne upošteva kombinacije hkrati prisotnih genetskih in okoljskih vplivov. To vodi do izgube moči pri odkrivanju pomembnih zvez med odzivno in neodvisnimi spremenljivkami.

Za analizo binarnih odzivnih spremenljivk je tako mnogo bolj primerna uporaba modela logistične regresije, v katerem je upoštevana informacija o vseh neodvisnih spremenljivkah hkrati, ki so lahko tako opisne kot tudi številske. Ocene parametrov modela, ki jih interpretiramo kot logaritem razmerja obetov, lahko dobimo z metodo največjega verjetja. V aplikacijah logistične regresije, predvsem v analizah majhnih vzorcev ali kadar so dogodki (v kateri od skupin, ki določajo neodvisno spremenljivko) redki (sparse data), pa se lahko zgodi, da algoritem po metodi največjega verjetja ne konvergira, ocene parametrov modela pa so posledično nesmiselne. Ta problem je v statistiki poznan kot »ločenost« (separation). Navadno nastane kot posledica (pre)majhnega števila enot v vzorcu, čeprav se lahko pojavi tudi pri analizah večjih vzorcev (van Smeden in sod., 2016). Ker programska oprema problema pogosto ne identificira, raziskovalci ločenost v praksi lahko prezrejo, in posledično rezultate analize povsem napačno interpretirajo. S člankom tako želimo: 1.) motivirati uporabo logistične regresije za preučevanje povezanosti binarne odzivne spremenljivke z množico neodvisnih spremenljivk; 2.) bralcem predstaviti problem ločenosti na konkretnem primeru; 3) pokazati, kako problem ločenosti premostiti.

2 LOGISTIČNA REGRESIJA

V praksi bi pogosto radi ocenili povezanost med naborom neodvisnih spremenljivk $X = \{X_1, X_2, \dots, X_K\}$ z binarno odzivno spremenljivko Y. Vrednosti, ki jih lahko zavzame Y lahko zapišemo kot 1 ali 0, pri čemer bomo z $Y = 1$ označili pojav dogodka, ki nas zanima. V najpreprostejšem primeru je v podatkih le ena neodvisna spremenljivka (preučevani dejavnik), ki prav tako zavzema le dve vrednosti. Takšne podatke lahko strnemo v kontingenčno tabelo dimenzije 2 × 2:

		X	
		0	1
Y	0	f_{00}	f_{01}
	1	f_{10}	f_{11}

(1)

pri čemer so f_{00}, f_{01}, f_{10}, f_{11} absolutne frekvence različnih kombinacij vrednosti spremenljivk X in Y. Tovrstne podatke bi lahko analizirali z računanjem deležev dogodkov (relativnih frekvenc) v obeh skupinah, ki določata X, in tako ocenili verjetnost proučevanega dogodka pogojno na X: verjetnost dogodka v skupini $X = 0$, $P^0 = P(Y=1|X=0)$, bi ocenili kot $f_{10}/(f_{00}+f_{00})$, verjetnost dogodka v skupini $X = 1$, $P^1 = P(Y=1|X=1)$, pa kot $f_{11}/(f_{01}+f_{11})$. Vpliv X na Y bi potem lahko kvantificirali kot razliko verjetnosti $P^1 - P^0$ ali pa kot razmerje verjetnosti P^1/P^0 (Agresti, 1990).

Tovrstna analiza postane problematična, kadar so neodvisne spremenljivke številske ali pa ko želimo opisati povezanost Y z več neodvisnimi spremenljivkami X hkrati. Zato se za analizo binarnih odzivnih spremenljivk raje uporablja model logistične regresije, ki omogoča vključitev večjega števila neodvisnih spremenljivk ne glede na tip spremenljivk. Model je formuliran v smislu verjetnosti proučevanega dogodka pogojno na vrednosti neodvisnih spremenljivk:

$$P(Y=1|X) = \frac{\exp(\beta_0 + \beta_1 X_1 + \beta_2 X_2 + \cdots + \beta_k X_K)}{1 + \exp(\beta_0 + \beta_1 X_1 + \beta_2 X_2 + \cdots + \beta_k X_K)},$$

(2)

pri čemer je β_0 presečišče, $\beta_1 \beta_{2,\dots,} \beta_K$ pa so regresijski parametri za neodvisne spremenljivke $X_1, X_2, \dots, X_k$. Model lahko zapišemo tudi v smislu logit transformacije verjetnosti $P(Y=1|X) = P$, ki je logistični regresiji dala ime. V tem primeru definiramo t. i. obete kot razmerje verjetnosti $P / (1 - P)$. Model za logaritem obetov je linearna kombinacija neodvisnih spremenljivk:

$$\text{logit}(P(Y=1|X)) = \text{logit}(P) = \log\left(\frac{P}{1-P}\right) = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \cdots + \beta_K X_K. \quad (3)$$

Regresijski parameter β_k lahko interpretiramo kot razliko logaritma obetov ob povečanju X_k za eno enoto ter ob upoštevanju ostalih spremenljivk v modelu. Lažе pa parametre interpretiramo v smislu $\exp(\beta_k)$, ki predstavlja razmerje obetov dveh enot, ki se razlikujeta le za eno enoto dane neodvisne spremenljivke X_k, medtem ko imata enake vrednosti ostalih neodvisnih spremenljivk. Kot tak je model logistične regresije primeren za dosego katerega koli od treh statističnih ciljev modeliranja: za opisovanje povezanosti nabora neodvisnih spremenljivk z odzivno spremenljivko Y; za ocenjevanje vpliva preučevanega dejavnika na odzivno spremenljivko Y ob upoštevanju ostalih spremenljivk v modelu; ter za napovedovanje verjetnosti dogodka za nove enote, pogojno na vrednosti neodvisnih spremenljivk (Shmueli, 2010).

V primeru kontingenčne tabele dimenzije 2 × 2 (1) lahko model logistične regresije zapišemo kot

$$\text{logit}(P(Y=1|X=0)) = \text{logit}(P^0) = \log\left(\frac{P^0}{1-P^0}\right) = \beta_0$$

$$\text{logit}(P(Y=1|X=1)) = \text{logit}(P^1) = \beta_0 + \beta_1.$$

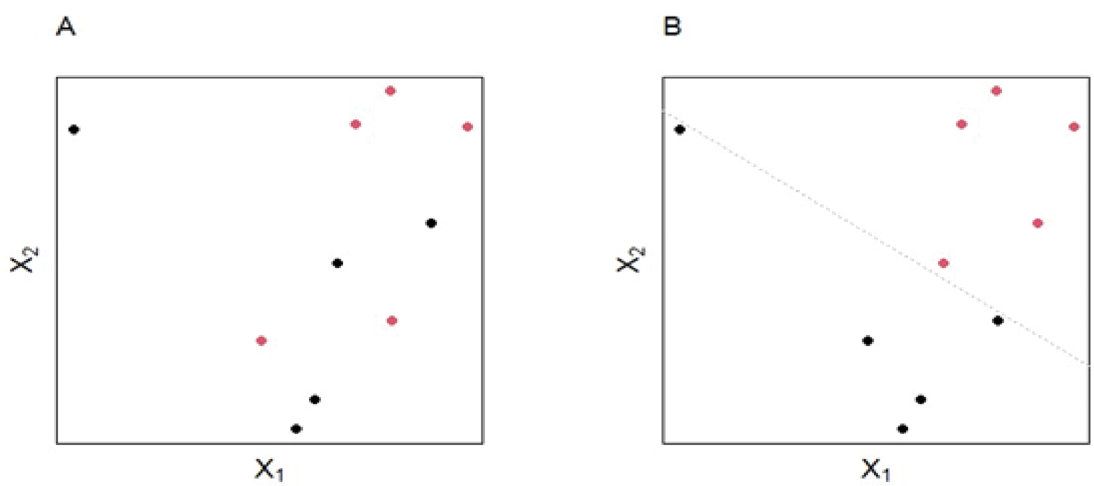
V danem primeru je β_0 logaritem obetov za Y= 1, ko X= 0, in β_1 razlika logaritma obetov, ko X= 1 v primerjavi z X= 0. Torej,

$$\beta_1 = \text{logit}(P^1) - \text{logit}(P^0) = \log\left(\frac{P^1}{1-P^1}\right) - \log\left(\frac{P^0}{1-P^0}\right) = \log\left(\frac{\left(\frac{P^1}{1-P^1}\right)}{\left(\frac{P^0}{1-P^0}\right)}\right).$$

Parametre modela logistične regresije po navadi ocenjujemo z metodo največjega verjetja. Za primer kontingenčne tabele dimenzije 2 × 2 (1) je ocena za regresijski parameter β_1 enaka

$$\hat{\beta}_1 = \log\left(\frac{f_{00} f_{11}}{f_{01} f_{10}}\right). \quad (4)$$

V večini primerov pa enačba zaprte oblike za ocenjevanje parametrov ne obstaja, ocene pa dobimo z uporabo iterativnih algoritmov; v praksi se pogosto upora-



Slika 1: Grafična ponazoritev situacije brez ločenosti (A) ter popolne ločenosti (B), ki nastane kot posledica linearne kombinacije dveh neodvisnih številskih spremenljivk X_1 in X_2. Črne in rdeče točke predstavljajo različne vrednosti odzivne spremenljivke Y, $y_i \in \{0,1\}$.
Figure 1: Graphical representation of situation without separation (A) and complete separation (B) by variables X_1 and X_2. Black and red circles represent different levels of the outcome Y, $y_i \in \{0,1\}$.

blja Newton-Raphsonova metoda (Newtonova metoda, 2023).

3 PROBLEM LOČENOSTI

Privlačnim lastnostim logistične regresije navkljub, ko je vzorec zadosti velik in naš model pravilno specificiran, pa ocene, dobljene z metodo največjega verjetja, lahko postanejo vprašljive. V primeru, ko je eden od dogodkov v kateri od skupin, ki določajo neodvisno spremenljivko, redek, kadar so vzorci majhni ali ko obstajajo močne povezave med odzivno spremenljivko Y ter neodvisnimi spremenljivkami X, se namreč lahko zgodi, da ocene parametrov po metodi največjega verjetja ne obstajajo, saj funkcija logaritma verjetja narašča v neskončnost. Tovrstno situacijo imenujemo ločenost (separation), saj sta na podlagi ene neodvisne spremenljivke ali linearne kombinacije več neodvisnih spremenljivk vrednosti odzivne spremenljivke Y ločeni. Čeprav problem pogosteje nastane, ko so neodvisne spremenljivke opisne, pa to ni izključno, temveč ločenost lahko povzroči tudi neodvisna številska spremenljivka oz. linearna kombinacija le-teh, kot je prikazano na Sliki 1B. Geometrijsko gledano ločenost torej nastane, ko v prostoru obstaja hiperravnina, ki vrednosti odzivne spremenljivke Y bodisi popolnoma loči (popolna ločenost, complete separation) (Slika 1B) ali pa jih popolnoma loči z izjemo nekaterih točk, ki ležijo točno na ravnini (kvazi-popolna ločenost, quasi-complete separation). To pomeni, da dobimo na podlagi neodvisnih spremenljivk v modelu popolne napovedi bodisi za vse enote v vzorcu bodisi za del enot v vzorcu (Albert in Anderson, 1984).

4 PRIMER: USPELOST PRIDOBIVANJA PODLAG ZA CEPLJENJE KONOPLJE

Globalni trg konoplje se širi (Rasera in sod., 2021), kar prinaša zahteve po izboljšanju agronomske prakse za povečanje učinkovitosti proizvodnje (García-Tejero in sod., 2019). Ena od novejših metod za pridobivanje sadik konoplje je cepljenje, starodavna tehnika vegetativnega razmnoževanja. Ta vključuje združitev vsaj dveh delov rastline – koreninskega sistema (podlage) in nadzemnega dela (cepiča), ki skupaj tvorita cepljeno rastlino (Salehi-Mohammadi in sod., 2009). Bitežnik in sod. (2024) so za konopljo prilagodili standardno dvo-stopenjsko metodo cepljenja, uporabljeno pri vrtnarskih kulturah. Študija je preučevala vpliv različnih podlag na stopnjo preživetja, morfološke parametre in biokemijsko sestavo ženskih socvetij konoplje.

V članku se bomo osredotočili na uspelost pridobivanja podlag za cepljenje konoplje (*Cannabis sativa* L.) (Bitežnik in sod., 2024). Odzivna spremenljivka Y (Uspelost podlage) zavzema dve vrednosti: podlaga se ukorenini, kar bomo označili z $Y = 1$, ali pa ne, $Y = 0$. V raziskavi so obravnavali naslednje podlage: sejance dvodomne sorte industrijske konoplje ‚Carmagnola' (CAR) in ‚Tiborszallasi' (TIB) ter sejance slovenske populacije dvodomne konoplje ‚Gorička Simba' (SIM). V poskusu je bilo 20 ponovitev za vsako podlago, skupno torej 60 podlag, pridobljenih iz sejancev. Obravnavali so tudi s potaknjenci razmnožene genotipe sort CAR, SIM in TIB, pri čemer je bil vsak genotip zastopan v 50 ponovitvah.

Za ilustracijo bomo primer sprva nekoliko poenostavili ter zanemarili razlike med sortami. Recimo, da nas zanimajo le razlike med uspelostjo pridobivanja podlag s potaknjenci razmnoženih rastlin v primerjavi s sejanci. Imamo torej eno neodvisno spremenljivko X (Sejanec), pri čemer bo $X = 1$ podlaga, pridobljena iz sejancev, ter $X = 0$ podlaga, pridobljena iz potaknjencev. Podatki so prikazani v Tabeli 1.

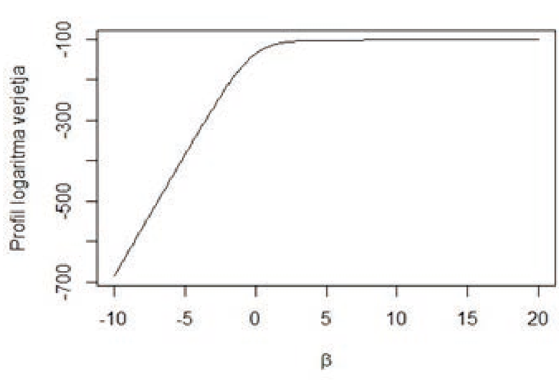
Tabela 1: Podatki o uspelosti pridobivanja podlag glede na potaknjence in sejance v študiji Bitežnika in sod., 2014.
Table 1: Data on the rootstock rooting success using seedling or stem cutting, as reported in Bitežnik et al., 2014.

		Sejanec (X)	
		Ne ($X = 0$)	Da ($X = 1$)
Uspelost podlage	Ne ($Y = 0$)	64	0
(Y)	Da ($Y = 1$)	86	60

Podatki pričajo o močni povezanosti med Y in X, kar povzroči, da je ena od štirih celic kontingenčne tabele (1) prazna, $f_{01} = 0$: uspešne so bile vse podlage, ki so bile pridobljene iz sejancev. Ocena za verjetnost uspelosti podlage za sejance po metodi največjega verjetja je enaka P^1=60/60=1. Vseeno pa nas v analizah pogosto zanima tudi inferenca – radi bi kvantificirali in interpretirali vpliv neodvisne spremenljivke X na odzivno spremenljivko Y. Kot je razvidno iz enačbe (4), ocena regresijskega parametra $\hat{\beta}$=log((64·60)/(0·86)) ni definirana. Da torej lahko ocenimo parametre modela logistične regresije, je potreben dodaten pogoj – ocenjene verjetnosti morajo biti na intervalu (0, 1), izključujoč 0 oz. 1. Ta pogoj je smiseln, saj v praksi nikoli ne predpostavljamo, da lahko na podlagi ene ali več neodvisnih spremenljivk popolnoma napovemo nek dogodek v populaciji.

V primeru ločenosti podatkov se lahko zgodi, da iterativni algoritmi za ocene parametrov logistične regresije, implementirani v različni programski opremi, dajo različne ocene parametrov ter njihovih standardnih napak. Analiza z R-ovo (R Core Team, 2022) funkcijo glm da $\hat{\beta}$ = 18,27 z ogromno standardno napako s_{β} = 842,07. Bega to, da program pri tem ne javi nobene napake in izgleda, kot da je algoritem po Newton-Raphsonovi metodi konvergiral. Neizkušen raziskovalec bi tako lahko poročal nesmiselne ocene razmerij obetov za ukoreninjenje podlage iz sejanca glede na podlago iz potaknjenca, na primer exp (18,27) > 999,999 z zelo širokim 95 % Waldovim intervalom zaupanja exp ($\hat{\beta} \pm 1{,}96 \cdot s_{\beta}$) = (0,001; > 999,999). IBM-ov SPSS (verzija 27) da oceno $\hat{\beta}$= 20,91 s standardno napako 5188,89. V kolikor izpis natančneje preučimo, SPSS za razliko od R-a raziskovalca opozori, da algoritem ni našel končne rešitve, saj se je ustavil preden je konvergiral, ko je bilo doseženo maksimalno število dvajsetih iteracij (`Estimation terminated at iteration number 20 because maximum iterations has been reached. Final solution cannot be found.`).

Čeprav oba programa vrneta oceno za parameter β, ki naj bi maksimirala logaritem verjetja, razlike v ocenah nastanejo zaradi drugačnih definicij konvergence; konvergenca je načeloma dosežena takrat, ko je sprememba ocen parametrov v naslednji iteraciji »minimalna«. V praksi spremembe ocen parametrov iz iteracije v iteracijo skoraj nikoli ne bodo natanko nič, temveč se v vsakem naslednjem koraku logaritem verjetja veča, čeprav po možnosti le malenkostno. Zato algoritem potrebuje definicijo minimalne spremembe, ki mora biti po absolutni vrednosti manjša od ε, ki pa je v različnih programih različno definiran (npr. 10^{-5}, 10^{-8}, 10^{-10}). Razlike v definicijah so po navadi irelevantne, v primeru ločenosti pa velike spremembe v ocenah parametrov privedejo do le majhne spremembe logaritma verjetja. Situacija je



Slika 2: Funkcija profila logaritma verjetja za logaritem razmerja obetov β spremenljivke X (Sejanec), dobljena s fiksiranjem vrednosti za presečišče, ki je v primeru ločenosti skoraj ravna za znaten obseg možnih vrednosti za β.
Figure 2: Profile log likelihood function for the log odds ratio β of X (Seedling), obtained by fixing the intercept, which is almost flat over a vast range of possible values for β.

ponazorjena na Sliki 2, kjer logaritem verjetja sicer monotono narašča, a je skoraj raven za znaten obseg možnih vrednosti za β; tako na primer sprememba β s 5 na 15 ne izboljša prileganja modela v smislu logaritma verjetja, ravnost funkcije pa povzroči, da je standardna napaka ogromna in Waldov interval zaupanja za $\hat{\beta}$ izjemno širok, neinformativen in za prakso neuporaben, tako rekoč raztezajoč se od minus do plus neskončno (Mansournia in sod., 2018).

5 REŠITEV − FIRTHOVA PENALIZACIJA

Pri reševanju problema ločenosti je bistveno, da raziskovalec problem prepozna. Včasih statistični program raziskovalca na težavo opozori. V primeru podatkov o konoplji funkcija `glm` v `R`-u ni dala nobenega opozorila, lahko pa se pojavi sporočilo, da so ocenjene verjetnosti enake 0 ali 1 (`glm.fit: fitted probabilities numerically 0 or 1 occurred`). IBM-ov SPSS nas je po drugi strani opozoril, da je bilo doseženo maksimalno število iteracij, preden je funkcija konvergirala. Bistveno je, da ločenosti ne moremo odpraviti z večanjem števila iteracij. V splošnem velja, da ima ločenost za posledico velike ocene parametrov ter ogromne standardne napake bodisi za presečišče bodisi za neodvisne spremenljivke, ki ločenost povzročajo. Če je katera od teh spremenljivk opisna spremenljivka, kot priročno diagnostično orodje služi tabelni pregled podatkov: frekvenca nič v kateri koli celici kontingenčne tabele pomeni ločenost. Načeloma lahko problem sami prepoznamo tudi na podlagi napovedanih verjetnosti, ki bodo v primeru ločenosti vsaj za nekatere enote zelo blizu (ali enake) 0 ali 1.

Včasih lahko problem ločenosti razrešimo s smiselno revizijo podatkov. Tipični strategiji modeliranja, ki pogosto privedeta do ločenosti, sta kategorizacija številskih spremenljivk ali klasifikacija nominalnih spremenljivk v preštevilne razrede. Kadar je to smiselno, lahko kategorije za nominalne spremenljivke združimo.

V situacijah, ko ločenosti ne moremo preprosto odpraviti z revizijo podatkov, kot v primeru o konoplji, solidno rešitev problema ponujajo metode, ki modificirajo funkcijo verjetja. V kolikor logaritem verjetja, ki ga maksimiramo, rahlo modificiramo tako, da mu dodamo penalizacijski člen, lahko ocene parametrov skrčimo proti 0 in tako preprečimo, da gredo v smeri proti ±∞. Motivacija za frekventistične metode penalizacije je bodisi zmanjševanje pristranskosti bodisi zmanjševanje srednje kvadratne napake ocen. Te metode pa lahko motiviramo tudi s perspektive Bayesove statistike, ki obstoječe podatke združi z neko apriorno informacijo o porazdelitvi parametrov, ki po navadi ne predvideva ekstremnih vrednosti parametrov (Greenland in Mansournia, 2015).

V tem članku bomo predstavili Firthov tip penalizacije, ki je bil sprva motiviran z vidika zmanjševanja pristranskosti ocen parametrov v posplošenih linearnih modelih (Firth, 1993). Heinze in Schemper (2002) pa sta pokazala, da ponuja tudi dobro rešitev za problem ločenosti v logistični regresiji tako, da napovedane verjetnosti potegne stran od skrajnosti 0 in 1 proti 0,5. Firthov tip logistične regresije je implementiran v vseh glavnih statističnih programih: R, SAS, Stata, Statistica. V `R`-u model Firthove logistične regresije lahko ocenimo z uporabo funkcije `logistf` iz paketa `logistf` (Heinze in sod. 2022). Funkcija `STATS_FIRTHLOG` v SPSS-u prav tako bazira na `R`-ovi funkciji `logistf`. Na splošno je v primeru, ko lahko podatke strnemo v kontingenčno tabelo dimenzije 2×2 (1) ocena za β po Firthu enaka oceni po metodi največjega verjetja po tem, ko smo vsaki frekvenci v štirih celicah tabele dodali 0,5. V našem ilustrativnem primeru je ocena za $\hat{\beta}$ = log ((64,5·60,5)/(0,5·86,5))= 4,5 s standardno napako 1,43. Za namen statističnega sklepanja Heinze in Schemper (2002) predlagata uporabo intervalov zaupanja, ki temeljijo na profilu funkcije verjetja (profile likelihood confidence intervals) ter zaradi asimetričnosti omogočajo boljšo pokritost ocen v primerjavi z Waldovimi intervali zaupanja, ki so simetrični. V našem primeru je 95 % interval zaupanja za (2,53; 9,35), kar izključuje vrednost 0, torej je vpliv Sejanca na Uspelost podlage močno statistično značilen. Z modelom tako ocenjujemo, da ima podlaga, pridobljena iz sejanca, exp($\hat{\beta}$) = 90-krat večje obete, da se ukorenini, kot pa podlaga, pridobljena iz potaknjenca; pripadajoči 95 % interval zaupanja za razmerje obetov je (exp(2,53); exp(9,35)) = 12,6; 11498,8) kar nedvomno priča o tem, da bo podlaga, pridobljena iz sejancev, po vsej verjetnosti zares uspela.

V kolikor pa nas poleg ocen parametrov zanimajo tudi napovedane verjetnosti, je pri uporabi Firthove logistične regresije potrebna previdnost, saj so napovedane verjetnosti pristranske napram verjetnosti 0,5. Firthova logistična regresija namreč penalizira tudi oceno za presečišče. Za premostitev problema so Puhr in sod. (2017) predlagali metodo FLIC, ki popravi oceno za presečišče tako, da napovedane verjetnosti postanejo

Tabela 2: Rezultati analize podatkov o uspelosti pridobivanja podlag za cepljenje konoplje (Bitežnik in sod., 2024) na podlagi modela logistične regresije (5) z neodvisnima spremenljivkama Sejanec in Sorta ter z njuno interakcijo, ki jih dobimo po metodi največjega verjetja ter s Firthovim tipom penalizacije.
Table 2: Analysis results on the rootstock rooting success (Bitežnik et al., 2024) based on the logistic regression model (5), including seedling, variety and their interaction as covariates, which were obtained by the method of maximum likelihood and Firth's penalized likelihood, respectively.

Metoda	Spremenljivka	β̂	Standardna napaka	95 % interval zaupanja	*p*-vrednost	Razmerje obetov
Metoda največjega verjetja	Presečišče	−0,08	0.28	(−0,63; 0,47)	0.777	
	Potaknjenec CAR – referenca					1,00
	Potaknjenec SIM	0,93	0,42	(0,11, 1,75)	0,03	2,53
	Potaknjenec TIB	0,24	0,40	(−0,55, −1,03)	0,55	1,27
	Sejanec CAR	18,65	1458,51	(−2840, −2877)	0,99	125286193
	Interakcija SIM	−0,93	2062,64	(−4044, −4042)	1	0,40
	Interakcija TIB	−0,24	2062,64	(−4044, −4042)	1	0,79
Firthov tip penalizacije	Presečišče	−0,08	0.28	(−0,63; 0,47)	0.779	
	Potaknjenec CAR - referenca					1,00
	Potaknjenec SIM	0,91	0,41	(0,11, 1,74)	0,026	2,48
	Potaknjenec TIB	0,24	0,40	(−0,54, 1,02)	0,523	1,27
	Sejanec CAR	3,79	1,46	(1,71, 8,66)	<0,001	44,35
	Interakcija SIM	−0,91	2,07	(−6,19, 4,38)	0,665	0,40
	Interakcija TIB	−0,24	2,06	(−5,52, 5,04)	0,909	0,79

nepristranske, medtem ko so ostale ocene parametrov enake kot pri Firthovi logistični regresiji.

Zdaj poglejmo rezultate analize podatkov o konoplji, pri čemer poleg spremenljivke Sejanec upoštevamo tudi spremenljivko Sorta s tremi kategorijami (CAR, SIM in TIB). Ker ima spremenljivka Sorta tri ravni, bo model vključeval dve umetni spremenljivki (dummy variables), ki sta dihotomni: vrednosti bodo torej enake 0 razen za sorto SIM pri X_1 oz. za sorto TIB pri X_2 bodo 1. Spremenljivka $X_3 = 1$ za sejance in $X_3 = 0$ za potaknjence. Poleg glavnih vplivov nas bo zanimalo tudi, ali obstaja interakcija med Sorto in Sejancem (torej, ali je vpliv sejanca drugačen glede na sorto). Naš model, na podlagi katerega lahko izračunamo razmerje obetov za 4 kombinacije vrednosti Sejanec in Sorta, zdaj zapišemo kot

$$\text{logit}(P) = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_1 X_3 + \beta_5 X_2 X_3, \quad (5)$$

kjer je potaknjenec CAR referenčna kategorija, β_1 in β_2 sta logaritma razmerja obetov za potaknjenca SIM oz. TIB glede na potaknjenec CAR, β_3 za sejanec CAR glede na potaknjenec CAR, β_4 in β_5 pa sta razliki logaritma razmerja obetov med sejancem SIM oz. TIB ter sejancem CAR.

Ocene parametrov modela (5), ki vključuje dva dejavnika ter njuno interakcijo, po metodi največjega verjetja ter z uporabo Firthovega tipa penalizacije so podane v Tabeli 2. Med metodama ni večjih razlik pri ocenah logaritma razmerij obetov za potaknjenca SIM in TIB. Zaradi ločenosti, saj je bila uspelost pridobivanja podlage za cepljenje konoplje za vse tri sejance 100 %, ocena logaritma razmerja obetov za sejanec CAR po metodi največjega verjetja ne obstaja. Statistični program R nam vrne neznačilne ocene razmerij obetov, ki pa se raztezajo tako rekoč od ±∞. Prav tako so tudi standardne napake za ocene interakcij ogromne. Po drugi strani Firthov tip penalizacije potrjuje, da ima spremenljivka Sejanec pomemben vpliv na uspelost pridobivanja podlage, in vrne močno značilno ($p < 0,001$) razmerje obetov za sejance sorte CAR v primerjavi s potaknjenci sorte CAR s precej asimetričnimi 95 % intervali zaupanja. Interakciji v modelu nista statistično značilni, kar nakazuje na to, da je vpliv Sejanca podoben ne glede na Sorto.

Pri načrtovanih poskusih modela navadno ne bi poenostavljali. V opazovalnih študijah pa bi se za voljo interpretabilnosti rezultatov raje odločili za bolj parsimoničen model, če sta ta dva enakovredna pri pojasnjevanju povezanosti med neodvisnimi spremenljivkami in odzivno spremenljivko. S testom razmerja verjetij z dvema stopinjama prostosti tako na primer lahko preverimo, da interakcija v modelu ni potrebna (p=0,17), ni torej pomembnih razlik med sejanci glede na sorto: vpliv sejanca je za vse sorte podoben. Model (5) tako lahko poenostavimo:

$$\text{logit}(P) = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3, \quad (6)$$

kjer je potaknjenec CAR referenčna kategorija, in sta logaritma razmerja obetov za potaknjenca SIM oz. TIB, pa kvantificira vpliv sejanca ob upoštevanju sorte. Rezultati modela (6), ocenjenega po Firthovi metodi, so podani v Tabeli 3.

Iz analize torej lahko sklenemo, da imajo potaknjenci sorte SIM 2,48-krat večje obete za uspelost podlage kot potaknjenci sorte CAR, pripadajoči 95 % interval zaupanja je (1,11; 5,67), medtem ko med potaknjenci sorte TIB in potaknjenci sorte CAR ni statistično značilnih razlik (p = 0,523). Primerjavo med sortama SIM in TIB lahko dobimo tako, da v modelu sorto SIM vzamemo

Tabela 3: Rezultati analize podatkov o uspelosti pridobivanja podlag za cepljenje konoplje (Bitežnik in sod., 2024) na podlagi modela logistične regresije (6) z neodvisnima spremenljivkama Sejanec in Sorta, ki jih dobimo po metodi največjega verjetja ter s Firthovim tipom penalizacije.
Table 3: Analysis results on the rootstock rooting success (Bitežnik et al., 2024) based on the logistic regression model (5), including Seedling and Variety as covariates, which were obtained by the method of maximum likelohood and Firth's penalized likelihood, respectively.

Metoda	Spremenljivka		Standardna napaka	95 % interval zaupanja	p-vrednost	Razmerje obetov
Firthov tip penalizacije	Presečišče		0.28	(,63; 0,47)	0.78	
	Potaknjenec CAR - referenca					1,00
	Potaknjenec SIM	0,91	0,41	(0,11, 1,74)	0,026	2,48
	Potaknjenec TIB	0,24	0,39	(0,54, 1,02)	0,523	1,27
	Sejanec	4,54	1,41	(2,56, 9,38)	<0,001	93,38

za referenčno kategorijo. Izkaže se, da med potaknjenci sorte TIB in potaknjenci sorte SIM ni značilnih razlik (p = 0,102). Za podlage iste sorte velja, da imajo sejanci 93-krat večje obete za uspelost podlage kot potaknjenci. Pripadajoči 95 % interval zaupanja je (13; 11870), močno asimetričen, a izraža pomemben vpliv sejancev v primerjavi s potaknjenci na uspelost pridobivanja podlag za cepljenje konoplje.

6 IMPLEMENTACIJA V R-U

V nadaljevanju dodajamo kodo za implementacijo logistične regresije po metodi največjega verjetja ter s Firthovim tipom penalizacije v `R`-u. Za ponovljivost najprej generiramo podatke, ki so prikazani v Tabeli 1.

```
R> podatki <- data.frame(sejanec = c(1,1,0,0), usp_podlage = c(1,0,1,0))
R> podatki <- podatki[c(rep(1,60), rep(2,0), rep(3, 86), rep(4, 64)), ]
```

Ocene za model logistične regresije po metodi največjega verjetja dobimo s funkcijo glm.

```
R> model <- glm(usp_podlage ~ sejanec, podatki, family = „binomial")
```

Izpis povzetka modela vrne ukaz `summary(model)`:

```
Call:
glm(formula = usp_podlage ~ sejanec, family = „binomial",
    data = podatki)
Coefficients:
              Estimate Std. Error z value Pr(>|z|)
 (Intercept)   0.2955     0.1651   1.790  0.0735 .
 sejanec      18.2706   842.0690   0.022  0.9827
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
(Dispersion parameter for binomial family taken to be 1)
    Null deviance: 258.23  on 209 degrees of freedom
Residual deviance: 204.71  on 208 degrees of freedom
AIC: 208.71
Number of Fisher Scoring iterations: 17
```

Napovedane verjetnosti za enote v podatkovnem okviru izračuna funkcija `predict(model, type = „response")`.

Po tem, ko smo z ukazom `library(logistf)` naložili ustrezni paket, lahko ocene za Firthov model logistične regresije dobimo s funkcijo `logistf`.

```
R> model_firth <- logistf(usp_podlage ~ sejanec, podatki)
```

Izpis povzetka modela prikažemo s `summary(model_firth)`, v katerem so zapisani tudi 95 % intervali zaupanja za parametre modela, ki temeljijo na profilu funkcije verjetja, s pripadajočimi p-vrednostmi:

```
logistf(formula = usp_podlage ~ sejanec, data = podatki)
Model fitted by Penalized ML
Coefficients: coef  se(coef)  lower 0.95  upper 0.95    Chisq            p method
(Intercept)    0.2934792    0.1645131  -0.02713328  0.6191539  3.216735 7.288887e-02      2
sejanec          4.5023114   1.4295431  2.53155543  9.3466073 49.421415 2.064793e-12      2

Method: 1-Wald, 2-Profile penalized log-likelihood, 3-None

Likelihood ratio test = 49.42143 on 1 df, p = 2.064793e-12, n=210
Wald test = 14.58793 on 1 df, p = 0.0001337684
```

V kolikor nas poleg ocen parametrov zanimajo tudi napovedane verjetnosti, je priporočeno v funkcijo `logistf` dodati argument `flic = TRUE`.

```
R> model_flic <- logistf(usp_podlage ~ sejanec, podatki, flic = TRUE)
```

Iz izpisa povzetka modela `summary(model_flic)` lahko vidimo, da se model `model_flic` razlikuje od modela `model_firth` zgolj v oceni za presečišče. Napovedane verjetnosti dobimo z ukazom `model_flic$predict`.

Podatkom dodamo še spremenljivko sorta:

```
R> podatki$sorta <- c(rep(c(‚CAR', ‚SIM', ‚TIB'), each = 20),
                      rep(‚CAR', 24), rep(‚SIM', 35), rep(‚TIB', 27),
                      rep(‚CAR', 26), rep(‚SIM', 15), rep(‚TIB', 23))
R> podatki$sorta <- factor
(podatki$sorta)
```

Model z obema neodvisnima spremenljivkama ter njuno interakcijo naredimo z naslednjim ukazom:

```
R> model_firth_int <- logistf(usp_podlage ~ sorta*sejanec, podatki)
```

Model lahko poenostavimo tako, da iz modela izpustimo interakcijo, če ta ni potrebna:

```
R> model_firth_2 <- logistf(usp_podlage ~ sorta+sejanec, podatki)
```

Ali je interakcija v modelu potrebna ali ne, lahko preverimo s testom razmerja verjetij z dvema stopinjama prostosti:

```
R> 1 - pchisq((-2)*(model_firth_int$loglik-model_firth_2$loglik)[1], df = 2)
```

Referenčno kategorijo iz sorte CAR v sorto SIM v modelu lahko zamenjamo z ukazom:

```
R> podatki$sorta <- relevel
(podatki$sorta, ref = ‚SIM')
```

7 SKLEPI

Pri analizah podatkov z modelom logistične regresije, ki omogoča preučevanje zveze med binarno odzivno spremenljivko ter eno ali več neodvisnimi spremenljivkami, se lahko zgodi, da ocene parametrov po metodi največjega verjetja ne obstajajo. Raziskovalce dobljene ocene razmerij obetov, ki jih ponujajo statistični programi in se praktično raztezajo od ±∞, lahko begajo, saj v praksi ne domnevamo, da je vpliv neke neodvisne spremenljivke na odzivno spremenljivko zares »neskončen«. Ločenost je tako prej posledica »smole pri vzorčenju« in jo lahko odpravimo z večanjem števila enot v vzorcu (Šinkovec in sod., 2019). Nenavadno se lahko zdi tudi to, da kljub očitni močni povezanosti med odzivno ter neodvisno spremenljivko zveza med njima ni statistično značilna. To je posledica ogromnih standardnih napak, ki vodijo do povsem neinformativnih Waldovih intervalov zaupanja. V članku smo ločenost predstavili na konkretnem primeru podatkov o konoplji, opisali njene posledice ter kot metodo, ki se je skozi čas že uveljavila kot dobra rešitev problema, predlagali Firthov tip penalizacije, ki prepreči, da ocene parametrov divergirajo.

Za konec naj še poudarimo, da je ločenost le skrajni primer, ko metoda logistične regresije odpove. Močno pristranske ocene parametrov (v smeri stran od 0) z velikimi standardnimi napakami lahko dobimo tudi pri analizah podatkov, ki niso nujno povsem ločeni (Greenland in sod., 2016). Podobno kot pri ločenosti težave (t. i. sparse data bias) navadno nastanejo pri analizah vzorcev, kjer so dogodki (v kateri od skupin, ki določajo neodvisno spremenljivko) redki, ali v analizah majhnih vzorcev. Podobno kot pri ločenosti lahko te težave omilimo oz. odpravimo z uporabo Firthove penalizacije. Predvsem je pomembno to, da se raziskovalci problemov zavedajo in jih lahko prepoznajo – le tako se bodo izognili poročanju nesmiselnih rezultatov svojih analiz.

8 VIRI

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