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Ovitek: Učinki različnih koncentracij $CdCl_2$ (0, 1, 1,5, 2 in 2,5 mM) na morfologijo in rast tobaka (*Nicotiana tabacum* L.) po 21 dneh izpostavitve (Foto: Maryam KOLAHI)
Cover: Effects of different concentrations $CdCl_2$ *(0, 1, 1.5, 2 and 2.5 mM) on morphology and growth of tobacco (*Nicotiana tabacum *L.) after 21 days of exposure. (Photo: Maryam KOLAHI)*

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Effect of growth media and plant growth promoting rhizobacteria (PGPR) on growth and flowering indices of China aster

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Effect of growth media and plant growth promoting rhizobacteria (PGPR) on growth and flowering indices of China aster

Abstract: he China aster used for various purposes like bouquets preparation, flower arrangements, bedding plants, edge, and herbaceous borders in gardens, flower shows and exhibitions. The study focused on using different growth media and plant growth-promoting rhizobacteria (PGPR) to enhance the growth of Chin aster. Four types of growth media garden loamy soil (S), soil + perlite (SP), soil + cocopeat (SC), and soil + cocopeat + perlite (SCP)), with three incubation conditions: inoculation with *Bacillus subtilis* (Ehrenberg 1835) Cohn 1872 and *Pseudomonas putida* Trevisan 1889, and a control group with no bacterial incubation, each with three replicates. Regardless of the growth media used, *P. putida* resulted in taller plants than those treated with *B. subtilis*. Among the growth media tested, SCP produced the tallest plants and most axillary shoots. SCP with *P. putida* had the highest chlorophyll content and leaf area. SCP also resulted in the most flowers, especially with *P. putida*. SCP with *P. putida* had the highest leaf nitrogen content, while SC and SP with *B. subtilis* showed high leaf phosphorus and potassium levels. The findings from our study highlight that utilizing SCP as a composite growth media, with or without bacterial incubation, produced the most favorable effects on the growth and flowering indices of China aster.

Key words: *Bacillus subtilis,* coco peat, perlite, *Pseudomonas putida,* soil

Učinek gojišča in rast zvpodbujajočih rizobakterij (PGPR) na rast in cvetne indekse kitajske nebine

Izvleček: Kitajska nebina se uporablja v različne namene kot so izdeleva šopkov, cvetnih aranžmajev, kot pokrovna cvetica in za obrobe v vrtovih, za cvetlične predstave in razstave. Raziskava se je osredotočila na uporabo različnih rastnih medijev in rast vzpodbujajočih rizobakterij (PGPR) na pospeševanje rasti kitajske nebine. Uporabljeni so bili štirje rastni mediji in sicer: vrtna zemlja (S), vrtna zemlja + perlit (SP), vrtna zemlja + ostanki kokosa (SC), in vrtna zemlja + ostanki kokosa + perlit (SCP)), s tremi režimi inokulacije: inokulaciji z bakterijama *Bacillus subtilis* (Ehrenberg (1835) Cohn 1872 in *Pseudomonas putida* Trevisan 1889 in kontrola brez bakterijske inokjulacije, vse v treh ponovitvah. Ne glede na rastne medije je inokulacija z bakterijo *P. putida* dala višje rastline kot tista z bakterijo *B. subtilis*. Med preiskušenimi rastnimi mediji so bile rastline na SCP najvišje, z največ stranskih poganjkov. Rastline na SCP z bakterijo *P. putida* so imele največjo vsebnost klorofila in največjo listno površino, imele so največ cvetov in največjo vsebnost dušika v listih. Rastline na gojiščih SC in SP, inokulirane z bakterijo *B. subtilis,* so imele v listih veliko vsebnost fosforja in kalija. Izsledki raziskave pojasnjujejo, da ima uporaba SCP kot sestavljenega gojišča, z ali brez inokulacije z bakterijami, najugodnejši vpliv na rast in cvetne indekse kitajske nebine.

Ključne besede: *Bacillus subtilis,* ostanki kokosa, perlit, *Pseudomonas putida,* tla

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1 INTRODUCTION

The China aster serves multiple functions, including for creating bouquets, flower arrangements, as bedding plants, and for edging herbaceous borders in gardens, as well as being a popular choice for flower shows and exhibitions. Plant growth-promoting rhizobacteria (PGPR) were proposed by Kloepper et al. (1980) for the first time, when he used fluorescent *Pseudomonas* as a growth promoter capable of resisting plant pathogens. Since then, the term PGPR has included all rhizobacteria effective in enhancing plants growth. PGPR plays a special role by slowing down plant contagion with disease, increasing nutrient absorption, and improving seed germination and plant resistance to environmental stresses. Rhizobacteria improve plant growth and synthesize some secondary metabolites such as phytohormone, enzymes, siderophores, and antibiotics (Ahmadi et al. 2020).

Bacillus and *Pseudomonas* are bacteria genera that frequently colonize the surface of the roots and the adjacent soil in the root zone (Ahemad and Kibret, 2014). These bacteria have two effects on plant growth. They produce stimulating plant growth compounds and help to absorb essential elements such as nitrogen by biological nitrogen fixation and phosphorous by solving phosphate. They also produce hormones including auxin, cytokinin, and gibberellin and the other effect is helping plants adaptable to biotic and abiotic stresses by inducing resistance systems (Sandhya et al. 2009; Orfanoudakis et al. 2010; Arzansh et al. 2012; Asif et al. 2019). Fernandez et al. (2020) stated that the use of growth- stimulating bacteria improved shoot and root length, stem diameter, dry mass, and absorption of nutritional elements. There are various beneficial effects of PGPRs on growth parameters in different plants. These effects include increasing leaf length, width, and leaf area, as well as enhancing flowering aspects like flower length, diameter, and numbers in chrysanthemum (*Chrysanthemum morifolium* L.) (Cipriano and Freitas, 2018). PGPRs also contribute to enhancing the fresh and dry mass of plants and flowers, flower stalk characteristics, stalk length, and the number of days to flowering in hyacinth (*Hyacinthus orientalis* L.) (Kumari et al., 2016). Moreover, they can magnify bulb length, diameter, and weight in certain plants such as hyacinths (Karagöz et al., 2019), and intensify plant height, vegetative and root mass, as well as leaf numbers in buttercup (*Ranunculus asiaticus* L.) (Domenico, 2020).

Growing media are an essential part of the propagation system because rooting competency depends on the type of medium used. The growing media should be porous, uniform in texture, hold sufficient moisture, and should be well drained, which provides physical support, aeration, and water. Different types of growth media such as rock-wool, perlite, vermiculite, peat, and coconut fiber (coco peat) have been used to grow many kinds of crops (Bar-Tal et al. 2019). The use of different organic and inorganic growth media leads to the best nutrient uptake and sufficient growth, water, and oxygen holding. A good growth medium provides enough support to the plants, serves as a reservoir for nutrients and water, and improves gaseous exchange between the roots and atmosphere (Mazahreh et al. 2015). Perlite is a glassy volcanic rock with a rhyolitic composition and high water-holding capacity, but it has no buffering capacity and contains no mineral nutrients. It is a stable material, that can last for several years, and its stability is not greatly affected by acids and microorganisms (Bar-Tal, 2019). Cocopeat has been formed from the middle layers or mesocarp of the coconut fruits, which helps to absorb nutritional elements by plants (Carlile et al. 2019). Cocopeat has a high water-holding capacity and prevents from soil compaction, decreases germination time and increases seed germination uniformity (Yan and murphy, 2008).

China aster (*Callistephus chinensis* Nees) belongs to the Asteraceae family comprising 152 species, which are widespread around the world. This plant is self-pollinated, propagated by seeds. The first flowering occurs within 55-60 days after planting. There is high diversity of flower colors in this ornamental plant including white, pale yellow, pink, red, blue, and violet. The most popular flower shape is a row of petals around the yellow center, but also with some rows of petals around the yellow center (Prasanth et al. 2020). This plant species is frequently used in green spaces as a bedding plant and in bouquets and floral compositions as a cut flower. Additionally, it is valued for its medicinal attributes, purportedly possessing anti-inflammatory, antioxidant, and anti-cancer qualities. (Bhargav et al. 2018). In order to the successful cultivation of some ornamental flowers such as marigold cultivars (Maślanka and Magdziarz, 2017), East lilium (Karagüzel, 2020) and gerbera (Sirin, 2011) growing media like perlite, peat moss, and cocopeat has been applied.

There is no literature for the application of PGPRs along with growth media on China aster as a bedding plant in landscaping and green spaces. Therefore, the objective of this study was to investigate the effects of two PGPRs of *B. subtilis* and *P. putida* and growth media of soil, soil + perlite, soil + cocopeat and their combination on China aster growth, and flowering indices.

2 MATERIAL AND METHODS

2.1 STUDY AREA, PLANT CULTIVATION AND TREATMENT APPLICATION

The research was done at the Research Greenhouse of the Horticultural Science Department of the Agricultural Faculty of Zanjan University, Zanjan, Iran. The treatments included four types of growth media (soil (S), soil + perlite (SP), soil + cocopeat (SC) and soil + cocopeat + perlite (SCP)) and three types of incubation included incubation with plant growth promoting rhizobacteria, *B. subtilis* (Ehrenberg (1835) Cohn 1872 and *P. putida* Trevisan 1889*,* and no-bacterial incubation with three replicates. The seeds of Aadya cultivar were initially planted in plastic trays and later transplanted at the two-leaf stage into plastic pots. These pots were filled with various types of growing media, with one plant being placed in each pot.

At first, the plants in pots were irrigated fully. Then irrigation was once every second days. The incubation was with two types of bacteria using injection by siring with 108 colony-forming units per milliliter (CFU ml^{-1}) into the growing media of the plants in the pots.

2.2 PLANT GROWTH AND FLOWERING INDICES

The height of plant, and flower diameter were measured by digital caliper. Leaf area was determined by leaf area meter. The number of axillary shoots and flower numbers were counted manually. The parameters of height and flowering were recorded once at the end of the experiment. The number of flowers were counted on the on the axillary stems.

2.3 TOTAL CHLOROPHYLL CONTENT

Total chlorophyll is defined as the sum of chlorophyll a and b. In this method 0.1 g of fully expanded leaf fresh sample was homogenized by 10 ml acetone 80%, and was centrifuged to obtain the extract. The absorbance of the extract was read by spectrophotometer at 645 and 663 nm wavelengths. Finally, the calculation of chlorophyll a and b was using equations (1) and (2) (Arnon, 1949).

$$Chl\ a = [(12/7 \times A663 - 2/69 \times A645)] \times V/100 \times M \quad (1)$$

$$Chl\ b = [22/9 \times A645 - 4/69 \times A663)] \times V/100 \times M \quad (2)$$

where Chl was chlorophyll (mg g^{-1} FM), A663 and A645 relate to absorption rate at 663 and 645 nm wave lengths, respectively, V is the sample volume, and M is the fresh mass of the sample.

2.4 LEAF NITROGEN, POTASSIUM, AND PHOSPHOROUS

Nitrogen, potassium, and phosphorous of leaf samples were measured with Kejeldal device, flame photometer, and spectrophotometer, respectively (Tekaya et al. 2014).

2.5 STATISTICAL ANALYSIS

The experiment followed a 3× 4 factorial design within a completely randomized design with four replications. Data analysis was performed using SAS software version 9.4, and mean values were compared using the Duncan test at a significance level of 0.05. The graphs were plotted by Excel software.

3 RESULTS AND DISCUSSION

3.1 GROWTH AND FLOWER INDICES

Based on the interaction effects of growth media and type of bacteria incubation (Table 1), *P. putida* incubation increased plant height by 22.47% in soil + perlite (SP) growth media and had the biggest plant height (27.25 cm) as compared to the no-bacterial incubation treatment. Also, in soil + cocopeat + perlite (SCP) media, *P. putida* incubation and no-bacterial incubation treatment showed the same plant height and higher than *B. subtilis* incubation. *B. subtilis* incubation did not show an increasing effect on plant height in the treatments. Based on Table 1, generally, *P. putida* induced greater plant height than *B. subtilis*, and the application of SCP alone had a higher effect on plant height than other applied growth media. The application of three growth media together possibly provided better plant growth conditions and nutritional status than the other growth media treatments, which led to more longitudinal growth of the plants. An increase in plant height in media amended with different constituents has also been reported by

Singh (2013) in *Alstroemeria* and Rajera and Sharma (2017) in lily. A similar trend of increase in plant height was due to a nutrient rich media that was also recorded by Singh (2013) in tuberose plants. *P. putida* incubation has also been the most effective in plant height trait probably because pseudomonas species cause lower pH in soils than bacillus species (Ng et al. 2022), and China aster grows better in the soils with pH around 6.0 (*Indian Institute of Horticultural Research (ICAR)*). pH values will decrease due to the release of hydrogen ions because of an increase in soluble phosphate concentrations, however, the ability of pseudomonas species in phosphate solubility is higher than bacillus species (Ng et al. 2022).

SC media showed the smallest plant height (19.75 cm) regardless of the type of bacteria in comparison to the no-bacterial incubation treatment. Cocopeat as a growth media has a higher amount of water-holding capacity than perlite. Since China aster grows better in well-drained soils (*Indian Institute of Horticultural Research (ICAR)*), therefore, the possible reason for the decrease in most of the plant growth and flowering indices in SC growth media in this study might be the aeration status for the plants and the bacteria. The aeration and gas exchange between the soil and the atmosphere could be restricted for both the bacteria and the plants due to the application of SC with high water holding capacity and perhaps over-irrigating conditions. If the gas exchange rolls as a growth-reducing factor, growth parameters would be affected. Since China aster is a shallow-rooted crop, it requires irrigation at an interval of 3 to 7 days depending on soil moisture (*Indian Institute of Horticultural Research (ICAR)*), it seems that watering every two days created over-irrigation conditions in SC growth media in our research. Lee et al. (2017) and Sharma and Godara (2017) reported that flowering time, fresh mass of plant, plant height, the number of flowers per bush, the size of flowers, and the earliest time of flowering was the highest in perlite + cocopeat media.

SCP media (with or without bacterial incubation) exhibited a greater number of axillary shoots compared to other media types (Table 1). Nevertheless, the combination of *P. putida* with S media resulted in the lowest number of axillary shoots, while SC media with *B. subtilis* and without bacterial incubation also displayed lower counts of axillary shoots. Due to the leaf production by axillary shoots, which require increased nutritional elements (Bredmose, 2003), the SCP media exhibited superior plant nutritional conditions compared to other media types, resulting in a higher number of axillary shoots.

SCP with or without bacterial incubation had the highest number and diameter of flowers among the treatments (Table 1), that is because SCP media had better plant nutritional condition than the other media (Bredmose, 2003) and led to more numbers and diameters of flowers. Also, this treatment had the highest number of axillary shoots as mentioned earlier, which could be considered rational because the more axillary shoots the more flowers. Axillary shoots serve as propagation mate-

Table 1: Effect of growth media and plant growth promoting bacteria on vegetative, flowering traits and chlorophyll content of China aster

Bacteria type	Culture media	PH (cm)	NAS	FN	FD (cm)	LA (cm²)	TChl (mg g^{-1} F.M)
Without bacteria		22 .25bc	14 .50ef	13 .75de	5 .50bc	701 .80d	0 .754d
Pseudomonas putida	Soil	23 .25b	12 .00g	15 .00de	5.62bc	1063 .98b	0 .543efg
Bacillus subtilis		21 .50bc	15 .50e	15 .00de	4 .87d	577 .37f	1 .233ab
Without bacteria		23 .00b	14 .00ef	12 .75d	5 .62bc	890 .60c	0 .763d
Pseudomonas putida	Soil + perlite	27 .25a	16 .25cd	16 .50d	5 .75bc	639 .61e	0 .670e
Bacillus subtilis		23 .62ab	18 .75c	6 .25g	5 .37bcd	670 .28e	0 .616ef
Without bacteria		20 .25bc	12 .75g	11 .25ef	6 .00ab	698 .22de	0 .523g
Pseudomonas putida	Soil + cocopeat	19 .75c	16 .75cd	17 .25c	6 .00ab	534 .51f	0 .340h
Bacillus subtilis		19 .75c	12 .75g	12 .50e	4 .75d	528 .85f	0 .541efg
Without bacteria		27 .00a	23 .75b	25 .90a	6 .25ab	1121 .46ab	1 .112b
Pseudomonas putida	Soil + cocopeat + perlite	27 .00a	29 .50a	26 .00a	6 .37a	1495 .05a	1 .296a
Bacillus subtilis		23 .25b	23 .25b	23 .00b	6 .25ab	1143 .19ab	0 .863c

Mean value followed by the same letters in each column are not significantly different at 5 % level using Duncan multiple range test. PH: plant height, NAS: number of axillary shoots, FN: flower number, FD: flower diameter; LA: leaf area; TChl: total chlorophyll.

rial and give rise to flowering shoots, thereby enhancing flower production in terms of both quantity and diameter (Bredmose, 2003).

As shown in Table 1, SCP with *P. putida* had the greatest chlorophyll content (1.29 mg g^{-1} FM) and leaf area (1495.05 mm^2). SC with both bacteria led to the lowest chlorophyll content and leaf area. That could be because of the aeration condition for plant growth in this media explained earlier. Reduction in chlorophyll content is directly related to environmental stresses such as low gas exchange between plant growth media and atmosphere, salinity etc. (Aslanpour et al. 2019). Consistently across the other treatments, a similar pattern was noted for chlorophyll content and leaf area as indicated in Table 1. This correlation aligns with the concept that greater leaf area tends to be associated with increased chlorophyll content (Aslanpour et al. 2019).

3.2 NITROGEN, PHOSPHOROUS, AND POTASSIUM

As demonstrated in Figure 1, SCP with *P. putida* had the greatest leaf nitrogen (N) (2.63 mg kg^{-1}DM), SC plus *B. subtilis* induced to the greatest phosphorous (P) content (0.64 mg kg^{-1} DM) and the highest potassium (K) content attributed to SP plus *B. subtilis* (2.44 mg kg^{-1} DM). Normally, rhizobacteria help in fixing atmospheric nitrogen, and provide nutritional uptake by solubilizing phosphate and producing biologically active molecules (Arshad and Frankenberger, 1992). According to Figure 1, the trends in leaf nutrients for the treatments were uncertain and variable, making it difficult to draw definitive conclusions or provide explanations for these observations.

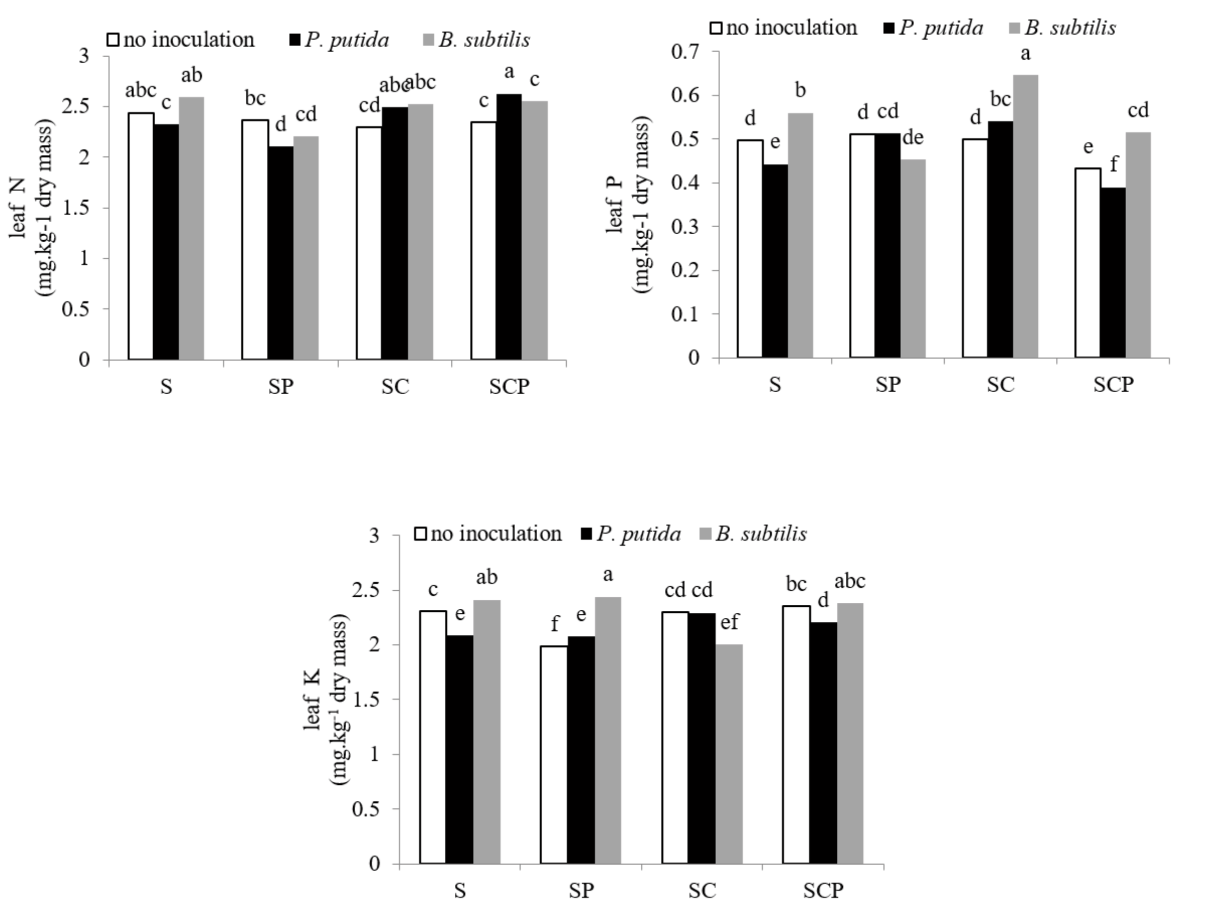

Figure 1: Effect of growth media and plant growth promoting bacteria on leaf nutrients (N, P, and K) of China aster. Mean value followed by the same letters in each column are not significantly different at 5% level using Duncan multiple range test. S: soil; SP: soil + perlite; SC: soil + cocopeat; SPC: soil + cocopeat + perlite

4 CONCLUSIONS

In summary, it can be inferred that SCP growth media, whether with or without bacterial incubation, had the most substantial influence on plant growth and flowering indices in China aster. The SCP media demonstrated superior performance in terms of plant height, number of axillary shoots, flower numbers and diameters, and leaf area.

5 DECLARATIONS

Author contributions. Conceived and designed the experiments: Arghavani, M and Aelaei, M. Performed the experiments: Mohammadi, S. Analyzed the data: Mohammadi, S and Sayyad-Amin, P. Wrote the paper: Sayyad-Amin, P, Edited the manuscript: Farahani, E and Esmaeili, S.

Compliance with ethical standards (Conflict interest): The authors declare that they have no conflict of interest.

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Improvement of germination, sprout weight and nutraceutical potential of mung bean (*Vigna radiata* (L.) R. Wilczek), sprouts by melatonin, as an elicitor

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Improvement of germination, sprout weight and nutraceutical potential of mung bean (*Vigna radiata* (L.) R. Wilczek), sprouts by melatonin, as an elicitor

Abstract: Using elicitors is one of the innovative techniques being currently applied for improving phenolic content, antioxidant capacity, and bioactive compounds in ready-to-eat sprouts. Therefore, this experiment was designed to evaluate the effect of melatonin (MT), as an elicitor, on mung bean germination and its sprout's yield and nutraceutical quality for a 5-day period during seed germination. Results showed that the processes involved on the 5 th day of germination led to an increase in total soluble protein (TSP), free amino acids (FAA), vitamin C, and antioxidant activity in the treated mung bean sprouts. In contrast, as time goes by, the rate of titratable acidity (TA), total phenolic content (TPC), flavonoid compounds, and reducing power decreased. Moreover, MT increased the percentage of seed germination (18.84 %) and sprout mass (25 %), as compared to the controls. Eliciting the seed of mung bean with MT resulted in enhancing total soluble solids (17.47 %) (TSS), TSP (23-37 %), FAA (20-38%), and reducing power (27-78%). In the sprouts of mung beans, similarly, it increased the TPC (13-28 %), flavonoid compounds (24-46 %), vitamin C (18-41 %), and antioxidant potential (13-34 %). In conclusion, using MT could change the phytochemical profiles of ready-to-eat sprouts and improve the health-promoting potential of produced sprouts during germination.

Key words: antioxidant activity, ascorbic acid, elicitation, flavonoids, legume

Izboljšanje kalitve, mase poganjkov in hranilne vrednosti kalčkov zlatega fižola (*Vigna radiata* (L.) R. Wilczek) z melatoninom kot elicitorjem

Izvleček: Uporaba elicitorjev je novejša tehnika, ki se v zadnjem času uporablja za izboljšanje vsebnosti fenolov, antioksidacijske sposobnosti in vsebnosti bioaktivnih snovi v kalčkih pripravljenih za prehrano. V ta namen je bil opravljen poskus za ovrednotenje učinka melatonina (MT) kot elicitorja na kalitev zlatega (mungo) fižola, pridelek kalčkov in njihove hranilne vrednosti v petdnevnem obdobju kalitve semen. Rezultati so pokazali, da je obravnavanje zlatega fižola peti dan kalitve vodilo k povečanju vsebnosti celokupnih topnih beljakovin (TSP), prostih amino kislin (FAA), vitamina C in antioksidacijske aktivnosti kolčkov. V nasprotju so se s časom parametri kot so titrabilna kislost (TA), vsebnost celokupnih fenolov (TPC) in flavonoidov ter redukcijska moč zmanjševali. Dodatek melatonina je povečal odstotek kalitve (18,84 %) in maso kalčkov (25 %), v primerjavi s kontrolo. Elicitiranje semen zlatega fižola z melatoninom je povečalo vsebnost topnih snovi (17,47 %), topnih beljakovin (23-37 %) in prostih amino kislin (20-38 %), a zmanjšalo redukcijsko moč (27-78 %). Podobno je obravnavanje z melatoninom v kalčkih zlatega fižola povečalo vsebnost celokupnih fenolov (13-28 %), flavonoidov (24-46 %), vitamina C (18-41 %), in antioksidacijski potencial (13-34 %). Zaključimo lahko, da bi uporaba melatonina med kalitvijo lahko spremenila fitokemični profil kalčkov pripravljenih za prehrano in s tem povečala njihov potencial za izboljšanje zdravja.

Ključne besede: antioksidacijska aktivnost, askorbinska kislina, elicitacija, flavonoidi, stročnice

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1 INTRODUCTION

Legumes are a valuable group of nutritious foods rich in carbohydrates, dietary fibers, proteins, lipids, minerals and antioxidant compounds (Lin and Lai, 2006). In legumes, mung bean (*Vigna radiata* (L.) R. Wilczek) because of its edible bean has remarkably been receiving the attention of consumers for a long time. The seed of mung beans is rich in nutrients and phytochemicals (Syed et al., 2011; Tang et al., 2014). In recent years, a great deal of research has shown that seed germination can enhance biological activities in the seed and increase its nutritional value (Shohang e al., 2012; Fouad and Rehab, 2015; Nkhata et al., 2018 Araújo et al., 2023). During the germination process, starch and complex proteins are converted into simple carbohydrates and free amino acids, and this facilitates digestive mechanisms in the human body (Nkhata et al., 2018). Also, seed germination improves the amount of vitamins, minerals, secondary metabolites and antioxidant compounds in the sprout (Xu et al., 2005; Khalil et al., 2007; Świeca et al., 2012), while reducing specific anti-nutrient compounds such as phytic acid (Khalil et al., 2007). Currently, there is increasing interest in consuming seed sprouts, either on their own or as ingredients in other products, due to their superior health benefits and nutritional composition (Ebert, 2020).

Elicitors are biotic and abiotic compounds which induced physiological changes in plants. By activating different defense mechanisms and responses, they affect the metabolism of plants and change the synthesis of phytochemicals (Baenas et al., 2014b). Accordingly, using various elicitors has reportedly improved plant growth and bioactive compounds in edible sprouts (Kim e al., 2006; Pérez-Balibrea et al., 2011; Limón et al., 2014; Świeca, 2015; Peñas et al., 2015). Treating edible chia sprouts with salicylic acid and hydrogen peroxide remarkably augments antioxidant compounds and conversely reduced obesity-related oxidative stress in laboratory mice (Gómez-Velázquez et al., 2021). Furthermore, phytohormones such as methyl jasmonate and jasmonic acid have reportedly acted as elicitors and assisted in increasing glucosinolate contents in edible *Brassica* sprouts (Baenas et al., 2014a). Melatonin (MT) is a bioactive molecule, with several biological roles in plants (Arnao and Hernández-Ruiz, 2018). In higher plants, MT is available in almost all tissues and organs. It is involved in various physiological processes such as seed germination, rooting, flower development, photosynthesis, maturity, senescence, osmotic regulation and resistance to abiotic stress (Arnao and Hernández-Ruiz, 2018). In plants, MT is served as an activator of antioxidant systems, and this mitigates the destructive effects of oxidative stress (Zhang et al., 2014; Lei et al., 2021). So far, exogenous MT has reportedly reduced chilling-induced damage, delayed aging, maintained sensory qualities and benefited nutritional values in fruits and vegetables in the postharvest stage (Jannatizadeh, 2019; Tan et al., 2020; Ma et al., 2021) and improved seed germination of *different* plant species under abiotic stressors (Zhang et al., 2014; Cao et al., 2019; Li et al., 2019; Lei et al., 2021; Yin et al., 2022). The current study aimed to determine the effects of exogenous MT, as an elicitor, on germination, sprout mass, qualitative features and nutritional characteristics of edible mung bean sprouts.

2 MATERIALS AND METHODS

2.1 PLANT MATERIALS AND TREATMENTS

One-year old seeds of mang bean (Parto cultivar) were obtained from Pakan Bazr Co. (Isfahan, Iran). Before germination, mung bean seeds were soaked in distilled water for 8 hours. The seeds were disinfected with 2 % sodium hypochlorite solution for two minutes and then washed with distilled water until a neutral pH was reached. Then, the seeds were divided into two groups: one group was placed in distilled water (as control) and another group was placed in 150 µM MT solution (elicitor solution) for 20 minutes. The concentration of 150 µM MT was selected according to preliminary experiments. The seeds were maintained at room temperature for 2 hours so that extra water would evaporate from their surface. The seeds (80 g) were placed in plastic containers and were allowed to germinate for 5 days. The conditions were made suitable for seed germination, with darkness, an appropriate temperature (25 °C) and a high level of relative humidity (80 %). In total, 30 plastic containers were used in the experiment, which included the control and MT treatments, each with 3 replications. Sampling was performed over a period of 5 days. The containers were irrigated daily with distilled water (25 ml). On each of the 5 days during the germination process, the samples were weighed (sprout mass), frozen in liquid nitrogen and stored at -80 °C for further measurements.

2.2 GERMINATION PERCENTAGE

After applying the MT treatment, which served as an elicitor, and having a control treatment, the germination rate was recorded separately. For this purpose, 5 batches of 100 seeds were considered for each treatment. The seeds were placed in Petri dishes (9 x 9 cm) containing two layers of Whatman filter paper, which were moistened with distilled water (10 ml). The Petri dishes were incubated for 5 days in the dark at 25 °C in a growth chamber (Memmert, Germany). The seeds were evaluated daily for germination, and germinated seeds were recorded. Each seed was considered as germinated when its seminal root emergence reached 2 mm. Germination rate was evaluated based on the number of germinated seeds from the total number of seeds (Peñas et al., 2015).

2.3 CHEMICAL ANALYSIS OF MUNG BEAN SPROUTS

2.3.1 Soluble solids and titratable acidity

Soluble solids (TSS) and titratable acidity (TA) were measured according to a method used by Chen et al. (2018) with slight modifications. For each treatment, a number of germinated mung beans (2 g) were randomly selected and their extract was prepared. The filtered extract was used for measuring TSS by a hand-held refractometer (N-50E, Atago, Japan), and the results were expressed as % Brix. Measuring the TA involved extracting one gram of sample using deionized water (3 ml). Then, the diluted extract (10 ml) was titrated using sodium hypochlorite (2 mM) and with phenolphthalein (1 %) as an indicator. Based on the consumed amount of solution during the titration process, the TA of the sprouts was reported as the percentage of malic acid.

2.3.2 Protein and free amino acids

The total soluble protein (TSP) was measured according to the Bradford method using bovine serum albumin as a standard (Bradford, 1976). Finally, the TSP was expressed as mg/g fresh mass. Free amino acids (FAA) were measured by the colorimetric method, using a ninhydrin reagent (Rosen, 1957). The amounts of FAA were calculated based on the glycine standard curve, and were ultimately reported as mg/g fresh mass.

2.3.3 Phenols and flavonoids

Total phenolic content (TPC) of sprouts was measured using Folin–Ciocalteu's reagent, as described by Singleton and Rossi (1965). Briefly, 2 grams of frozen sprouts were crushed using HCl-methanol-water solution (10 ml) at a ratio of (1:80:19) and placed on a shaker for 16 hours at room temperature. Then, the samples were centrifuged at 12,000 rpm for 15 minutes. The resultant extract was used for measuring TPC, flavonoids and antioxidant capacity.

Flavonoids content was determined according to a method used by Hasperué et al. (2016) with slight modifications. Accordingly, 100 µl NaNO2 (5 %) was added to 1 ml extract, and the solution was stored at room temperature for 5 minutes. Then, 50 µl $AlCl_3$ was added to the solution and, after 5 minutes, 250 µl NaOH (1M) was added. The absorbance of the solution was read at 510 nm using a spectrophotometer (Analytik Jena, Germany), and the flavonoid content was calculated based on a quercetin standard curve. The results were reported as mg g FM^{-1}.

2.3.4 Antioxidant activity

Determining the reducing power involved measuring the efficiency of extracts in reducing Fe^{+3} by relevant evaluations according to a method used by S'wieca et al. (2012). Briefly, 1 ml extract was mixed with 2.5 ml phosphate buffer (0.2 M) and 2.5 ml potassium ferricyanide (1 %). This solution was incubated for 30 minutes in a hot water bath (50 °C). Then, 2.5 ml trichloroacetic acid (10 %) (w/v) was added to the solution, and the samples were centrifuged at 3000 rpm for 10 minutes. The supernatant (2.5 ml) was mixed by 2.5 ml distilled water and 0.5 ml iron chloride (0.1 %), the absorption of samples was read at 700 nm. Higher absorption values per sample indicate greater reducing potential, which was expressed as quercetin equivalent (Q) in micrograms per gram of fresh mass.

In determining antiradical activity, each sample was evaluated in terms of free radical scavenging capacity, using the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) method. 100 µl of extract was mixed with 2900 µl of DPPH solution. Immediately, the absorbance of each sample was read at 517 nm using a spectrophotometer. Then, the samples were placed in the dark for 30 minutes, and their absorbance values were reread. Using the following equation, the antiradical activity (%) was calculated (Khang et al., 2016).

$$\text{Antiradical activity} = [(Abs_{conrol} - Abs_{sample}) / Abs_{conrol})] \times 100$$

$Abs_{extract}$ is absorbance of extracts, and Abs_{blank} is absorbance of water.

2.3.5 Ascorbic acid

Vitamin C content in mung bean sprouts was measured using 2,6-dichloroindophenol (DIP) reagent, according to a method used by Nielsen (2017) with slight modifications. Briefly, 1 gram of mung bean sprout was pulverized using 10 ml of cold metaphosphoric acid (2 %). The samples were centrifuged at 3000 rpm for 5 minutes, and the extract solution was filtered. The filtered extract was titrated with 2,6-dichloroindophenol solution until the solution began to turn pink. The ascorbic acid content was reported as mg/g of fresh samples.

2.3.6 Statistical analysis

This experiment was conducted in a completely randomized design (CRD) with factorial arrangement of treatments and 3 replications. All data were statistically analyzed by analysis of variance (ANOVA) using SAS software (version 9.1). Means comparisons were assessed by Duncan Multiple Range test at the significance level of $p < 0.05$. All experiments were performed in triplicate.

3 RESULTS AND DISCUSSION

All measured traits of the mango bean sprouts were affected following 5-day period during seed germination. The analysis of variance (ANOVA) for all traits indicates significant difference among sprouting time, MT elicitation and their interaction, except for TSS (data not shown).

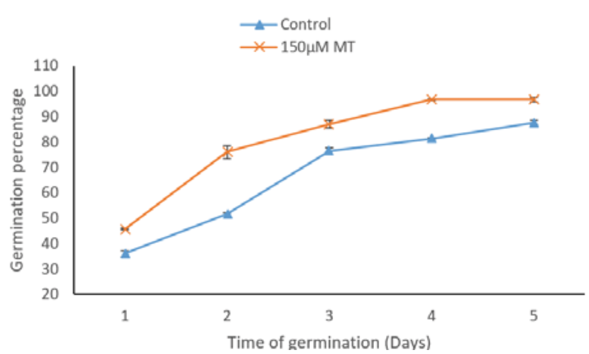

Figure 1: Impact of germination time and melatonin (MT) elicitation on germination percentage of mung bean sprouts. Error bars in the figure were standard deviations of triplicate experiments.

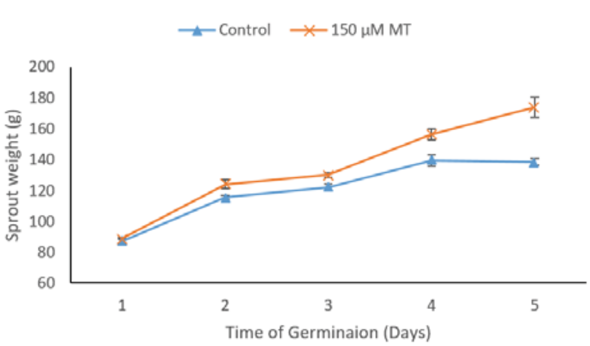

Figure 2: Impact of germination time and melatonin (MT) elicitation on weight of mung bean sprouts. Error bars in the figure were standard deviations of triplicate experiments.

3.1 EFFECTS OF MELATONIN AS AN ELICITOR ON SEED GERMINATION AND SPROUT MASS

Changes in seed germination and mass of mung bean sprouts are shown on different days of the experiment (Fig. 1 and 2). Through time, the percentage of seed germination increased and, thus, the mass of mung bean sprouts increased. Meanwhile, elicitation by MT increased the germination percentage and mass of mung bean sprouts. On the 5th day of elicitation, MT caused the sprout mass to increase by 25.57 %, compared to the control treatment (Fig. 1 and 2). Results from previous research showed that MT treatment not only increased the germination percentage, but also promoted subsequent growth when plants were exposed to environmental stresses (Li et al., 2019; Lei et al., 2021; Yin et al., 2022). The lipophilic and hydrophilic nature of MT, associated with its fluidity in crossing morpho-physiological barriers, usually result in rapid transfers and effectiveness of MT in all biological tissues (Garcia et al., 2014). Meanwhile, seed priming with MT can lead to optimal starch metabolism in seeds, thereby improving food supply in young seedlings while maintaining turgor pressure for tissue expansion during the germination process (Cao et al., 2019). MT is also known for its auxin-like activity, similar to IAA, which stimulates vegetative growth in plants (Arnao and Hernández-Ruiz, 2018). According to the results above presented, parallel to the increase in germination and initial seedling growth, the mass of sprouts also increases, and therefore, sprout production would stage higher levels of economic output.

3.2 EFFECTS OF MELATONIN AS AN ELICITOR ON TSS, TA AND VITAMIN C

Contents of TSS and TA were affected by MT and germination times treatments (Table 1). The results showed that the amount of TSS increased until the second day of germination, but then decreased significantly. On the other hand, as germination progressed, there was an increase in TA (Table 1). During the germination process, carbohydrates in the seed are metabolized into simpler sugars by digestive enzymes and are made available for the energy consumption of growing seedlings (Fouad and Rehab, 2015). There was a decrease in the TSS content of mung bean seeds after germination (Table 1), which can be explained by the consumption of sugars as an energy source for seedling growth. Results showed that on the 2th day of the experiment, elicitation by MT increased the TSS content and TA of mung bean sprouts, compared to the control (Table 1). Similarly, Bai et al. (2020) showed that cotton seeds pre-soaked with 100µM MT caused an increase in the amounts of soluble sugars in both normal and drought-stressed conditions. Another report indicated that MT regulated reserve mobilization (Lei et al., 2021) and that MT treatment increased the activity of starch hydrolyzing enzymes (α-amylase and β-galactosidase), thereby providing nutrients for seed germination (Chen et al., 2021). These results showed that MT elicitation provided energy for more efficient seed germination, which involved changing the metabolic patterns of sugars and organic acids in seeds and seedlings.

According to the results, as the germination time progressed, the vitamin C content increased significantly and reached its highest level (1.93 mg g FM^{-1}) up to the fourth day, although it decreased from the 4h to 5th day of germination. Compared to the control treatment, MT elicitation caused a significant increase in vitamin C content in mung bean sprouts on all days of germination, except the first day (Fig. 3). It has been reported that the application of exogenous MT in various species improved vitamin C as well as plant growth and development (Sardar et al., 2023; Iqbal et al., 2023). Using specific elicitors such as salicylic acid and chitosan (Pérez-Balibrea et al., 2011) and light (Xu et al., 2005) increased the vitamin C content in edible sprouts. Seemingly, the activity of key enzymes in ascorbic acid biosynthesis can be regulated by elicitors and, thus, could lead to changes in ascorbic acid content in edible sprouts (Xu et al., 2005).

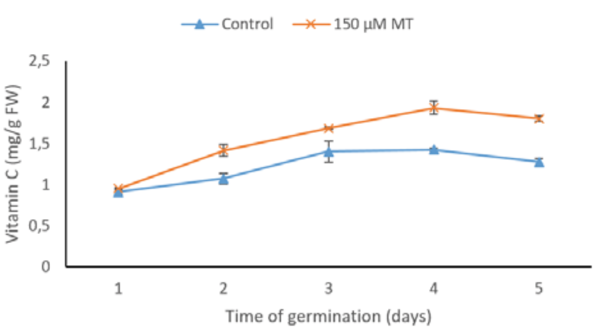

Figure 3: Impact of germination time and melatonin (MT) elicitation on vitamin C content of mung bean sprouts. Error bars in the figure were standard deviations of triplicate experiments.

3.3 EFFECTS OF MELATONIN AS AN ELICITOR ON TOTAL PROTEIN AND FREE AMINO ACIDS

Through the germination process, metabolic enzymes such as proteases are usually activated and, with the release of specific amino acids, new proteins begin to be synthesized (Diaz-Mendoza et al., 2019). As a result, sprouting may increase the nutritional quality of legumes, since the mechanism of sprouting is comprised of pathways that increase protein digestibility and increase biological value of proteins (Khalil et al., 2007; Nkhata et al., 2018). In the current research, as germination gradually progressed, especially on the 4th and 5th day, the TSP

Table 1: Influence of germination time and melatonin (MT) elicitation on total soluble solid (TSS), titratable acidity (TA), total soluble protein (TSP) and free amino acids (FAA) of mung bean sprouts.

Time of germination (Days)	Elicitor	TSS (% Brix)	TA (%)	TSP (mg g^{-1} FM)	FAA (mg g^{-1} FM)
1-day old	Control	11.33de	0.238e	8.74de	2.92e
	150 µM MT	13.00bcd	0.280e	7.78e	3.24de
2-day old	Control	13.33bc	0.293e	10.32cd	3.62cd
	150 µM MT	15.66a	0.393d	9.37cde	3.56d
3-day old	Control	13.33bc	0.413d	8.17de	3.47d
	150 µM MT	14.33ab	0.486ab	9.79cde	4.17b
4-day old	Control	10.33ef	0.433bcd	9.51cde	4.05bc
	150 µM MT	11.66cde	0.533a	13.04ab	5.59a
5-day old	Control	8.66fg	0.480abc	11.67bc	3.56d
	150µM MT	8.33g	0.426cd	14.43a	4.49b

Values are the means of three replicates, and different letters differ significantly by Duncan test. Mean significant at the $p < 0.05$ probability level.

and FAA increased significantly in mung bean sprouts (Table 1). Similarly, previous research indicated an increase in the amounts of protein and amino acids during the germination process, which may be caused by the synthesis of some enzymes (such as proteases), changes in the seeds composition or hormonal levels during seed swelling (Syed et al., 2011; Limón, et al., 2014; Fouad and Rehab, 2015).

The results showed that the MT elicitor had a significant effect on TSP and FAA of mung bean sprouts. In the early days of germination (until the 3rd day), the MT treatment had no significant effect on TSP, compared to the control. However, elicitation with MT caused an increase in TSP (37.11 and 23.65 %) on the 4th and 5th day, respectively, compared to the control. On all days of the experiment, except for the first and second days, MT-treated mung bean sprouts had higher FAA than non-elicited sprouts of the control (Table 1). This finding is in agreement with previous results by Li et al. (2019) where 200 µM of MT increased the efficiency of nutrient utilization and synthesis of new proteins to increase the germination of *Limonium bicolor* (Bag.) Kuntze seeds under salt stress conditions. Also, it has been reported that MT pretreatment improved α-amylase activity, soluble sugars and released amino acids, as well as, enhanced reserve mobilization, increased the germination rate and initial growth of wheat seeds under chromium stress (Lei et al., 2021). Therefore, it can be concluded that the MT treatment can provide energy for seed germination by changing amino acid contents and by regulating the degradation and biosynthesis of proteins.

3.4 EFFECTS OF MELATONIN AS AN ELICITOR ON PHENOLIC COMPOUNDS AND FLAVONOIDS

Phenolic compounds in plants have received much research focus because of their antioxidant properties and potential for human health (Świeca and Gawlik-Dziki, 2015). During the seed germination process, various changes occur in phenolic compounds, which depend not only on the genotype of the seed but also on the environmental conditions and germination time (Baenas et al., 2014b). The results showed that through germination time, TPC decreased (Fig. 4). The flavonoid content increased until the third day after germination and then suddenly decreased (Fig 5). Likewise, a significant decrease in phenolic compounds (e.g. tannin, phenolic acids, flavonoids and total phenolics contents) occurred in lentils seed during germination (Świeca et al., 2012). Similar conditions led to a gradual decrease in phenolic content in lentil sprouts after germination (Świeca, 2015), which are consistent with the results of the present research. In contrast, some cases of research indicated an increase in phenolic compounds in legumes during germination (Świeca and Gawlik-Dziki, 2015, Fouad and Rehab, 2015). In another relevant study, Shohag et al. (2012) reported that when the results were expressed based on fresh mass, germination caused a decrease in phenolic compounds because of the dilution effect, and on the contrary, the results were expressed based on dry mass, the phenolic content increased with the germination process.

Chemical elicitors provide a practical approach for increasing the nutritional quality and phytochemical compounds of edible sprouts (Baenas et al., 2014b). In the present research, using MT for the seed elicitation increased phenolic and flavonoid compounds in mung bean sprouts. Through the process of germination, the efficiency of elicitation increased. Accordingly, the highest MT effect on TPC and flavonoids content were observed on the fifth day of the experiment (Fig. 4 and 5). In pre-

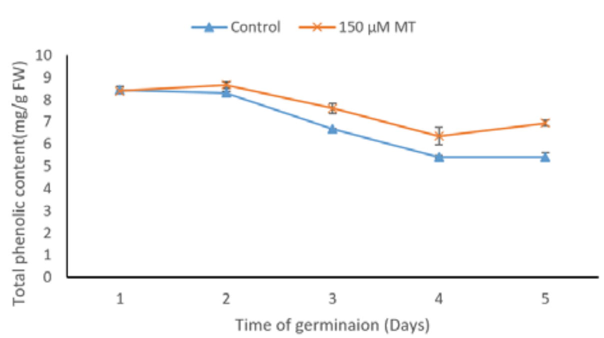

Figure 4: Impact of germination time and melatonin (MT) elicitation on total phenolic compounds of mung bean sprouts. Error bars in the figure were standard deviations of triplicate experiments.

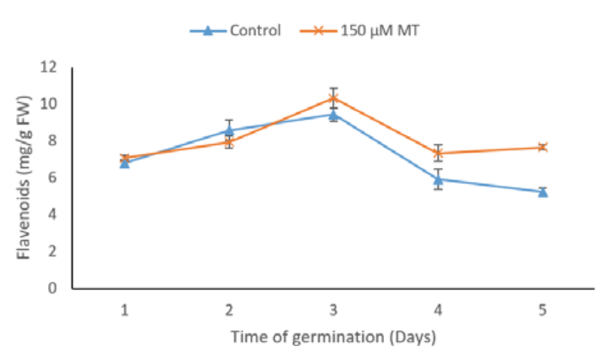

Figure 5: Impact of germination time and melatonin (MT) elicitation on flavonoids content of mung bean sprouts. Error bars in the figure were standard deviations of triplicate experiments.

vious research, several chemicals were used as elicitors, e.g. ascorbic acid, folic acid and chitosan/glutamic acid (Peñas et al., 2015), salicylic acid and hydrogen peroxide (Gómez-Velázquez et al., 2021) and methyl jasmonate (Kim et al., 2006), which enhanced the biosynthesis of phenolic and flavonoid compounds in the edible sprouts. While the current research indicated similar outcomes, Yin et al. (2022) reported that MT, can protect sprouts from NaCl stress by increasing antioxidant enzyme activities and phenolic acids accumulation (Yin et al., 2022). The application of MT in plants usually results in an accumulation of phenols by activating the phenylpropanoid pathway, which is actively associated with phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO) activities (Jannatizadeh, 2019). Also, the application of exogenous MT on barley reportedly increased gene expression and activity of phenylalanine ammonia-lyase and cinnamate-4-hydroxylase. Since these enzymes are involved in the biosynthesis of phenols, the exogenous MT triggered an increase in the amounts of phenolic compounds and phenolic acids in barley sprouts (Yin et al., 2022). It is commonly apparent that a higher level of phenolic compounds can contribute to the antioxidant capacity and, thus, would improve the nutritional value of edible sprouts (Świeca et al., 2012).

3.5 EFFECTS OF MELATONIN AS AN ELICITOR ON ANTIOXIDANT CAPACITIES

With the aim of evaluating the roles of time and elicitors on biological changes in mung bean sprouts, the antioxidant properties of the sprouts were measured by two methods, DPPH and reducing power. In the control treatment, as the germination time progressed, the reducing potential showed a trend of decrease, but the antioxidant activity (DPPH) increased until the 4th day, but thereafter decreased until the 5th day of germination (Table 2). Changes in the antioxidant content of edible sprouts during germination have reportedly occurred in previous experiments. In lentil sprouts, for example, free radical scavenging, metal chelating and reducing properties gradually decreased through time in the germination process (S´wieca et al., 2012). In several cultivars of soybean and mung bean, the FRAP method was used for measuring the antioxidant capacity which decreased through germination time (Shohag et al., 2012). In contrast, other leguminous species showed an increase in antioxidant capacity during germination (Aguilera et al., 2015). In contrast, other species showed an increase in antioxidant compound during seed germination (Aguilera et al., 2015; Araújo et al., 2023). Such results may depend on the genotypes of each species and on the conditions of cultivation in each experiment.

The results showed that MT caused a significant increase in the antioxidant capacity and reducing power in edible mung bean sprouts, compared to the control treatment (Table 2). The results of similar research showed that using chemical agents such as salicylic acid, methyl jasmonate and hydrogen peroxide, as elicitors, increased the antioxidant potential of edible legume sprouts (Kim et al., 2006; Świeca, 2015; Gómez-Velázquez et al., 2021). In other words, MT is regarded as a powerful antioxidant that scavenge free radicals (Arnao and Hernández-Ruiz, 2018). In cucumber plants, exogenous MT reduced oxidative damage by increasing antioxidant gene expression

Table 2: Influence of germination time and melatonin (MT) elicitation on reducing power and antiradical activity of mung bean sprouts.

Time of germination (Days)	Elicitor	Reducing power (µg g FM^{-1})	Antiradical activity (%)
1-day old	Control	4.61ab	51.97de
	150 µM MT	4.49b	50.20e
2-day old	Control	4.70ab	59.80d
	150 µM MT	5.43a	80.58ab
3-day old	Control	3.10cd	77.45bc
	150 µM MT	3.96b	87.95a
4-day old	Control	2.13e	84.64ab
	150 µM MT	3.81bc	85.19ab
5-day old	Control	2.52de	70.83c
	150 µM MT	4.18b	83.76ab

Values are the means of three replicates, and different letters differ significantly by Duncan test. Mean significant at the $p < 0.05$ probability level.

and improved seed germination under salt stress conditions (Zhang et al., 2014). In another study, Aguilera et al. (2015) reported that enriching sprouts with MT caused antioxidant functions and free radical scavenging. Enhancements in antioxidant activity in plants, via elicitors, can be related to improvements in bioactive compounds that neutralize free radicals and protect plants against oxidative damage. Enhancements in antioxidant activity in plants, via elicitors, can be related to improvements in bioactive compounds that neutralize free radicals and protect plants against oxidative damage (Guru et al., 2022). In previous researches, it was observed that phenolic compounds, vitamin C and flavonoids had significant, positive relationships with antioxidant capacity in plants (Shohag et al., 2012, Peñas et al., 2015). Thus, the function of MT in increasing the antioxidant activity of mung bean sprouts can be attributed to the increase in flavonoids and ascorbic acid.

4 CONCLUSIONS

In conclusion, the results of this research showed that several biological features such as antioxidants, amino acids, proteins, soluble solids and vitamin C increased significantly through mung bean sprouting. As an elicitor, melatonin affected amino acid content and total protein, increased the germination percentage of seeds and, thus, improved the fresh mass of mung bean sprouts, compared to the control treatment. Also, using melatonin on mung bean seeds increased the measurable values of phenolic compounds, total flavonoids, antioxidant capacity, reducing power and vitamin C content, thereby improving the nutritional value of edible mung bean sprouts.

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Evaluation of the distribution of cadmium and its toxic effects on the biological responses of *Nicotiana tabacum* L.

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Evaluation of the distribution of cadmium and its toxic effects on the biological responses of *Nicotiana tabacum* L.

Abstract: The environmental pollutant cadmium (Cd) contributes to cell destruction in plants by elevated ROS. This study investigated the effects of Cd on tobacco plants (*Nicotiana tabacum* L.) to understand their response to oxidative stress induced by Cd, with a focus on morphological and biochemical characteristics. The finding revealed that the increased concentration of Cd reduced the values of PLSTI, PDSTI, and RWC compared with the control. In addition, the functions of CAT, POD, and APX enzymes were increased, while SOD enzyme activity decreased. Cd significantly increased proline, antioxidant capacity, MDA, and H_2O_2 levels. As the concentration of Cd in the environment elevates, its accumulation in roots and shoots increases. However, the amount of Cd accumulated in the roots was greater than in the shoots. Furthermore, the accumulation of Cd in the roots reduced amount of elements such as zinc, calcium, manganese, copper, and iron in the tissues of plants. The high potential of tobacco to absorb heavy metals makes it a suitable Cd accumulator, and its non-edible nature allows its use in phytoremediation. This study helps to better understand the interaction of different antioxidant pathways with Cd toxicity and the biochemical changes resulting from oxidative stress pathways in tobacco plants.

Key words: antioxidant enzymes, cadmium, oxidative stress, tobacco

Ovrednotenje razporeditve kadmija in njegovi toksični učinki na biološki odziv v tobaku (*Nicotiana tabacum* L.)

Izvleček: Okoljsko onesnaženje s kadmijem (Cd) prispeva k uničenju celic in povečanju reaktivnih zvrsti kisika v rastlinah (ROS). V raziskavi so bili preučevani učinki Cd na rastline tobaka (*Nicotiana tabacum* L.) za razumevanje njihovega odziva na oksidacijski stres, ki ga vzpodbudi Cd s poudarkom na morfoloških in biokemičnih lastnostih. Izsledki so pokazali, da je povečana koncentracija Cd zmanjšala vrednosti PLSTI, PDSTI in RWC v primerjavi s kontrolo. Dodatno se je povečalo delovanje encimov kot so CAT, POD in APX med tem, ko se je aktivnost encima SOD zmanjšala. Povečenaje Cd je značilno povečalo vsebnosti prolina, MDA, H_2O_2 in antioksidacijsko aktivnost. S povečevanjem koncentracije Cd v okolju se povečuje njegovo kopičenje v koreninah in poganjkih. Povečevanje vsebnosti Cd je večje v koreninah kot v poganjkih. Kopičenje Cd v koreninah je zmanjšalo vsebnosti elementov v rastlinskih tkivih kot so zink, kalcij, mangan, baker in železo. Zaradi velikega potenciala tobaka za absorbcijo težkih kovin bi lahko bil ta primeren akumulator Cd in ker ni užiten bi bil primeren tudi za fitoremediacijo. Raziskava prispeva tudi k boljšemu razumevanju interakcij različnih antioksidacijskih mehanizmov s strupenostjo Cd in biokemijskih sprememb, ki nastanejo zaradi oksidacijskega stresa v rastlinah tobaka.

Ključne besede: antioksidacijski encimi, kadmij, oksidacijski stres, tobak

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1 INTRODUCTION

Tobacco (*Nicotiana tabacum* L.) is an herbaceous species belonging to the Solanaceae family. Tobacco is produced commercially as a major ingredient in cigarettes and as a nicotine extract for medicinal purposes on a large scale. Nicotine, which is the specific and major alkaloid of the tobacco plant (95 % of total alkaloid content), is used to improve symptoms of Parkinson's and Alzheimer's diseases also manufactures e-cigarettes, nicotine patches, and gum. In addition, nicotine derivatives, including N-acyl nornicotine, have potent insecticidal properties (Barreto et al., 2015). This plant is used as a template organism in botanical research such as plastid studies, tissue culture, genetic engineering, genetic transformation, and the production of secondary metabolites with therapeutic value and transgenic proteins (Weathers et al., 2010).

The persistence and high tendency to accumulate heavy metals (HM) cause damage to the cellular structure of plants. Among HMs, plant uptake of Cd has been the subject of various research due to its excellent water solvability, high toxicity, and mobility (Pal and Maiti, 2019). The threshold level of Cd in agricultural soils (approximately 100 mg kg^{-1}) is increasing due to human activities, including the application of phosphate fertilizers and sewage sludge for soil amendment (Zulfiqar et al., 2022). Since the beginning of the 21^{st} century, approximately 30,000 tons of Cd are produced annually, with around 13,000 tons resulting from human activities. Levels exceeding 3 mg kg^{-1} in soil can cause toxicity in plants (Asgher et al., 2015). Other reasons for soil contamination with Cd include forest fires and industrial activities, including refining, ore mining, leaks of contaminated water (warehouses and waste), pesticides, and tire wear (Zulfiqar et al., 2022). Cd competes for uptake by plants due to its similarity in physicochemical properties to micronutrients, utilizing similar transporters. Cd ions are transported across membranes by a special type of metal carrier, so roots are the first organs that are directly in contact with toxic metal ions and will also have more metal content than shoots (Chen et al., 2018). Cd toxicity leads to reduced plant growth due to disruptions in cell division, chlorosis, leaf necrosis, and accelerated leaf senescence. These symptoms are attributed to alterations in the biochemical components of the plant, including the induction of lipid peroxidation, deficiencies in nutrient uptake, disruptions in water absorption, and the plant's hydrological relationships. These modifications are often the result of oxidative stress due to an increase in cellular reactive oxygen species (ROS) (Burzyn´ski and Zurek, 2007). In ordinary condition, ROS is produced at some point of cellular metabolism, and numerous approaches, which includes the mitochondrial electron delivery chain, using the reaction of semiquinone with oxygen, fatty acid β-oxidation, and enzymatic reactions such as peroxidase, NADPH oxidase, and xanthine oxidase. At the same time, ROS is elevated in abiotic stress situations, including HM, due to an imbalance between ROS production and elimination (Zhang et al., 2007). Cd can elevate ROS production, including superoxide radicals, at complex III of the mitochondrial electron delivery chain. Furthermore, plasma membrane NADPH oxidase (NOX) may be involved in Cd toxicity-induced ROS production after short-term exposure to HM (Garnier et al., 2006). Plant tolerance to Cd is supported by different mechanisms such as Cd chelation by cell wall components, vacuolar compartmentalization, and Cd chelation by cytosolic organic acids or peptides (Rizwan et al., 2016).

Here, we investigat the impact of cadmium chloride ($CdCl_2$) toxicity on tobacco plant growth, biochemical parameters, oxidative damage, and antioxidant enzyme activity during stress periods, as well as how Cd interacts with iron (Fe), zinc (Zn), calcium (Ca), copper (Cu), and manganese (Mn). Those results offer data on the defense mechanisms of tobacco, Cd accumulation, and the tolerance of tobacco, which may be grown as crops and medicinal plants on Cd-infected soils.

2 MATERIALS AND METHODS

2.1 PLANT CULTURE AND CD TREATMENT

Tobacco seeds (*Nicotiana tabacum* 'Samsun') were obtained from the Institute of Plant Molecular Biology, University of Strasbourg, France. Seeds were surface-sterilized with ethanol and sodium hypochlorite solution. Seeds were grown in sterilized coco peat and perlite (in a 1:2 ratio) in a greenhouse (25-30 °C, 16/8 h light/dark, light intensity 100 mmol m^{-2} s^{-1}, relative humidity 60–75 %). After germination, the seedlings were irrigated with Hoagland 25 %, 50 %, and 100 % solution (pH 5.8–6) consisting of micronutrients: KCl, H_3BO_3, $MnSO_4.H_2O$, $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$, H_2MoO_4, and macronutrients: KNO_3, Ca $(NO_3)_2.4H_2O$, $NH_4H_2PO_4$, $MgSO_4$, and iron solution: $FeSO_4.H_2O$, EDTA once a week, and distilled water every 3 days. At the start of the trifoliate phase, $CdCl_2$ was irrigated in three replicates at five different concentrations (0, 1, 1.5, 2, and 2.5 mM) once a week for 21 days (to determine the above concentrations, a concentration determination was conducted, which indicated that tobacco plants do not survive at $CdCl_2$ concentrations of 4 mM and above). After the treatment, the plants were harvested for the relevant experiments.

2.2 TOLERANCE INDICES (%)

Stress tolerance indices were calculated by the following formula Amin et al. (2014):

PLSTI: (Plantlet length (shoot + root) for stressed plant/ plantlet length (shoot + root) for control plant) × 100

PDSTI: (Plantlet dry mass (shoot + root) for stressed plant/plantlet dry mass (shoot + root) for control plant) × 100

2.3 LEAF RELATIVE WATER CONTENT (RWC)

Relative water content (RWC) was analyzed according to the formula (Ritchie et al., 1990):
RWC (%) = (FM-DM)/(TM-DM) .

Leaf samples from each treatment were weighed to measure fresh mass (*FM*), placed in distilled water for 24 h, and weighed to determine the turgid mass (TM). Then the samples were dried at 65 ˚C for 24 h, to measure dry mass (*DM*).

2.4 ENZYME ASSAYS

Activities of antioxidative enzymes were estimated according to the protocols described by Hajiboland et al. (2010). The activity of the enzyme superoxide dismutase (SOD) was performed using a monoformazan formation at 560 nm. Ascorbate peroxidase (APX) activity was assayed by measuring the oxidation of ascorbic acid at 290 nm. Catalase (CAT) activity was determined by investigating H_2O_2 reduction at 240 nm. Peroxidase (POD) activity was investigated using the guaiacol at 470 nm.

2.5 MEASUREMENT OF OXIDATIVE STRESS INDICATORS

Proline was investigated via the method of Bates et al. (1973). Lipid peroxidation was determined utilizing thiobarbituric acid at 532 nm. The H_2O_2 concentration was estimated utilizing potassium iodide at 390 nm (Hajiboland et al., 2010). Radical scavenging ability was assayed by the DPPH method at 517 nm (Miliauskas et al., 2004).

2.6 DETERMINATION OF CD, CA, CU, FE, MN, AND ZN CONTENT AND CD DISTRIBUTION

The Cd content of the samples and other mineral elements was measured by the method of Dániel et al. (1997). For this purpose, the roots were immersed for 10 minutes in EDTA-Na_2 solution (0.1 M) to facilitate the removal of Cd from the root surfaces, followed by rinsing with distilled water. The shoot and root portions of the plants were dried at 25 °C and then ground into a powder. To 0.5 g of the dried samples, 10 ml of nitric acid (65 %) were added for acid digestion, and the samples were placed under a fume hood (24 h). Subsequently, the samples were heated to 90 °C to facilitate the evaporation of acidic vapors from the solution. After cooling, 1 ml of H_2O_2 (30 %) was added to the digested samples and heated until the solution became clear. The digested extract was then diluted with distilled water to a final volume of 25 ml. The Cd content and other mineral elements in the samples were measured using atomic absorption spectrometry and reported as mg g^{-1} dry mass. The translocation factor (TF), bioaccumulation factor (BF), and transfer coefficient (TC) were determined by the following formula (Eid and Shaltout, 2016).

$$TF = Cd_{shoot}/Cd_{root}, BF = Cd_{root}/Cd_{medium}, TC = Cd_{shoot}/Cd_{medium}$$

2.7 STATISTICAL ANALYSES

Data analysis was enforced with the SPSS 24.0 software package. Experimental data was released as the mean ± SD. One-way ANOVA was employed to define differences between means. Duncan's test and the significance level at $p \leq 0.05$ were used.

3 RESULTS AND DISCUSSION

3.1 TOLERANCE INDICES OF TOBACCO PLANTS UNDER CADMIUM STRESS

The PDSTI (plantlet dry mass stress tolerance index) and PLSTI (plantlet length stress tolerance index) tolerance indices decreased significantly with increasing $CdCl_2$ concentration. PLSTI values of 42.50 % and 19.51 % were the highest and lowest when $CdCl_2$ was applied at 1 and 2.5 mM, respectively. In addition, similar results were obtained for PDSTI. Treatments 1 and 2.5 mM had the highest and lowest rates of PDSTI, 30.79 %, and 9.20 %, respectively ($p \leq 0.05$) (Figure 1, Figure 2A-B).

Plant growth characteristics are considered sensitive parameters to measure their resistance to metal toxicity (Imtiaz et al., 2015). Our experimental data showed that Cd stress inhibited the growth of tobacco plants. Similarly, a reduction in DWSTI and PHSTI indices was reported in *Cicer arietinum* L. under Cd stress (Mohajel Kazemi et al., 2020). Additionally, some researchers have reported a decrease in the dry mass of both roots and shoots of plants with increased exposure to Cd (Yazdi et al., 2019). In the present study, it is presumed that the inadequate absorption and transfer of essential minerals, including calcium, phosphate, potassium, and iron, along with the inhibition of cell division and abnormal mitosis -which are direct outcomes of root metabolism disruption -are likely factors contributing to the reduction in plant height and dry mass (Muradoglu et al., 2015; Kolahi et al., 2020). In addition, by disrupting water absorption, Cd reduces cell water potential and cell wall elasticity, reduces hydraulic conductivity through aquaporin, which causes cells to remain small, reduces intercellular space, and inhibits plant length growth (Karcz and Kurtyka, 2007; Ehlert et al., 2009). A similar scenario was observed by Monteiro et al. (2012).

Figure 1: Effects of different concentrations CdCl2 (0, 1, 1.5, 2 and 2.5 mM) on morphology and growth of *Nicotiana tabacum* for 21 days.

They attributed the greatest reduction in plant growth under Cd stress to oxidative damage. The production of ROS due to the presence of heavy metals leads to the degradation of cell biomolecules and organelles and membrane lipids, resulting in decreased plant growth and ultimately plant mortality (Monteiro et al., 2012: Yazdi et al., 2019).

3.2 LEAF RELATIVE WATER CONTENT (RWC) OF TOBACCO PLANTS UNDER CADMIUM STRESS

The changes of water content in plants under $CdCl_2$ stress showed RWC decreased significantly with an increase in the concentration of $CdCl_2$ (Figure 3). Similar result was obtained in potato due to Cd-influenced water imbalance (Li et al., 2019). The change in root structure caused by Cd stress (such as increased root suberization and lignification and loss of endoderm integrity) disrupts the root-soil relationship, ultimately reducing water uptake (Barcelo and Poschenriedr, 1990). Elevated Cd concentrations in plant tissues cause membrane damage, electrolyte leakage, and cytoplasmic thickening. Therefore, the presence of Cd reduces the ability to absorb water and inhibits short-range migration in the apoplast and symplast pathways, ultimately reducing its availability to physiological processes. Cd reduces root hydraulic conductivity and turgor pressure by interfering with aquaporin function and altering gene expression. Furthermore, Cd disrupts stomatal conductivity and water balance in plant cells by reducing stomata, leaf transpiration rate and RWC, and limiting water availability to plants for cell expansion (Gall et al., 2015).

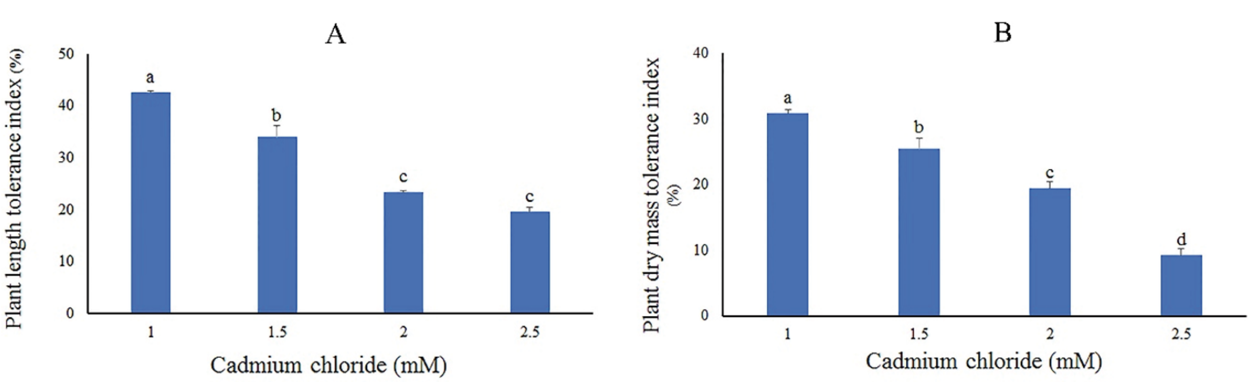


Figure 2: Effects of different concentrations CdCl2 on A) Plant length tolerance index (PLSTI), B) Plant dry mass tolerance index (PDSTI) for 21 days. Values with different letters are statistically significantly different at p ≤ 0.05.

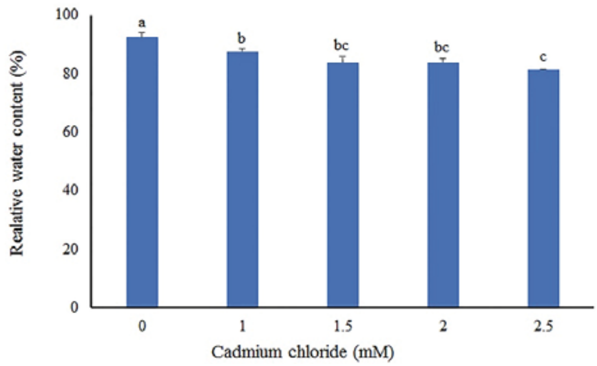


Figure 3: Effects of different concentrations CdCl2 on Relative water content (RWC) for 21 days. Values with different letters are statistically significantly different at $p \leq 0.05$.

3.3 CHANGES IN ANTIOXIDANT ENZYMES ACTIVITIES OF TOBACCO PLANTS UNDER CADMIUM STRESS

The activity of SOD enzyme considerably decreased in the plants under stress. The smallest enzyme activity (64.88 % decrease compared to control) was obtained by 1 mM $CdCl_2$ (equivalent to 9.15 U mg protein^{-1}) (Figure 4A). With the increase in $CdCl_2$ concentration, the activity of the ascorbate peroxidase enzyme increased, and the highest activity of this enzyme was in the treatment of 2.5 mM $CdCl_2$ with a value of 9.41 U mg^{-1} protein. An increase in APX enzyme activity was observed with increasing $CdCl_2$ and its peak activity was shown at 2.5 mM $CdCl_2$, reaching 69.54 % of controls (Figure 4B). A considerable change in CAT enzyme activity was also shown. Plants treated with 2 mM $CdCl_2$ showed the highest CAT enzyme activity (0.4226 U mg^{-1} protein) (Figure 4C). POD enzyme activity showed a considerable difference in all treatments except the 1 mM $CdCl_2$ treatment. The highest level of enzyme activity was observed (87.96 %) in 2 mM $CdCl_2$ (13.21 U mg^{-1} protein) ($p \leq 0.05$) (Figure 4D).

The binding of HM to the sulfhydryl group of enzymes inhibits their activity and disrupts their structure in plants under Cd stress. Moreover, other reports have revealed that protein denaturation and inactivation are vital in the plant reaction to HM toxicity (Emamverdian et al., 2015). In addition, the plant's resistance to Cd-related stress is closely related to the ability of the antioxidant system to eliminate ROS; also, the synergistic antioxidant enzymes in response to Cd treatment, which actively eliminates oxidative conditions. In this research, increased activity of CAT, POD, and APX enzymes was observed under Cd stress, although decreased SOD enzyme activity in tobacco leaves suggested the involvement of other protective factors. Overall, this condition may be a direct result of the enhanced production of ROS compounds and, ultimately the stimulation of antioxidant enzyme defense systems to neutralize these compounds. The results showed that the activities of POD, APX, and CAT enzymes were improved in contrast to the decrease of SOD enzyme activity, which could confirm that the H_2O_2 removal was favorable. Similar results were obtained for the antioxidant enzymes activity of *Dittrichia viscosa* (L.) Greuter affected by Cd (Fernandez et al., 2013).

APX enzymes remove excess H_2O_2 using ascorbate; so can act as ROS modulation for signal transmission. Alternatively, H_2O_2 can be degraded to water and oxygen by the POD enzyme in the cell wall and cytoplasm or by the CAT enzyme in peroxisomes and mitochondria (Chen et al., 2003). POD enzymes have heme groups in their structure; they preferentially oxidize aromatic electron donors such as guaiacol and pyragallol with the help of H_2O_2. Peroxidase enzymes are associated with some vital cellular processes, such as growth, differentiation, and resistance to various abiotic and biotic stresses. Due to the role of this enzyme in lignin biosynthesis, it can create a physical barrier against HMs. Different stress states, including heavy metals, herbicides, ozone, and polycyclic aromatic hydrocarbon, changed the function of the GPX enzyme (Bhaduri and Fulekar, 2012). In recent research, Cd led to the continuous production of H_2O_2 and increased POD activity; resulting in the breakdown of excess H_2O_2 at the cytoplasmic level. Similar results are consistent with our study; antioxidant enzymes activity (*e.g.*, POD, APX, and CAT) was increased in sugarcane affected by Cd (Yousefi et al., 2018). In this study, the induction of CAT, APX, and POD enzymes under Cd stress may indicate a role for these three enzymes in enhancing tobacco defense mechanisms against oxidative damage.

3.4 OXIDATIVE STRESS INDICES OF TOBACCO PLANTS UNDER CADMIUM STRESS

3.4.1 Evaluation of proline content

A significant increase in proline was shown in the treated tobacco compared to the control, which had the highest levels at 2 and 2.5 mM $CdCl_2$ and equal to 1.21 and 1.20 mg g^{-1} FM, respectively ($p \leq 0.05$) (Figure 5A).

Under Cd stress, proline reduces the destructive effect in plants by cheating Cd, creating a non-toxic

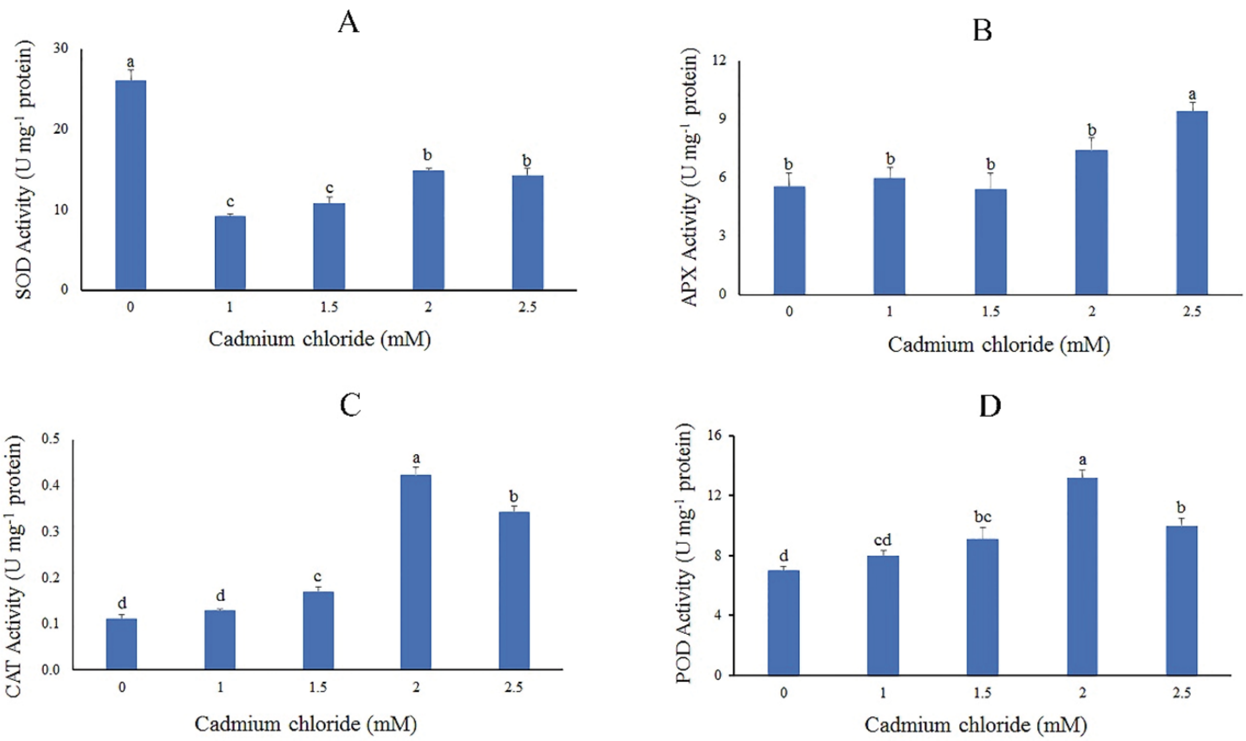


Figure 4: Effects of different concentrations CdCl2 on the activities of A) SOD (Superoxide dismutase), B) APX (Ascorbate peroxidase), C) CAT (Catalase), D) POD (Peroxidase) for 21 days. Values with different letters are statistically significantly different at $p \leq 0.05$.

proline-Cd complex, stabilizing macromolecules and organelles, stabilizing protein synthesis, and preventing enzyme denaturation. In addition, proline, either free or bound to polypeptides, can react directly to H_2O_2, hydroxide ions, dioxygen, and inhibit free radicals induced by Cd toxicity (Rejeb et al., 2014). It has been suggested that the increase of proline in Cd-treated plants is not directly associated with high HM concentrations but rather reduced water capacity and disturbances in water balance in the cells. Therefore, proline accumulation may be directly related to plant water balance (Clemens, 2006). According to the present study, tobacco plants under $CdCl_2$ stress accumulate more proline, which is attributed to ROS detoxification and elevated resistance to Cd. Comparable findings have additionally been noted in some Cd-affected crops, such as *Arabidopsis thaliana* (L.) Heynh. (Xiao et al., 2020).

3.4.2 Estimation of malondialdehyde (MDA)

The MDA amount in tobaccos increased significantly, getting the top level at 2.5 mM of $CdCl_2$ with a value of 3.63 μmol g^{-1} FM ($p \leq 0.05$) (Figure 5B). The significant ion in MDA amount (lipid peroxidation) causes damage to membrane nature, increased cell permeability, dysfunction of enzymes, ion leakage, and cell death. An increase in the amount of MDA can be an ideal detector for assessing metal toxicity and determining Cd tolerance in tobacco (Nazar et al., 2012). HM stress stimulates free radical production, including superoxide radicals, by increasing lipoxygenase enzymatic activity, followed by lipid peroxidation and elevated MDA levels (Ahmad et al., 2009). Indeed, due to the increase in free radicals under stress, the antioxidant system cannot remove them effectively, ultimately damaging the cellular composition of the plant. Various studies are consistent with the present research. High amounts of inner membrane peroxidation influenced by Cd were observed in sassafras (Zhao et al., 2021). Furthermore, an increase in ROS and MDA in Cd treatment has blended in crops such as cotton, confirming the present study's finding (Khan et al., 2013). In a recent study, Cd accumulation in tobacco plants also elevated the production of free radicals such as H_2O_2, increased MDA, and induced oxidative stress.

3.4.3 Evaluation of H_2O_2 content

The H_2O_2 content increased significantly with the rising $CdCl_2$ concentration except for the treatment of 1 mM $CdCl_2$. This increase was more noticeable (90 %) in 2.5 mM $CdCl_2$ treatment with 0.38 µmol g^{-1} FM of H_2O_2 ($p \leq 0.05$) (Figure 5C).

HM, directly and indirectly, leads to ROS production and subsequent oxidative stress in plant tissues. Cd induces ROS production indirectly by disrupting the structure of leaf chloroplasts. Furthermore, the inhibition of electron transport by Cd toxicity leads to the photoinactivation of photosystem II (Farooq et al., 2016). It has been suggested that during plant treatment, which includes rice and peas, Cd increases the production of ROS such as H_2O_2 by indirectly activating NADPH oxidase enzyme bound to the membranes of peroxisomes and leading to an oxidative burst (Tran and Popova, 2013). Also, an elevated H_2O_2 has been stated in *Vaccinium corymbosum* L. under the influence of Cd (Manquián- Cerda et al., 2016).

Furthermore, Cd toxicity causes a rise in ROS in the mitochondrial electron transport chain, and ROS overproduction leads to ATP depletion and decreased respiration (Singh et al., 2016). An increase in H_2O_2, after disrupting the harmony between its production and removal, induces senescence, lipid peroxidation, and disruption of integrated membranes in plants (Shiyu et al., 2020). It has been advised that to protect plant cells against H_2O_2 accumulation caused by various environmental stresses, different proteins and compounds act as ROS scavengers, i.e. antioxidant defense mechanisms (Shahid et al., 2014).

3.4.4 Evaluation of the antioxidant content by the DPPH assay

The antioxidant content of plants revealed a considerable enhancement in tobacco plants under $CdCl_2$ treatment. The maximum antioxidant capacity was stated in 2 mM $CdCl_2$ with a value of 99.35 % ($p \leq 0.05$) (Figure 5D).

The DPPH test defines phenolic compounds' free radical stabilizing capacity (Müller et al., 2011). With the increase of phenolic acids under the influence of Cd, the antioxidant capacity of the plant also increased, as well as the ability to resist oxidative damage caused by HM (Shan, 2022). Research has shown that phenolic compounds such as flavonoids, phenolic acids, and flavonolignans act as antioxidants in plants. High concentrations of polyphenols induce hydrogen from the hydroxyl groups of the aromatic circle to ROS (Sharififar

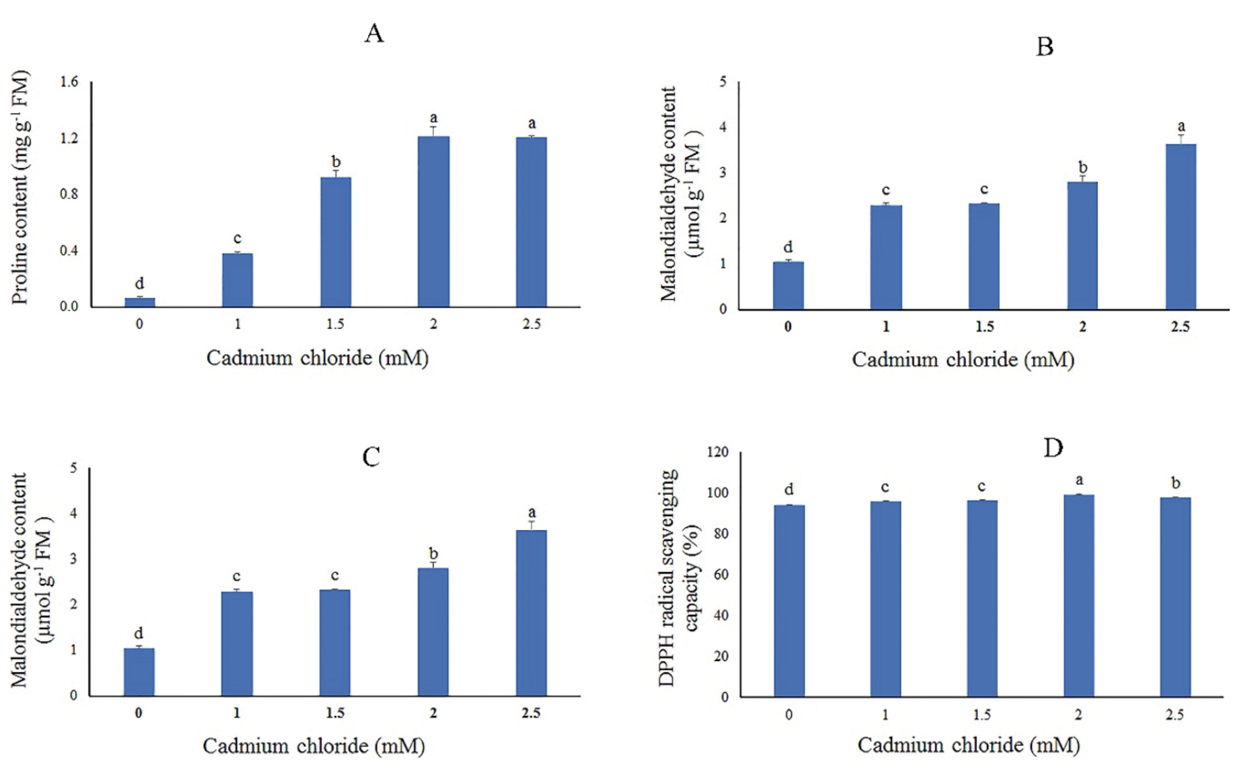


Figure 5: Effects of different concentrations CdCl2 on A) Proline, B) Malondialdehyde (MDA), C) Hydrogen peroxide (H_2O_2), D) DPPH radical scavenging capacity for 21 days. Values with different letters are statistically significantly different at $p \leq 0.05$.

et al., 2009). Under Cd stress, tobacco plants stimulate phenolic pathways, enhancing the plant's antioxidant capacity to fight oxidative harm. These effects are similar to data on *Vaccinium corymbosum* L., treated with HM, confirming an increase in antioxidant activity after exposure to HM (Manquián-Cerda et al., 2018). Antioxidant capacity was increased in *Gynura procumbens* (Lour.) Merr. and *Ocimum basilicum* L. under HM stress (*e.g.*, Cd, Cu, and Al), confirming a positive correlation between DPPH activity and the amount of metabolic compounds, such as phenols and flavonoids (Ibrahim et al., 2017).

3.5 CD ACCUMULATION, TRANSLOCATION FACTOR (TF), BIOACCUMULATION FACTOR (BF), AND TRANSLOCATION COEFFICIENT (TC) OF TOBACCO PLANTS UNDER CADMIUM STRESS

Based on our data, the amount of Cd in the roots and stems of tobacco plants changed significantly under the influence of different Cd treatments. As shown in Figure 6, Cd accumulation in roots and stems elevated steadily with the addition of Cd concentration in the medium. The concentration of Cd in the roots was remarkably higher than in the shoots. Compared with the control, the lowest and highest Cd concentrations in

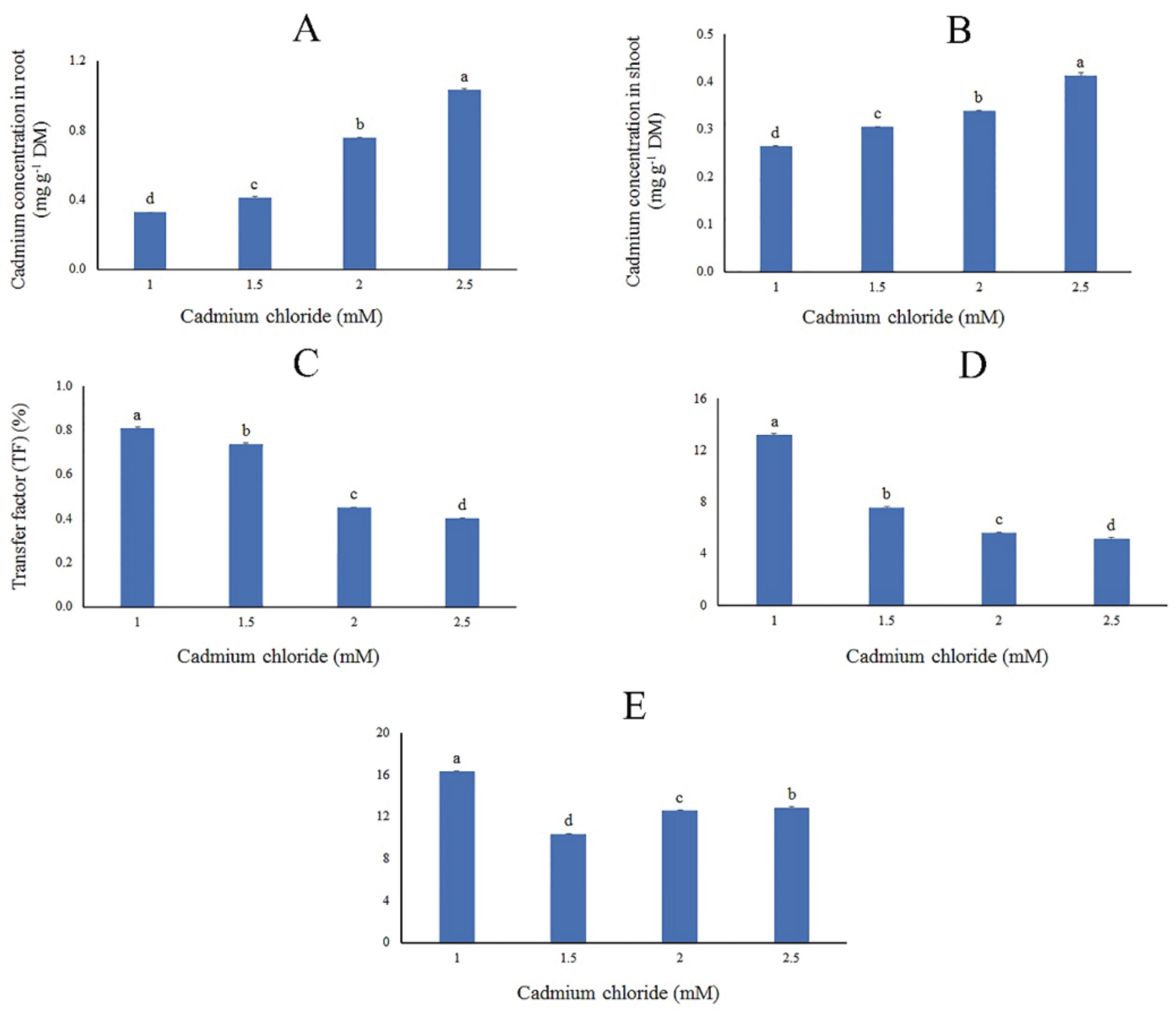


Figure 6: Effects of different concentrations $CdCl_2$ on accumulation of Cd in A) roots and B) shoots. Study of C) Transfer factor (TF), D) Transfer coefficient (TC) and E) Bioaccumulation factor (BF). Values with different letters are statistically significantly different at $p \leq 0.05$.

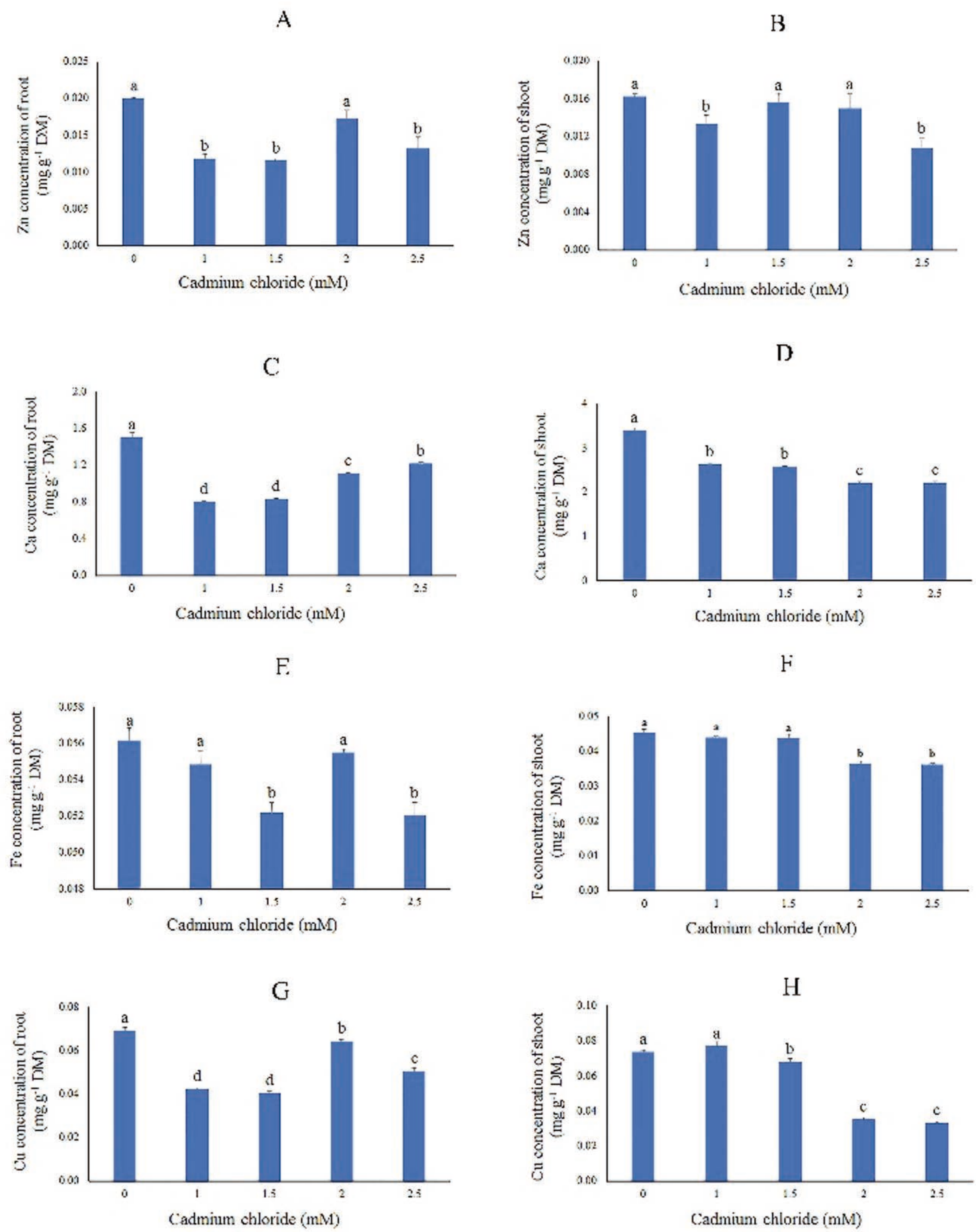
the roots and the shoots were observed in the 1 and 2.5 mM $CdCl_2$ treatments, respectively (Figure 6A-B). In contrast, the TF index continuously decreased with increasing $CdCl_2$ concentration, indicating a decrease in Cd transfer from the roots to the shoots of the tobacco plant (Figure 6C). BF and TC indices also showed a significant decrease with increasing $CdCl_2$ concentration (Figure 6 D-E) ($p \leq 0.05$).

Because Cd contacts the roots and prevents their transfer to the shoots, the roots contain a larger amount of Cd. In addition, Cd accumulates in the underground parts relative to the aerial organs by entering the root apoplastic pathway, where it penetrates and cumulates in root tissue. Furthermore, Cd accumulation in vacuoles of root cells prevents the xylem loading, thus reducing the toxicity symptoms and protecting the plant from stress (Lux et al., 2011). Akhter (2012) reported the reduction of root-to-shoot Cd transfer at high Cd levels was associated with elevated biosynthesis of phytochelatins (PCs) and relative compounds (Cys, Glu, and γ-Glu-Cys) that bind to Cd. The PC gene expression increased in the roots of *Saccharum officinarum* L. during the duration of Cd treatment, resulting in higher PC levels in roots compared with the shoots (Yousefi et al., 2018). The plant's ability to decrease in TF and TC indices has also been reported in plants such as *Saccharum officinarum,* and *Satureja hortensis* L. as Cd concentrations increase (Yousefi et al., 2018; Azizollahi et al., 2019). The capacity of plants to compile metals in shoots and transfer them from root to stem was determined by TF and TC indices, respectively. A TF index higher than 1 indicates the greatest efficiency of the seedling in transferring metals from the root to the aerial organs (Eid and Shaltout, 2016). In the recent search, the TF amount was less than 1 due to Cd concentration in tobacco roots contrasted to stems and leaves. The TF and TC values decreased with increasing Cd concentration, indicating an improved resistance barrier. Root endurance prevents the transfer of Cd to tobacco shoots. Niu et al. (2007) showed a reduction in BF in plants likewise alfalfa, castor bean, and mustard with increasing Cd concentrations; this index's value varied depending on the concentration, nature of HM, and environmental conditions. The value of the BF index evaluates the capacity of plant roots to uptake Cd from the ground; this index is higher than 1 in HM-accumulating plants and less than 1 in HM-remover plants (Yanqun et al., 2005). In recent research, the value of the BF index is greater than 1, which indicates a higher Cd absorption capacity of tobacco roots; therefore, tobacco is considered a Cd accumulator. Equal findings have also been stated in chickpeas (Mohajel Kazemi et al., 2020).

3.6 ZN, CA, FE, CU, AND MN CONCENTRATIONS OF TOBACCO PLANTS UNDER CADMIUM STRESS

Zn concentration was notably decreased by treatment of tobacco roots with $CdCl_2$, except for the 2 mM treatment. Zn concentration in the shoots decreased remarkably at 1 and 2.5 mM while increasing the $CdCl_2$ levels in the medium (Figure 7A-B). In addition, increasing $CdCl_2$ levels in the treatments resulted in decreased Ca^{2+} concentrations in tobacco tissues compared with controls (Figure 7C-D). Also, Fe concentrations reported a remarkable induction in tobacco roots at 1.5 and 2.5 mM treatments. As the $CdCl_2$ concentration increased, the iron concentration decreased in the tobacco shoots in treatments 2 and 2.5 mM (Fig. 7E-F). Cu concentrations decreased at all tested $CdCl_2$ concentrations except 1 mM (Fig. 7G-H). Furthermore, the levels of Mn in the aerial parts also decreased significantly in all treatments except 1.5 mM treatment ($p \leq 0.05$) (Figure 7I-J).

By disrupting membrane transport proteins, Cd causes membrane permeability changes and affects nutrient absorption and accumulation, thereby causing nutritional deficiencies and disorders (Yao et al., 2009). Possible evidence for tobacco essential element reduction is lipid peroxidation due to Cd toxicity, which can alter cell membrane function and ultimately disrupt the plant's nutritional balance; however chemically similar to Cd, the concentration of each element decreases for other specific reasons under the influence of Cd. For example, Cu, the electron transporter in photosynthetic organisms, is adversely affected by Cd because it serves as a cofactor for enzyme structure and occupies the active site of the enzyme cofactor. Cd tends to react with compounds containing the -SH functional group, thus competing with copper for this position and reducing the copper concentration (Qian et al., 2009). As another micronutrient, Mn is required for important metabolic processes such as the photolysis of H_2O by photosystem II and the uptake of NO_2^- in chloroplasts (Wu et al., 2003). The amount of Mn decreases because Cd and Mn compete for a membrane transporter (Ramachandran and D'Souza, 2002). The presence of Cd in the medium can affect the nutritional processes of leaves and roots by preventing the loading of ions into the branches of the plant and affecting PC production. Fe transport is dependent on PC production, and Cd pollution affects Fe transport to the shoots by increasing PC production and occupying Fe transporters such as IRT1 (iron-regulated-transporter^{-1}, belongs to the ZIP family of transporters) as well as Nramp (natural-resistance-associated-macrophage protein) family transporters (Takahashi et al., 2011). The decrease in Fe concentration caused by Cd is always associated with


A
Zn concentration of root (mg g-1 DM)
0.025
0.020
0.015
0.010
0.005
0.000
0
1
1.5
2
2.5
Cadmium chloride (mM)
B
Zn concentration of shoot (mg g-1 DM)
0.020
0.016
0.012
0.008
0.004
0.000
Cadmium chloride (mM)
C
Ca concentration of root (mg g-1 DM)
2.0
1.6
1.2
0.8
0.4
0.0
Cadmium chloride (mM)
D
Ca concentration of shoot (mg g-1 DM)
4
3
2
1
0
Cadmium chloride (mM)
E
Fe concentration of root (mg g-1 DM)
Cadmium chloride (mM)
F
Fe concentration of shoot (mg g-1 DM)
0.05
0.04
0.03
0.02
0.01
0.00
Cadmium chloride (mM)
G
Cu concentration of root (mg g-1 DM)
0.08
0.06
0.04
0.02
0.00
Cadmium chloride (mM)
H
Cu concentration of shoot (mg g-1 DM)
0.10
0.08
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Cadmium chloride (mM)

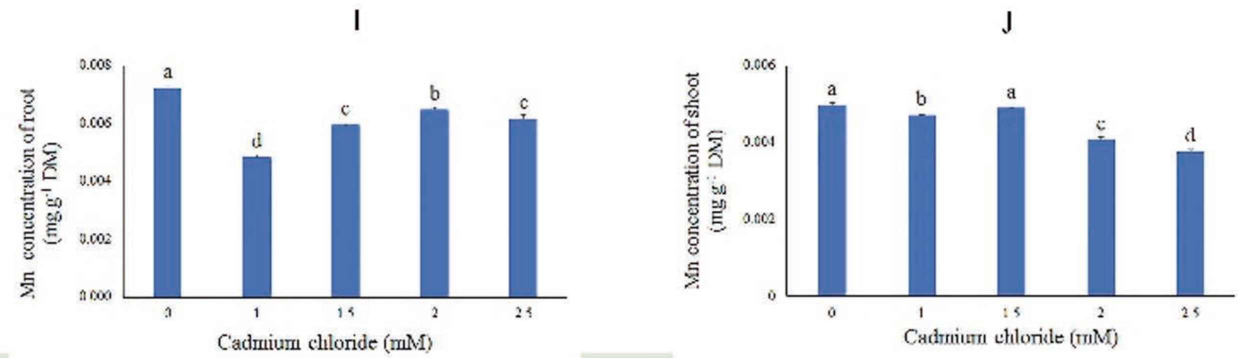

Figure 7: Effects of different concentrations $CdCl_2$ on A) Zn root, B) Zn shoot, C) Ca root, D) Ca shoot, E) Fe root, F) Fe shoot, G) Cu root, H) Cu shoot, I) Mn root, J) Mn shoot concentrations. Values with different letters are statistically significantly different at $p \leq 0.05$.

the antagonistic effects of these two elements. In addition, Fe and Zn play a role in the formation of protective enzymes, likewise CAT and SOD enzymes. SOD is present in the cell in three different forms, FeSOD (chloroplasts), MnSOD (mitochondrial and peroxisomes), and Cu/ZnSOD (chloroplasts and cytoplasm). Cd can replace Fe and Zn in various macromolecules and inactivate this form of the enzyme. On the other hand, the release of free Fe due to the presence of Cd can lead to redox reactions and induce oxidative stress (Cuypers et al., 2010). Many studies have reported a reduction of Zn absorption in the Cd treatment, suggesting an opposite relationship in the transportation of Cd and Zn. In *Gynura pseudochina* (L.) DC., Cd uptake by the roots decreased as Zn concentration increased. Therefore, Cd entry is attributed to Zn transporters, which tend to occupy more Zn than Cd (Panitlertumpai et al., 2013). In plants, both Ca and Cd compete for the same Ca channel. Cd can enter the plasmalemma of root tissues via Ca channels, and depolarization of the membrane potential can also inhibit Ca uptake during Cd processing. (Li et al., 2012). One of the reasons for limiting Ca movement into the aerial parts of the plant during Cd treatment could be the presence of calcium oxalate crystals in the plant's vessels (Barcelo' et al., 1988). Increasing the Ca concentration in the growth medium remarkably decreased Cd harmful in *Mesembryanthemum crystallinum* L., sea purslane, and *Arabidopsis*, which is consistent with the idea of an opposition between two cations all through uptake (Suzuki et al., 2005). With Ca application, Cd tolerance was also increased in *Nicotiana tabacum* due to the formation and elimination of Cd and Ca-containing crystals through the trichome head cells. In addition, adding the amount of Ca in the culture medium increased the expression of the LCT1 gene in tobacco, which is a non-selective transmembrane transporter for K, Na, and Ca and blocks Cd absorption to reducing its toxicity (Antosiewicz and Hennig, 2004).

4 CONCLUSION

The present study investigated growth indices, oxidative damage, antioxidant mechanisms, and Cd accumulation in tobacco plants under Cd stress. Tobacco plants' growth and biochemical responses under Cd stress reveal their high resistance to HM. Raising the level of H_2O_2 (as a signaling molecule) increased MDA production; therefore, oxidative stress is triggered in tobacco seedlings under Cd treatment. Also the CAT, POD, and APX enzymes are enhanced to deal with ROS toxicity. Enzymatic and non-enzymatic antioxidant systems appeared to be initiated in tobacco reacting to Cd toxicity, with different roles in these responses. In reaction to toxicity due to Cd accumulation, tobacco plants enhanced proline, decreased water potential, and disrupted water balance.

Furthermore, adding Cd to the culture medium resulted in Cd penetration into the roots and shoots of tobacco plants. Accumulation of Cd in roots and lipid peroxidation disturbs the plant nutrient balance and reduces mineral nutrients such as Zn, Ca, Mn, Cu, and Fe in roots and shoots. Finally, the reduction in tobacco plant growth properties may be due to the chemical similarity of some ions with Cd and their antagonistic relationships and competition for inhibit absorption and transfer. The reduction of these nutrients by Cd stress can lead to decreased growth rate and dysfunction of several enzymes involved in growth and defense responses. A BF value greater than 1 indicates that tobacco roots can uptake Cd from the environment, so tobacco as an inedible and Cd-accumulating plant can be used in Cd-contaminated soil.

Changes in oxidative stress indices and activation of antioxidant pathways are valuable for understanding cellular mechanisms and processes implicated in plant cell reaction to Cd in cell biological stress and the development of environmental targets. Tobacco provides a valuable model for assessing plants' metabolic and cytoprotective responses under Cd.

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Growth and quality of 'Gulfblaze' plums subjected to fertigation

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Growth and quality of 'Gulfblaze' plums subjected to fertigation

Abstract: This work aimed to evaluate the effect of applying different doses of fertilizers through fertigation on the production, fruit quality, and nutritional status of the plum tree' Gulfblaze'. The experiment was conducted in Antônio Prado, RS, during the 2019/2020 harvest in a 'Gulfblaze' plum orchard established in 2014, with a density of 800 plants per hectare. The treatments applied were T1: Irrigation only, without fertilizer application; T2: Fertigation with 50 % of the dose recommended by CQFS-RS/SC; T3: Fertigation with 100 % of the recommendation; T4: Fertigation with 150 % of the recommendation, and T5: Fertigation at 200 % of the recommendation. A randomized block experimental design was used, with five treatments and three replications, with each plot consisting of 26 plants, and of these, five were used for evaluations. The diameter, length, and average mass of fruits increased as the fertigation doses increased. The firmness of the fruit pulp, as well as the leaf area of the plant, performed better at doses of 150 % and 200 % of the recommended amount. The highest and lowest fertigation doses promoted a higher soluble solids content and SS/AT ratio, indicating an improvement in the qualitative parameters of the plums.

Key words: fruticulture, nutrition, plant growth, Prunus salicina, soluble solids

Rast in kakovost sliv 'Gulfblaze' obravnavanih s fertigacijo

Izvleček: Namen raziskave je bil ovrednotiti učinke različnih odmerkov gnojil pri fertigaciji na obrod, kakovost plodov in stanje prehranjenost sliv 'Gulfblaze'. Poskus je potekal v Antônio Prado, RS, v rastni sezoni in obiranju 2019/2020 v nasadu sliv 'Gulfblaze', ki je bil osnovan 2014, z gostoto 800 dreves na hektar. Obravnavanja so bila sledeča: T1- samo namakanje, brez dodatka gnojil; T2 –fertigacija s 50 % odmerkom, kot jih priporoča CQFS-RS/SC; T3- fertigacija s 100 % odmerkom, kot je priporočeno; T4- fertigation s 150 % odmerkom priporočenega in T5- fertigation z 200 % odmerkom priporočenega. Uporabljen je bil naključni bločni poskus s petimi obravnavanji in tremi ponovitvami, kjer je vsak blok sestavljalo 26 dreves, od katerih je bilo pet uporabljenih za ovrednotenja. Premer, dolžina in poprečna masa plodov so naraščali z naraščanjem odmerka gnojil v fertigaciji. Čvrstost pulpe plodov in listna površina dreves sta bili boljši pri odmerkih 150 % in 200 % priporočene količine. Največji in najmanjši odmerki v fertigaciji so povečali vsebnost topnih snovi v plodovih kot tudi razmerje SS/AT, kar kaže na izboljšanje kakovostnih parametrov sliv.

Ključne besede: sadjarstvo, prehrana in rast rastlin, Prunus salicina, topne snovi

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1 INTRODUCTION

The plum tree is a plant that is part of the botanical family Rosaceae and the genus *Prunus*. Commercially, two species of this plant stand out: *Prunus salicina* Lindl., known as the Japanese plum, and *Prunus domestica* L., known as the European plum. However, the Japanese variety is more common in Brazil due to its adaptability to milder climates (Dalbó et al., 2021), while the European hexaploid variety (2n = 48) requires colder climatic conditions to thrive (Mayer et al., 2019; Castro et al., 2008).

In Brazil, it is not known precisely when this crop was introduced (Eidam et al., 2012), but it has been gaining prominence in recent years due to consumers' good acceptance of the fresh fruit (Castro et al., 2008). Data from IBGE (2017) show that the country produced 45,614 t of plums in an area of 3,837 ha. Of this production, 76.6 % is concentrated in the South region. The State of Rio Grande do Sul is the largest national producer, with 21,097 t produced in an area of 1,623 ha. In the state, the municipality of Caxias do Sul is the leading producer, responsible for 7,949 t, followed by Antônio Prado and Ipê, which produce 3,143 t and 1,326 t, respectively.

Among the most important cultivars is 'Gulfblaze', an early maturing cultivar, generally beginning to mature in mid-November. The plants require cross-pollination and are semi-vigorous, with an open growth habit and low winter cold requirements. The fruits are round and medium in size, around 49 mm in diameter, with an average mass of 50–65 g. The pulp is yellow, and the skin is red, with a sweet-acid flavor but with acidity and bitterness in the skin (Dalbó et al., 2021; Simonetto et al., 2007).

Farmers have adopted new technologies in their cultivation to meet consumer market demand and improve fruit production and quality. These innovations have proven especially relevant to mitigate the challenges arising from climate fluctuations observed in recent years (Junges et al., 2020). In Rio Grande do Sul, a state known for its high levels of rainfall, it is typical for periods of drought to occur due to the irregular distribution of rainfall, especially in crucial phases of the cultivation cycle, such as the growth until complete maturation of the fruits, harming fruit quality (Nachtigall & Hawerroth, 2023).

Water scarcity during fruit growing can affect fruit size, bud formation for the following year, and nutrient absorption (Nachtigall et al., 2012). Therefore, when the amount of water from rainfall is insufficient relative to potential evapotranspiration, it becomes essential to resort to irrigation to provide the water necessary for the crop cycle (Vélez, 2012). This practice ensures adequate availability of moisture, which is essential for plant development and fruit quality (Cechinel et al., 2022; Testezlaf, 2017).

Aiming to optimize the use of this system, many producers have adopted the fertigation technique, which consists of applying fertilizers with irrigation water (Pramanick et al., 2022; Septar & Stoli, 2019). This technique improves cultivation efficiency and contributes to the preservation of the environment, promoting sustainability in agriculture. Furthermore, fertigation represents a relevant alternative to dealing with the labor shortage in the field (Nachtigall, 2016; Soto & Parra et al., 2016).

Coelho et al. (2011) emphasize that localized irrigation, frequently used in fruit growing, restricts the root system of plants to the area wetted by the emitter. Therefore, it is necessary to provide nutrients to maintain production regularly. In this sense, fertigation stands out, as it allows the application of fertilizers to be divided into parts and more effective distribution in the area with a greater concentration of roots, reducing losses compared to the conventional system.

Furthermore, according to Silva et al. (2016), in the States of Rio Grande do Sul and Santa Catarina, the determination of nitrogen, phosphorus, and potassium doses is based on the levels present in the leaves and the expected productivity. However, Brunetto et al. (2011) highlight some drawbacks, such as not considering the increase in planting density and the nutritional requirements of the plants, the effects on the chemical and physical characteristics of the fruits, and the recommendation of fixed doses for productivity ranges when considering specific aspects of the location, like previous cultures. According to Mayer et al. (2019), the trend in contemporary fruit growing is to increase planting density in orchards, significantly increasing productivity. However, it is important to highlight that few studies in the specific literature are dedicated to adapting fertilizer levels, especially in high-density orchards. These factors can reduce the efficiency of applied fertilizers and exceed the plant's nutritional needs, which can increase production costs and cause future problems in the orchard.

Agricultural practices that combine irrigation and nutrition offer significant potential to increase productivity and quality, making production viable even in areas with low soil fertility and limited water availability. However, despite its evident benefits, its use is restricted due to inadequate technical training. Furthermore, farmers frequently have doubts regarding the economic return on this investment (Nachtigall, 2016).

In this context, the objective was to evaluate the effect of applying different doses of fertilizers, using fertigation, on fruit quality and nutritional status of the plum tree cultivar 'Gulfblaze' located in the Serra Gaúcha region, Southern Brazil.

2 MATERIAL AND METHODS

The experiment was conducted from July to November 2019 in a commercial orchard located in the municipality of Antônio Prado, in the ecoclimatic region of the Serra do Nordeste of Rio Grande do Sul, Southern Brazil (geographical coordinates are 28º50' S and 51º24' W, with an altitude of 673 m). The climate of this region is characterized as subtropical of the Cfb type, with mild summers, cold winters, and uniform rainfall throughout the year. The average annual rainfall is 1,700 mm, and the average temperature is 16.6 °C (Wrege et al., 2012).

The plum cultivar used was 'Gulfblaze', also known as Fla, in a five-year-old orchard on Okinawa rootstock. The plants were spaced 5.0 m between rows and 2.5 m between plants, with a planting density of 800 plants per hectare, cultivated in a cup system. The pollination system was based on using the Polli Rosa pollinator cultivar, implemented in a proportion of 10 %, being used exclusively as a pollinator. The orchard's production was intended for fresh consumption.

The following treatments were evaluated: T1 – Irrigation only, without fertilizer application; T2 – Fertigation with 50 % of the dose recommended by CQFS-RS/SC (2016); T3 – Fertigation with 100 % of the dose recommended by CQFS-RS/SC (2016); T4 – Fertigation with 150 % of the dose recommended by CQFS-RS/SC (2016); T5 – Fertigation with 200 % of the dose recommended by CQFS-RS/SC (2016). The experimental design was in randomized blocks with five treatments and three replications. Each plot consisted of 26 plants; five were used for evaluations. In these, two branches per plant were previously marked for later evaluations.

Before implementing the experiment, soil samples were collected in the 0–20 cm and 20–40 cm layers and were sent for chemical and fertility analysis. These analyzes presented the following average results: pH in water: 6.7; SMP Index: 6.5; H + Al: 2.5 $cmol_c·dm^{-3}$; H: 2.46 $cmol_c·dm^{-3}$; organic matter content: 3.2 % w/v; clay: 29 % w/v; P: 230.7 $mg·dm^{-3}$; Na: 11.0 $mg·dm^{-3}$; K: 279.0 $mg·dm^{-3}$; Ca: 12.9 $cmol_c·dm^{-3}$; Mg: 1.9 $cmol_c·dm^{-3}$; Al: zero; base saturation: 15.6 $cmol_c·dm^{-3}$; effective cation exchange capacity (CTC): 15.6 $cmol_c·dm^{-3}$; CTC at pH 7: 18.03 $mg·dm^{-3}$; base saturation (V): 86.4 %; Al saturation (m): zero; S: 7.4 $mg·dm^{-3}$; Cu: 2.3 $mg·dm^{-3}$; Zn: 22.0 $mg·dm^{-3}$; B: 0.5 $mg·dm^{-3}$; Mg: 1.2 $mg·dm^{-3}$; Ca/Mg: 6.7; Ca/K: 18.2; Mg/K: 2.7; Ca/CTC: 71.8; Mg/CTC: 10.7 and K/CTC: 3.9. The nutrient doses for fertigation were defined based on the results of the soil analysis and the recommendations of the Soil Chemistry and Fertility Commission of Rio Grande do Sul and Santa Catarina (CQFS-RS/SC, 2016) for the plum tree. The dose recommended by CQFS-RS/SC (2016), based on organic matter content in the soil, and, thus, N content, was 80 $kg·ha^{-1}$ N, 60 $kg·ha^{-1}$ K, 40 $kg·ha^{-1}$ P, 30 $kg·ha^{-1}$ Ca, and 10 $kg·ha^{-1}$ Mg, corresponding to a 100 % dose (T3).

Fertigation was carried out every two weeks from August 4, totaling eight applications, 14 days apart, and the fertilizers were distributed equally in each application. The sources of nitrogen (N), phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg) used were mono ammonium phosphate – Krista MAP (61 % P_2O_5 + 12 % N); potassium sulfate – Krista SOP (51 % K_2O + 18 % S); calcium nitrate – Calcinit (18 % Ca + 15.5 % N); magnesium nitrate – Krista MAG (11 % N + 9.3 % Mg) and mixed mineral fertilizer – Krista K (43 % K_2O + 12 % N + 1.0 % S + 1.0 % Mg).

The orchard had a drip irrigation system with two hoses per row and a spacing of 0.50 m per dripper. Irrigation management was carried out based on soil moisture, measured using two tensiometers installed at 0–20 cm, 20–40 cm deep, and 50 cm away from the plants. Irrigations occurred when the average soil water tension was lower than -10 kPa. The average water tension in the soil was considered to be between zero and -10 kPa, suitable for the crop, and irrigation was not carried out when it was within these established limits.

Phytosanitary treatments (fungicides, insecticides, and acaricides) were applied during the production cycle and based on weekly monitoring. Winter pruning was carried out in June, and manual fruit thinning was carried out in October, aiming to maintain 700 fruits per plant.

Evaluations were carried out during the experiment until fruit harvest, covering the period from October to November 2019. Two fruits were selected and identified on previously marked branches, totaling ten fruits per plot, for weekly evaluation of the diameter and length of the fruits after harvesting. Winter pruning was carried out in June, and manual fruit thinning was carried out in October, aiming to maintain 700 fruits per plant. The fruits' longitudinal and sagittal diameters and length were measured with a digital caliper, expressing the results in millimeters (mm). The daily fruit growth rate was calculated as the difference between the final and initial values divided by the number of days passed, and the results were expressed in millimeters per day ($mm·day^{-1}$).

Plant tissue samples were collected on November 22, 2019. One hundred complete leaves were collected per treatment from previously marked plants in the middle portion of the branches emitted during the year on the four legs of the plant. Subsequently, the samples were sent to the Soil Chemistry and Fertility Laboratory of the University of Caxias do Sul to verify the levels of macro and micronutrients in the leaf tissue according to the methods described by Tedesco et al. (1995).

Thirty complete leaves were collected per treatment from previously identified plants to determine the average leaf area. These leaves were selected from the middle part of the branches that grew in the year in question on the four legs of each plant. Leaf area measurement was carried out by digitizing leaf images using an AM350 leaf area meter. The results obtained were expressed in square millimeters (mm^2).

A representative sample of ten fruits per plot was collected for fruit quality analyses on November 25, 2019. With a precision electronic scale, the average mass obtained was evaluated by the quotient between the mass and the number of 10 fruits, with the data expressed in grams per fruit ($g \cdot fruit^{-1}$).

The firmness of the pulp was determined using a digital penetrometer with an 11 mm tip. After removing the epidermis with a superficial cut of two discs of approximately 1.0 cm in diameter on diametrically opposite sides in the equatorial portion of the fruit, the reading was carried out, and the results were expressed in kilograms-force (kg f). The diameter and length of fruits were measured with a digital caliper, positioning the fruits in a vertical position to measure length and horizontally for diameter. The results were expressed in millimeters (mm).

Afterward, the fruit juice was extracted with an electric centrifuge to determine soluble solids (SS), titratable acidity (TA), and SS/TA ratio. The soluble solids content was determined with an analog refractometer, and the data were expressed in degrees Brix (°Bx). The titratable acidity was determined by titrating 2.0 ml of the sample juice diluted in 50 ml of distilled water and adding three drops of phenolphthalein indicator to acquire a pink color, proceeding to titrate the sample with sodium hydroxide solution (NaOH 0.1 N), with a glass burette until reaching pH 8.1 (turning point) and the results were expressed as a percentage (% w/v) of malic acid. The ratio was calculated as the quotient between the soluble solids content and the titratable acidity. The determinations followed methods described by the Instituto Adolfo Lutz (1985).

The data obtained were subjected to analysis of variance (ANOVA), and the means were compared using the Scott-Knott test, with a significance level of 5 % probability of error, using the statistical program System for Analysis of Variance (SISVAR).

3 RESULTS AND DISCUSSION

The data regarding the growth rates of plum fruits depending on the fertigation doses applied are compiled in Figure 1.

For the growth rate regarding the diameter of the longitudinal and sagittal axis of the fruits, the plants subjected to the fertigation dose of 200 % of the recommendation by CQFS-RS/SC (2016), presented higher values than the other treatments, followed by treatment 150 % of recommendation. However, the lowest daily growth rate values were observed in plants that did not receive any fertilization throughout the production cycle. The daily fruit growth rate for length was higher at the two

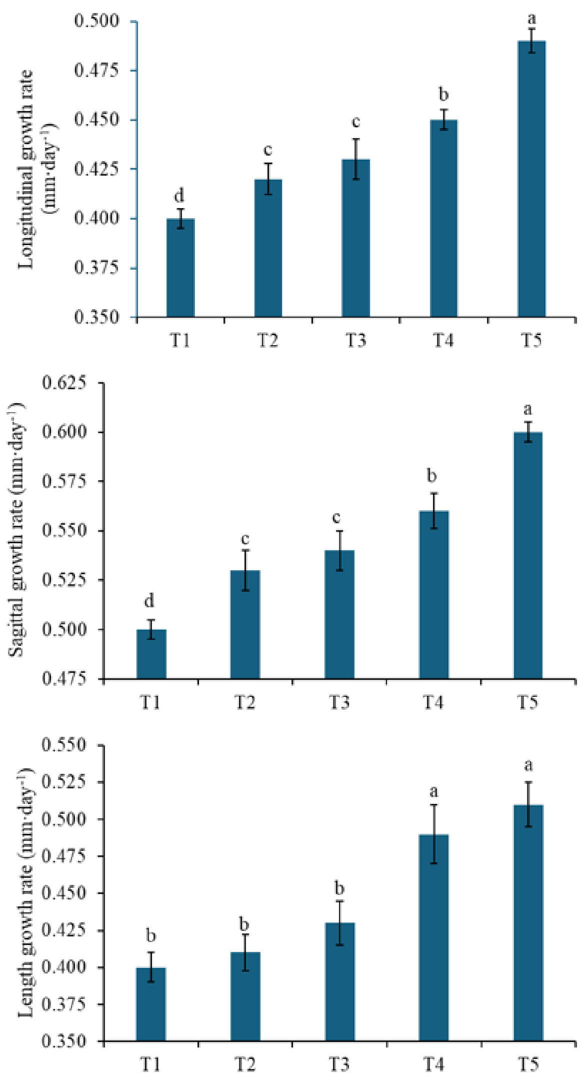

Figure 1: Daily growth rate of ‚Gulfblaze' plum fruits subjected to different doses of fertigation during the 2019/2020 cycle, Antônio Prado, RS. Means followed by the same lowercase letter do not differ statistically using the Scott-Knott Test at a 5 % error probability. T1 – control; T2 – 50 % of the recommendation; T3 – 100 % of recommendation; T4 – 150 % of recommendation; T5 – 200 % of recommendation. Coefficient of variation: Longitudinal: 3.14 %; Sagittal: 2.93 %; Length: 4.37 %.

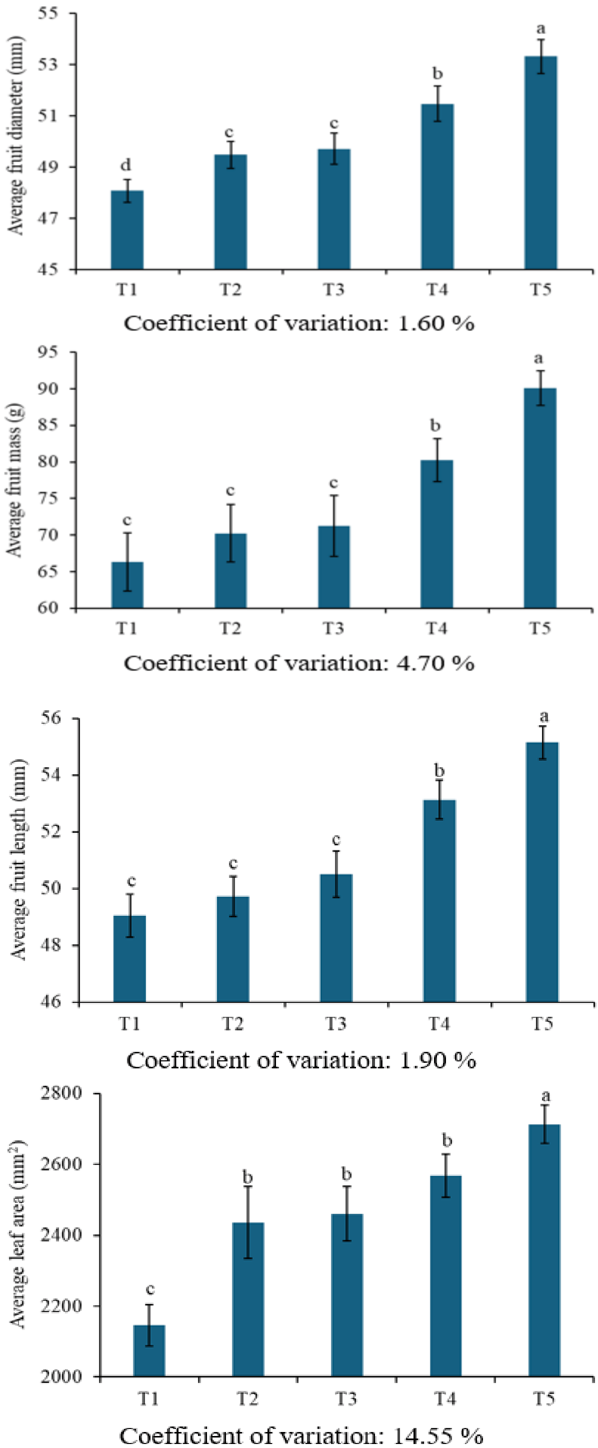

Figure 2: Biometric parameters of plums and leaf area of 'Gulfblaze' plum trees subjected to different doses of fertigation during the 2019/2020 cycle, Antônio Prado, RS. Means followed by the same lowercase letter do not differ statistically using the Scott-Knott Test at a 5 % error probability. T1 – control; T2 – 50 % of the recommendation; T3 – 100 % of recommendation; T4 – 150 % of recommendation; T5 – 200 % of recommendation.

highest doses, with 0.51 mm·day^{-1} and 0.49 mm·day^{-1}, respectively. In the control, doses of 50 % and 100 % of the recommendation showed the lowest growth rates.

The increase in the concentration of mineral nutrients, combined with the water availability provided by drip irrigation, increased the fruit growth rate. This phenomenon appears to be directly linked to the increase in the photosynthetically active surface of the plant, that is, to the expansion of the leaf area, which, in turn, resulted in greater production and availability of photoassimilates for the growth of plums (Pramanick et al., 2022). Data relating to biometric parameters of fruit diameter and length, average mass, and plant leaf area are compiled in Figure 2.

At harvest time, the fruit diameter, length, and leaf area variables were affected by fertigation treatments. For all these variables, it was observed that the increase in nutrient doses applied via fertigation increased these variables, positively affecting them. The fertigation treatment with 200 % of the dose recommended by CQFS-RS/SC (2016) was superior to the others. The control had a smaller fruit diameter and length at harvest than the other fertigation treatments, with 48.08 mm and 49.05 mm, respectively. On the other hand, the performance of 200 % and 150 % doses were 10.9 % and 7.1 %, higher than the control for fruit diameter and 12.4 % and 8.3 % for length, respectively. Chagas (2008) reported, for the cultivar ‚Gulfblaze‘, 48.3 mm in diameter and 46.1 mm in length of fruits in the 2007/2008 cycle in Jundiaí, SP, when characterizing cultivars with low cold requirements for subtropical regions of the state. For diameter, this result was greater than that observed in the control but lower than the treatments subjected to fertigation.

Fertigation treatment with 200 % of the dose recommended by CQFS-RS/SC (2016) resulted in the largest average leaf area (2,713 mm^2). In contrast, in the irrigation-only treatment, the plum trees had a smaller leaf area (2,146 mm^2). There was no difference between treatments with 50 %, 100 %, and 150 % doses. These results demonstrate a directly proportional relationship between leaf area and the quality parameters of fruit diameter and length, considering that an increase in leaf area was associated with an increase in the values of these parameters. Such a result may be related to the greater leaf area per plant, which may have provided greater availability of photoassimilates for the fruits. According to Franganito (2014), the leaf area and the functioning of the stomata influence productivity, as the first determines the interception of light, and the second controls the absorption of CO_2, an important drain of water and nutrients for the fruits.

However, contrary to what was observed in the present study, Chagas et al. (2016), when evaluating the effect of irrigation and fertigation on the leaf area of ‚Maxi Gala' and ‚Fuji Suprema' apple trees in the 2015/2016 harvest using conventional fertilization as treatments; conventional fertilization plus irrigation; irrigation plus fertigation and fertigation, no significant difference was observed between the treatments applied. Ferreira et al. (2018), when evaluating the effect of nitrogen fertilization (zero, 30 kg·ha^{-1}, 60 kg·ha^{-1}, and 120 kg·ha^{-1}) on peach trees ‚Cascata 1543' and ‚Cascata 1067' in an experiment conducted between 2012 and 2015 in the municipality of Pelotas, RS, found that the increase in nitrogen application promoted an increase in leaf area, being observed at a dose of 120 kg·ha^{-1} the largest average leaf area (1,882 mm^2) compared to the control (1,230 mm^2).

The leaf area is important for understanding photosynthesis, respiration, nutrient use, flowering, fruiting, and productivity (Demirsoy et al., 2004). Nitrogen is the nutrient most plants require, and those that most influence this variable since it is incorporated into the plant in the form of amino acids once absorbed from the soil solution. Thus, as the supply of this nutrient increases, proteins synthesized through amino acids promote leaf growth, increasing the photosynthetic area. Furthermore, nitrogen is involved in chlorophyll synthesis, and its deficiency decreases this synthesis. In situations with nutrient deficiency, there are losses in the photosynthetic process, reducing the efficiency of using sunlight and, consequently, the execution of essential functions, such as nutrient absorption (Dechen & Nachtigall, 2007).

From the results, it can be seen that, with the increase in the amount of nutrients made available to the plants, an expansion of the leaf area was obtained due to the greater photosynthetic area, resulting in a greater capacity to reallocate photosynthates and provide nutrients to the fruits, promoting greater potential for their growth.

For the variable average fruit mass, it was found that the plants that were subjected to fertigation doses of 200 % and 150 % of the recommendation had a mass increase of 35.8 % and 28.2 %, respectively, relative to the control (66.35 g·fruit^{-1}), which did not differ from the recommended doses of 50 % and 100 % (Figure 2). These results were superior to those obtained by Oliveira et al. (2019), who found fruits with an average mass of 60.0 g and 41.1 g, evaluating plum trees in Veranópolis, RS, classified plums with an average mass of less than 50 g as small, fruits with an average mass between 50–65 g as medium and fruits with an average mass of more than 65 g as large. With this classification, the fruits of the present study were considered large.

The greater average fruit mass increased fruit production and productivity, as observed in treatments with 150 % and 200 % of the CQFS-RS/CS (2016) recommendation. Considering the commercial scale of production, the average size of the fruits plays a crucial role in the viability of an orchard, as it directly impacts productivity and profitability. This is due to the potential to increase production per plant and positively influence the prices negotiated for larger caliber fruits on the market (Petri et al., 2016).

Considering that the average quantity of fruits kept on the plant after manual thinning is 700, it is possible to estimate the average production and productivity of the treatments. Using 100 % of the fertilizer recommendation according to CQFS-RS/SC, the estimated production and productivity are 49.8 kg·plant^{-1} and 39.8 t·ha^{-1}, respectively. On the other hand, with 200 % application of the recommendation, estimated production and productivity reach 63.1 kg·plant $^{-1}$ and 50 t·ha^{-1}, respectively, increasing 10.6 t·ha^{-1}, a 26.6 % increase relative to the recommended dose (T3). This result demonstrates the high productive capacity of the ‚Gulfblaze' cultivar, providing fruits with a high caliber when subjected to increased nutrient supply and under adequate water availability.

Data on the nutrient contents in the leaf tissue of the plum trees as a function of each fertigation regime is compiled in Table 1.

It is essential to highlight the increase in leaf potassium and nitrogen content, which, in high availability,

Table 1: Contents of nutrients in the leaf tissue of ‚Gulfblaze' plum trees subjected to different doses of fertigation during the 2019/2020 cycle, Antônio Prado, RS.

Treatment	N	P	K	Ca	Mg	S	Zn	Cu	Mn	Fe	B
			g·kg^{-1}						mg·kg^{-1}		
Control – T1	29.9	2.1	28.5	26.7	6.1	1.1	27.6	81.4	81.8	116.9	64.6
50 % – T2	29.3	2.3	24.9	25.7	6.2	0.7	28.1	96.8	84.8	120.2	60.5
100 % – T3	30.8	2.0	26.5	25.1	5.5	2.0	27.3	93.4	77.0	121.3	60.5
150 % – T4	29.8	1.7	20.3	18.7	4.2	1.5	20.1	76.4	72.2	94.1	57.7
200 % – T5	31.1	2.2	31.6	23.1	5.1	1.0	33.3	99.5	101.6	148.9	62.4

are related to the increase in fruit size. This is an important qualitative characteristic, as it adds value to the product, especially for fresh consumption. According to Dechen and Nachtigall (2007), adding nitrogen increases the availability of photosynthates to the fruits, providing greater plant growth and productive potential. For this, potassium is essential, as it helps in photosynthesis and, consequently, in cellular respiration and the accumulation of carbohydrates in the plant's tissues.

Furthermore, fruit size is determined by the genetic characteristics of each cultivar. Other management factors also influence it in the orchard besides nutrition and irrigation, such as pruning and thinning practices and applying phytoregulators. It is also important to consider the environmental and climatic conditions to which the orchard is subjected (Giovanaz et al., 2014).

The results of the present work differ from those observed by Cechinel et al. (2022) in ‚Kinkas' apple trees grown in São Joaquim, SC, during the 2014/2015 productive season. When evaluating the effect of irrigation and fertigation (conventional solid fertilization; conventional solid fertilization plus irrigation; fertigation plus irrigation and fertigation), they observed no difference in the treatments relative to the average fruit mass. Similarly, Dolinski et al. (2007), when evaluating the effect of different doses of nitrogen fertilizer (40 kg·ha^{-1}·year^{-1}, 80 kg·ha^{-1}·year^{-1}, 120 kg·ha^{-1}·year^{-1}, 160 kg·ha^{-1}·year^{-1}, and 200 kg·ha^{-1}·year^{-1}) and potash (55 kg·ha^{-1}·year^{-1}, 110 kg·ha^{-1}·year^{-1}, and 200 kg·ha^{-1}·year^{-1}) in the ‚Reubennel' plum tree in the region of Araucária, PR, in three consecutive years (2003, 2004, and 2005), the variable of average mass per fruit showed no difference between the evaluated treatments, with general averages in the range from 53 – 65 g·fruit^{-1}. The authors associated the lack of response to the high concentration of potassium in the soil in the study area and the supply of part of the nitrogen demand by soil organic matter, the natural presence of white clover, and rainwater. In the present study, it was observed that, even in fertile soil, this variable was affected by the treatments applied.

Nitrogen fertilization increases growth potential by increasing individual fruit mass at maturity and total fruit production (Saenz et al., 1997). On the other hand, the primary influence of potassium fertilization on the increase in average fruit mass is poorly understood. However, this nutrient is believed to participate in metabolic activities related to synthesizing and transporting carbohydrates and water to fruits, resulting in greater fruit mass (Aular & Natale, 2013). This may explain the greater average fruit mass with increasing nutrient doses in fertigation.

The results regarding the quality parameters of the evaluated fruits are presented in Figure 3.

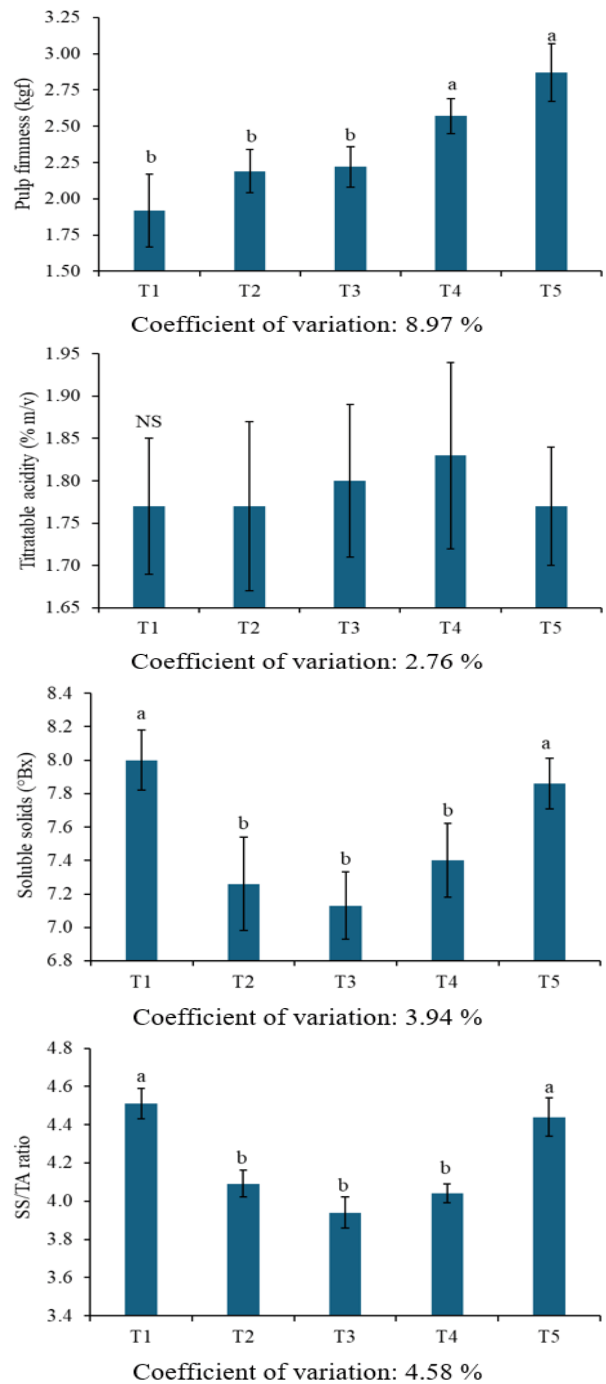

Figure 3: Results of pulp firmness, soluble solids content, titratable acidity, and SS/TA ratio of fruits from ‚Gulfblaze' plum trees subjected to different doses of fertigation during the 2019/2020 cycle, Antônio Prado, RS. Means followed by the same lowercase letter do not differ statistically using the Scott- Knott Test at a 5 % error probability. NS: not significant by the ANOVA. 1 – titratable acidity expressed as gram equivalents of malic acid per 100 ml of juice. T1 – control; T2 – 50 % of the recommendation; T3 – 100 % of recommendation; T4 – 150 % of recommendation; T5 – 200 % of recommendation.

There was no difference between treatments concerning the physical-chemical parameter of titratable acidity. However, pulp firmness, soluble solids content, and SS/TA ratio were influenced by fertigation doses. The highest values of pulp firmness were observed in 200 % fertigation treatments, followed by treatment 150 % of the recommendation, which was superior to the other treatments and did not differ from the control. The two highest doses promoted an increase of 49.5 % and 33.9 %, respectively, relative to the control. According to Crisosto et al. (2001), the minimum pulp firmness for harvesting plums is 2.65 kg f to avoid damage due to impact during handling and storage. Considering the classification and subsequent commercialization stages, fruits with pulp firmness in the 1.32–2.65 kgf range are deemed suitable. For consumption, plums must have firmness in the 0.81–1.32 kgf range, not less than 0.81 kgf.

A divergent result was observed by Branco et al. (2016), who found greater pulp firmness in the control when evaluating , Kinkas , apple trees in the 2013-2014 harvest in São Joaquim, RS, under the effect of applying the same amounts of nutrients between conventional fertilization, irrigation plus conventional fertilization, irrigation plus fertigation and in fertigation. The results were associated with the smaller fruit size in the control.

A likely explanation for the results obtained in the present study regarding pulp firmness was the observation that the control treatment was at a more advanced stage of maturation at the harvest stage. For this, the color of the fruit epidermis observed in the field during the experiment, the firmness of the pulp, and the soluble solids content obtained in the analyses were considered. Pessoa (2016) highlighted that it is natural to reduce the firmness of the pulp as the plum matures since the degradation of complex carbohydrates, such as pectic substances, cellulose, and hemicellulose, results in changes to the cell wall and causes the pulp to soften. Therefore, this quality attribute is vital for the post-harvest conservation of fruits.

Regarding the soluble solids content, the control (T1) and the dose of 200 % of the fertigation recommendation (T5) performed similarly, while the other treatments did not differ. Anzanello and Menin (2018), when evaluating the phenology, production, and quality of fruits of potential plum cultivars for the Serra Gaúcha region in the 2014/2015 harvest, reported a soluble solids content of 9.5 °Brix for the ‚Gulfblaze' cultivar, this value being higher than that found in the present study. On the other hand, in a study conducted by Vargas et al. (2017), this variable was not affected when evaluating the effect between rainfed, irrigated, fertigation A treatments (based on a nutrient extraction rate of 10 kg·ha^{-1}, 15 kg·ha^{-1}, and 20 kg·ha^{-1} of N, P, and K) and fertigation B (based on a nutrient extraction rate of 53 kg·ha^{-1}, 13 kg·ha^{-1}, and 55 kg·ha^{-1} of N, P, and K) in apples ‚Galaxy', in the Campos de Cima da Serra region, RS.

The smaller leaf area in the control may have provided greater sunlight on the fruits and, consequently, greater accumulation of sugars. According to Feliciano et al. (2010), fruits with greater exposure to the sun tend to increase the soluble solids content. Another factor observed was the higher leaf potassium content in the control and the higher dose treatment (Table 1), which may have influenced this variable. K fertilization plays a fundamental role in the biosynthesis of sugars and carbohydrates, favoring an increase in the concentration of soluble solids in the fruit pulp since this nutrient helps transport carbohydrates (Jawandha et al. 2017). However, Barreto et al. (2020) did not observe any influence of increasing doses of potassium on color, pulp firmness, soluble solids, juice pH, total phenolic compounds, and antioxidant activity of peaches ‚Sensation' in soils with a high content of this nutrient.

Furthermore, the more advanced maturation observed in the control may have affected the soluble solids content since, according to Argenta (2006), as the fruit ripens, the soluble solids content increases as a result of starch hydrolysis and other reserve substances present in the fruits.

Titratable acidity was not influenced by fertigation treatments, obtaining an average of 1.8 % w/v of malic acid. Chagas (2008), when evaluating the development of cultivars with low cold requirements for subtropical regions of the state of São Paulo in the 2007/2008 harvest, obtained for the cultivar ‚Gulfblaze' 1.0 % w/v of malic acid for the same harvest time of the present study.

Similar behavior to the soluble solids content was observed for the SS/TA ratio, in which the control and fertigation treatment of 200 % of the recommendation presented superior results. In the other treatments, the lowest results were obtained, not differing. Queiroz (2014) observed higher values, reporting an SS/TA ratio of 5.98 for the ‚Gulfblaze' cultivar when characterizing plum tree genotypes in the Central Depression physiographic region of the State of Rio Grande do Sul in 2013.

The SS/TA ratio is one of the leading indicators of fruit quality, as it is related to the flavor of the fruit. This relationship indicates that the higher the value, the sweeter and less acidic the fruits are (Chitarra & Chitarra, 2005). Therefore, as the titratable acidity values were not affected by the treatments, but the soluble solids values were, the SS/TA results' behavior was arranged the same way as those found for the soluble solids content.

Therefore, it is crucial to understand the productive performance and quality of plum trees subjected to fertigation. This is because a combination of physical and chemical characteristics of the fruits supplied determines consumer acceptance and commercial value of plums. Buyers and consumers prefer larger fruits with redder skin and a sweeter flavor. Consequently, obtaining larger fruits, as observed in fertigation treatments, can result in higher profitability for the producer, making this management a crucial practice in the orchard. Furthermore, the incorporation of new driving systems and the increase in plant density in the most recent production areas indicate the need to increase the amount of fertilizers supplied throughout the Japanese plum production cycle. This aims to achieve fruits with superior caliber and high productivity. Furthermore, ensuring available water during critical periods, even in low rainfall, promotes nutrient absorption and adequate plant growth.

4 CONCLUSIONS

Increasing the dose of nutrients in fertigation for the ‚Gulfblaze‘ plum tree increased the daily fruit growth rate. Fertigation doses of 150 % and 200 % of the dose recommended by CQFS increased production and improved fruit quality, while plants that were not subjected to fertigation and those subjected to a dose of 200 % of the recommendation had a higher content of soluble solids and SS/AT ratio, indicating better performance concerning consumer preferences.

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Pesticide residues in vegetables-validation of the gas chromatography-tandem mass spectrometry multiresidual method and a survey of vegetables on Slovenian market

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Pesticide residues in vegetables-validation of the gas chromatography-tandem mass spectrometry multiresidual method and a survey of vegetables on Slovenian market

Abstract: An analytical method for determining pesticide residues in vegetables was introduced and validated. The extraction was conducted using acetone, dichloromethane and petroleum ether, to enable extraction of active substances with a wide range of polarity. Determination was conducted using gas chromatography coupled with tandem mass spectrometry. Method is according to extraction efficiency and determination sensitivity comparable to other methods for determination of pesticide residues such as QuEChERS. The method was applied in practice. A total of 35 active substances (pesticides) were sought in 50 vegetable samples gathered from Slovenian stores. The active substances sought were not determined in 86.0 % of the samples analysed. Among positive samples in carrot boscalid and fluopyram were found. 21.4 % of carrot samples were positive. In lamb`s lettuce boscalid and fludioxonil were determined. 50 % of lamb`s lettuce samples were positive. In pepper boscalid, fluopyram and pyraclostrobin were found. 16.7 % of pepper samples were positive. In tomato flonicamid, fluopyram and tebuconazole were determined. 11.1 % of tomato samples were positive. The results were compared with those from the literature and the outcome was that vegetables from Slovenia contained boscalid, fluopyram, pyraclostrobin and tebuconazole, which were found also in China, Italy and Turkey.

Key words: vegetables. GC-MS/MS, pesticide residues. multiresidual method

Ostanki fitofarmacevtskih sredstev v zelenjavi - validacija multirezidualne metode s plinsko kromatografijo sklopljeno s tandemsko masno spektrometrijo in preiskava zelenjave na trgu v Sloveniji

Izvleček: Uvedli in validirali smo analizno metodo za določanje ostankov fitofarmacevtskih sredstev v zelenjavi. Ekstrakcijo smo izvedli z acetonom, diklormetanom in petroletrom, ter s tem omogočili ekstrakcijo aktivnih snovi s širokim razponom polarnosti. Določitev smo izvedli s plinsko kromatografijo sklopljeno s tandemsko masno spektrometrijo. Metoda je glede na učinkovitost ekstrakcije in občutljivost pri določitvi, primerljiva z drugimi metodami za določevanje ostankov fitofarmacevtskih sredstev kot je QuEChERS metoda. Metodo smo uporabili v praksi. V 50 vzorcih zelenjave iz slovenskih trgovskih polic smo določali skupno 35 aktivnih spojin (pesticidov). Iskanih aktivnih snovi nismo določili v 86,0 % analiziranih vzorcev. Med pozitivnimi vzorci smo v korenju našli boskalid in fluopiram. 21,4 % vzorcev korenja je bilo pozitivnih. V motovilcu smo določili boskalid in fludioksonil. 50 % vzorcev motovilca je bilo pozitivnih. V papriki smo našli boskalid, fluopiram in piraklostrobin. 16,7 % vzorcev paprike je bilo pozitivnih. V paradižniku smo določili flonikamid, fluopiram in tebukonazol. 11,1 % vzorcev paradižnika je bilo pozitivnih. Rezultate smo primerjali z literaturnimi podatki in ugotovili, da je zelenjava v Sloveniji vsebovala boskalid, fluopiram, piraklostrobin in tebukonazol, ki so jih določili tudi na Kitajskem, v Italiji in Turčiji.

Ključne besede: zelenjava, GC-MS/MS, ostanki fitofarmacevtskih sredstev, multirezidualna metoda

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1 INTRODUCTION

Vegetable is important source of nutrients, vitamins and fibers and is consumed daily by human population. To produce it in quantities large enough for whole population, farmers have to use plant protection products (PPPs) to protect it against numerous diseases and insects attacking vegetables. But demanding consumers require not only healthy but also safe food. Therefore it is important to monitor PPPs residues in food products on the market.

Numerous analytical methods have been developed to analyse PPPs residues in food. In the past there were three main routes for extraction procedure: acetone (Díez et al., 2006, Pizzutti et al., 2009), ethyl acetate (Sharif et al., 2006) and acetonitrile (Anastassiades et al., 2003, Lehotay and Maštovska, 2005, Lehotay, 2007). Nowadays most of laboratories use Quick Easy Cheap Effective Rugged and Safe method also called QuEChERS method, where acetonitrile is used (Calderon et al., 2022, Ngabirano and Birungi, 2022, Sahyoun et al., 2022, Tankiewicz and Berg, 2022). The advantage of this method is, that it is less time consuming and needs lower volumes of organic solvent. In our laboratory we are using method with acetone, to which dichloromethane and petroleum ether were added so that active substances of wide range of polarity can be extracted (Baša Česnik and Gregorčič, 2003, Baša Česnik et al., 2006) from very polar (for instance, flonicamid) to non-polar (for instance, cyhalothrin-lambda). In this paper we present simplified extraction procedure with the same three solvents, which is similarly as the QuEChERS method less time consuming and needs lower volumes of organic solvents as previous one.

Determination of PPPs residues is nowadays usually performed using gas chromatography coupled with mass spectrometry (GC-MS) (Knežević and Serdar, 2009, Santarelli et al., 2018), gas chromatography coupled with tandem mass spectrometry (GC-MS/MS) (Calderon et al., 2022, Ngabirano and Birungi, 2022, Sahyoun et al., 2022, Tankiewicz and Berg, 2022) and/or liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) (Balkan and Yilmaz, 2022, Qin et al., 2021). The most sensitive is tandem mass spectrometry, which was also used in our laboratory.

Numerous authors have analysed pesticide residues in vegetables with GC-MS/MS. Calderon et al. (2022) analysed 22 active substances in vegetables from Chile and Mexico. Ngabirano and Birungi (2022) analysed 1 active substance in vegetables from Uganda. Sahyoun et al. (2022) tested vegetable samples from France and Lebanon for 14 active substances. Tankiewicz and Berg (2022) introduced a method for determining 31 active substances in Polish vegetables. Up to 11 of active substances sought in these studies were introduced in our study as well. Our selection of active substances was based on both, those authorised for use in Slovenia (94.3 %) and those not authorised for use in Slovenia, but authorised in previous years (5.7 %), the latter to cover misuse of PPPs Of those selected, 57.2 % were fungicides, 25.7 % were acaricides and/or insecticides and 17.1 % were herbicides.

The purpose of this paper is to present the multiresidual GC-MS/MS method introduced for identifying 35 active substances in vegetables using acetone, dichloromethane and petroleum ether as the extraction solvent. The validation parameters for lettuce, potato and tomato are summarised, as well as the practical use of the method on 50 samples of vegetables gathered from Slovenian stores. The most problematic were carrot, where boscalid and fluopyram were found, lamb`s lettuce where boscalid and fludioxonil were determined, pepper where boscalid, fluopyram and tebuconazole were found and tomato where flonicamid, fluopyram and tebuconazole were determined. Concentrations of active substances were in range 0.005–0.060 mg kg^{-1}. All concentrations were below valid Maximum Residue Levels (MRLs). The contents of pesticide residues were compared with those from the literature. Finally, a risk assessment for consumers was conducted.

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

The certified standards were supplied by Dr. Ehrenstorfer (Augsburg, Germany). The acetone - p.a. grade, dichloromethane – p.a. grade, petroleum ether – p.a. grade (used for the extraction procedure) and acetone

HPLC-grade (used for preparation of standards) were supplied by J.T.Baker (Deventer, Netherlands). All other chemicals used were supplied by Sigma-Aldrich (Steinheim, Germany). The water used was MilliQ deionised water.

2.1.2 Preparation of the solutions

Stock solutions in acetone of individual active substances were prepared with the concentrations of 625 µg

Table 1: The active substances sought, their activity type, MRM transitions, dwell time and collision energy

Active substance	Activity type[a]	MRM transitions (Q1, Q2, Q3, Q4)[b]	Dwell (ms)	CE (V)[c]
azoxystrobin	F	**344→329**.1, 344→171.9, 344→155.8	40	10, 40, 40
benthiavalicarb-isopropyl	F	**181→180**, 181→126.9, 181→83.1	20.3, 17.6	20, 40, 40
boscalid	F	**140→112**, 140→76	45.7	10, 30
clomazone	H	204→107, **125→99**	87.2	20, 20
cyflufenamid	F	412→118.1, 412→89.9, **118→90.1**, 118→63	8.2, 8.6	30, 40, 10, 40
cypermethrin	A, I	181→152.1, **181→126.9**, 181→76.9	24.2, 19.7, 19.1, 22.1	30, 40, 40
cyprodinil	F	**225→223.7**, 224→208.1	17.3	20, 20
deltamethrin	I	253→171.9, **253→93.1**, 253→77	26.9	10, 20, 40
fenhexamid	F	301→176.9, **301→97**, 301→54.8	13.5	10, 10, 40
flonicamid	I	**174→146**, 174→126, 174→69	77.6	10, 20, 40
fluazifop-p-butyl	H	383→282.1, **254→146**	8.2	10, 20
fludioxonil	F	248→182.1, 248→154.1, **248→127.1**	9.7	10, 20, 30
flufenacet	H	**151→136.1,** 151→95.1	30.2	10, 30
fluopicolide	F	347→172, **209→182**, 173→145	14.5	30, 20, 10
fluopyram	F	**173→145**, 173→95.1	15.3	20, 30
flutolanil	F	**172.8→145**, 172.8→95, 172.8→75	12.6	15, 35, 55
indoxacarb	I	**264→176**, 264→147.9, 264→112.9	23.7	10, 30, 40
iprovalicarb	F	158→98, **158→72.1**, 158→55.1	8.6, 8.1	10, 10, 20
kresoxim-methyl	F	206→131.1, **206→116.1**	12.7	10, 10
lambda-cyhalothrin	I	**181→152.1**, 181→127.1, 181→77.1	18.6, 17.6	20, 30, 40
metazachlor	H	**209→132.1**, 209→117.1, 133→131.7	14	20, 40, 20
metrafenone	F	408→393, 393→378, **379→364**	29.9	10, 10, 10
myclobutanil	F	**179→125**, 179→90, 179→63	8.6	10, 40, 40
penconazole	F	248→206.1, 248→192.1, **248→157.1**	12.7	10, 10, 30
pendimethalin	H	252→191.1, **252→162.1**, 252→106.1	12.2	10, 10, 40
pirimicarb	I	**238→166.1**, 166→96.1	33.4	10, 10
proquinazid	F	**288→245**, 288→217, 272→216	13.5	10, 30, 20
prosulfocarb	H	**251→128.1**, 162→91.1, 162→65	32.5	10, 10, 40
pyraclostrobin	F	**164→132.1**, 164→104, 132→104	34.1	10, 30, 10
pyrimethanil	F	198→183.1, **198→118**	63.4	20, 40
pyriproxyfen	I	226→186.1, **226→77.1**	21.1	10, 40
tebuconazole	F	250→153, **250→125**, 250→70	10.2	10, 30, 10
tebufenpyrad	A	335→319.9, 333→318.2, **333→276.1**	21.3	10, 10, 10
tefluthrin	I	177→137, **177→127,** 177→87.1	36.6	20, 20, 40
tetraconazole	F	**336→218.1**, 336→164	24.7	20, 30

a A = acaricide, I = insecticide, F = fungicide, H = herbicide
b Q = qualifier ion, bold qualifier was used for integration
c CE = collision energy

pesticide ml^{-1}. From 35 stock solutions, three mixed solutions of all 35 active substances were prepared with a concentration of 5 μg ml^{-1}, 1 μg ml^{-1} and 0.1 μg ml^{-1}.

2.2 EXTRACTION PROCEDURE

To 20 g of sample in the beaker, 30 ml of acetone : dichloromethane : petroleum ether = 1 (v) : 2 (v) : 2 (v) was added. The mixture was homogenised for 2 minutes with a mixer. 10 g of anhydrous Na_2SO_4 was added. The mixture was homogenised for 2 minutes with a mixer. The whole content was filtered through filter paper black ribbon, which contained 20 g of anhydrous Na_2SO_4, into a 500 ml Soxhlet flask. Matrix was returned to the same beaker, 30 ml of acetone: dichloromethane: petroleum ether = 1 (v): 2 (v): 2 (v) was added, mixture was homogenised for 2 minutes with a mixer and afterwards filtered through the same filter paper as previously. Last step was repeated twice. Then solvent solution in Soxhlet flask was evaporated to approximately 2 ml on a rotavapor and dried with nitrogen flow. The dry eluate was dissolved in 2 ml of acetone for HPLC using ultrasound in order to prepare a sample. Extract was filtered with 0.2 μm pore size filter.

2.3 DETERMINATION

The samples were analysed using a gas chromatograph (Agilent Technologies 8890, Shanghai, China) coupled with tandem mass spectrometer (Agilent Technologies 7010B, Santa Clara, USA), equipped with a Gerstel 20PRE0795 multipurpose sampler (Gerstel, Sursee, Switzerland) and a HP-5 MS UI column (Agilent Technologies, 30 m, 0.25 mm i.d., 0.25 μm film thickness) with a constant flow of helium at 1.2 ml min^{-1}. The GC oven was programmed as follows: 55 °C for 2 min, from 55 °C to 100 °C at 20 °C min^{-1}, from 100 °C to 280 °C at 4 °C min^{-1}, held at 280 °C for 19.75 min. The temperature of the ion source was 230 °C, the auxiliary temperature was 280 °C and the quadrupoles temperature was 150 °C. For qualitative and quantitative determination, the MRM transitions were used. For each active substance two to four transitions, presented in Table 1, were used. The calibration was performed to matrix match standards.

2.4 VALIDATION OF METHODS

Method was validated on three representatives of vegetables: lettuce, which contains a lot of chlorophyll, potato, which contains a lot of starch and tomato which is acidic matrix.

2.4.1 LOQ and linearity

The linearity was verified using the matrix match standards (two repetitions for one concentration level, four to eight concentration levels for the calibration curve). The linearity and range were determined by linear regression, using the F test.

LOQs were estimated from the chromatograms of matrix match standards. LOQs were chosen at a minimum of S/N = 10.

2.4.2 Precision

Blank lettuce, potato and tomato were bought in store and analysed to prove that they contain no pesticide residues. For the determination of precision (ISO 5725), i.e. repeatability and reproducibility, the extracts of spiked blank lettuce, potato and tomato were analysed at LOQ. Within a period of 10 days, two parallel extracts were prepared each day for each concentration level. Each one was injected once. Then the standard deviation of the repeatability of the level and the standard deviation of reproducibility of the level were both calculated.

2.4.3 Uncertainty of repeatability and uncertainty of reproducibility

The uncertainty of repeatability and the uncertainty of reproducibility were calculated by multiplying the standard deviation of repeatability and the standard deviation of reproducibility by the Student's t factor, for nine degrees of freedom and a 95 % confidence level ($t_{95;9}$ = 2.262).

$U_r = t_{95;9} \times s_r$; $U_R = t_{95;9} \times s_R$

The measurement uncertainty for PPPs residues should be 50 %, as proposed in SANTE/11312/2021. When validating, analysts must prove that their measurement uncertainty is below or equal to the proposed measurement uncertainty.

Table 2: Vegetable samples collected from stores in Slovenia in 2023 .

No. of sample	Crop	Type of production	Origin	State of crop	Sample mass (kg)
1	brussels sprouts	conventional	Netherlands	fresh	1.1
2	carrot	conventional	Slovenia	frozen	0.9
3	carrot	conventional	Slovenia	frozen	0.9
4	carrot	conventional	Slovenia	fresh	1.3
5	carrot	conventional	Austria	processed	1.2
6	carrot	conventional	Slovenia	processed	2
7	carrot	organic	Italy	fresh	1
8	carrot	organic	Italy	fresh	1
9	carrot	conventional	Slovenia	fresh	1
10	carrot	conventional	Slovenia	fresh	1.1
11	carrot	conventional	Slovenia	fresh	1.2
12	carrot	conventional	Slovenia	fresh	2.5
13	carrot	conventional	Slovenia	fresh	2.5
14	carrot	conventional	Slovenia	fresh	2.5
15	carrot	organic	Italy	fresh	2
16	cauliflower	conventional	unknown	frozen	1
17	cauliflower	conventional	Netherlands	fresh	1.7
18	cauliflower	organic	Italy	fresh	1
19	cauliflower	conventional	Croatia	fresh	1.8
20	kale	conventional	Croatia	fresh	1.2
21	lamb's lettuce	conventional	Italy	fresh	0.5
22	lamb's lettuce	organic	Italy	fresh	0.5
23	lamb's lettuce	conventional	Italy	fresh	0.5
24	lamb's lettuce	conventional	Croatia	fresh	0.5
25	lettuce	conventional	Slovenia	fresh	1
26	lettuce	conventional	Slovenia	fresh	1
27	pepper	organic	Italy	fresh	1.6
28	pepper	organic	Italy	fresh	1.2
29	pepper	organic	Italy	fresh	1.8
30	pepper	conventional	Macedonia	fresh	1.3
31	pepper	conventional	Poland	fresh	1.5
32	pepper	conventional	Italy	fresh	1.2
33	spinach	organic	Italy	frozen	1.2
34	spinach	conventional	Slovenia	frozen	0.9
35	spinach	organic	Italy	fresh	0.5
36	spinach	conventional	Croatia	fresh	0.5
37	spinach	organic	Italy	fresh	1
38	tomato	organic	Italy	processed	1
39	tomato	conventional	Italy	processed	1
40	tomato	conventional	Italy	processed	1
41	tomato	conventional	Croatia	fresh	1.5
42	tomato	conventional	Slovenia	fresh	1.1
43	tomato	conventional	Croatia	fresh	1.2
44	tomato	conventional	Croatia	fresh	1
45	tomato	conventional	Croatia	fresh	1.1
46	tomato	organic	Italy	fresh	1
47	zucchini	conventional	Italy	fresh	1.3
48	zucchini	organic	Italy	fresh	1
49	zucchini	conventional	Croatia	fresh	1.5
50	zucchini	organic	Italy	fresh	1

2.4.4 Accuracy

The accuracy was verified by checking the recoveries. The average of the recoveries from the tests for precision (10 days, 2 parallel samples each day) was calculated. According to the requirements for method validation procedures (SANTE/11312/2021), acceptable mean recoveries are those within the range of 70 % to 120 %, with an associated repeatability of RSDr ≤ 20 %.

According to the guidelines for single-laboratory validation (Alder et al. 2000), acceptable mean recoveries at level > 0.001 mg kg^{-1} ≤ 0.01 mg kg^{-1} are those within the range of 60 % to 120 %, with an associated repeatability RSDr ≤ 30 %.

2.5 CONSUMER RISK ASSESSMENT

Long-term exposure was calculated using the EFSA PRIMo model revision 3.1 (EFSA, 2024). Input values were supervised trial median residues (STMRs) and Acceptable Daily Intakes (ADIs). Chronic consumer exposure was expressed in % of the ADI. The acceptable limit for long-term exposure is 100 % of the ADI.

Short-term exposure was calculated using the EFSA PRIMo model revision 3.1. Input values were the highest residues (HRs) and Acute Reference Doses (ARfDs). Where ARfDs were not allocated, ADIs were used instead. Acute consumer exposure was expressed in % of the ARfD. The acceptable limit for short-term exposure is 100 % of the ARfD.

2.6 SAMPLING

A total of 50 vegetable samples were collected in September 2023 on Slovenian market. The sampling distribution is presented in Table 2. Processed carrot was cooked carrot in salt solution and processed tomato was tomato paste.

3 RESULTS AND DISCUSSION

3.1 COMPARISON OF PREVIOUS AND PRESENT EXTRACTION METHOD WITH ACETONE, DICHLOROMETHANE AND PETROLEUMETHER

In our previous method (Baša Česnik and Gregorčič, 2003, Baša Česnik et al., 2006) separation of water and organic phase was conducted in separatory funnels, which is time consuming and physically demanding. In present method this phase is no longer required. Water is eliminated by adding anhydrous Na_2SO_4 directly to the mixture of matrix and solvents.

Also, in previous method (Baša Česnik and Gregorčič, 2003, Baša Česnik et al., 2006) 74 ml of acetone p.a., 148 ml of dichloromethane p.a. and 148 ml of petroleum ether p.a. were used per sample. In present method only 18 ml of acetone p.a., 36 ml of dichloromethane p.a. and 36 ml of petroleum ether p.a. were used per sample. Therefore approximately 4-times lower amounts of solvents were used.

3.2 VALIDATION OF METHOD

3.2.1 LOQ and linearity

The linear model is valid for all active substances presented in Tables 3-5. Linearity was proven in the range of 0.005 mg kg^{-1} to 0.04 mg kg^{-1} for all active substances for lettuce, potato and tomato. R^2 ranged from 0.953 to 0.999 for lettuce, from 0.970 to 0.999 for potato and from 0.960 to 0.997 for tomato. Results are presented in Tables 3-5.

3.2.2 Accuracy

The results for the recoveries are given in Tables 3-5. The recoveries at LOQs for the active substances scanned with GC-MS/MS are in the range of 73.4 % to 94.3 %, with RSDs of 11.7 % to 17.8 % for lettuce, 75.0 % to 89.0 %, with RSDs of 8.6 % to 18.3 % for potato and 81.8 % to 100.9 %, with RSDs of 9.6 % to 16.7 % for tomato.

All recoveries and RSDs are within the required ranges from the literature (Alder et al., 2000; SANTE/11813/2017).

3.2.3 Uncertainty of repeatability and uncertainty of reproducibility

Table 3: Validation parameters for lettuce

Active substance	Linearity range (mg kg^{-1})	R^2	LOQ (mg kg^{-1})	Recovery (%)	RSD (%)[a]	U_r (mg kg^{-1})[b]	U_r (%)[c]	U_R (mg kg^{-1})[d]	U_R (%)[e]
azoxystrobin	0.005-0.04	0.985	0.005	80.6	12.3	0.0008	15.9	0.0011	22.7
benthiavalicarb-isopropyl	0.005-0.04	0.995	0.005	84.6	16.1	0.0007	14.3	0.0016	31.5
boscalid	0.005-0.04	0.984	0.005	77.1	16.3	0.0010	19.8	0.0014	28.8
clomazone	0.005-0.04	0.953	0.005	88.7	16.7	0.0014	27.1	0.0017	33.9
cyflufenamid	0.005-0.04	0.983	0.005	81.0	13.0	0.0006	12.2	0.0012	24.2
cypermethrin	0.005-0.04	0.994	0.005	78.8	16.2	0.0009	17.5	0.0015	29.4
cyprodinil	0.005-0.04	0.999	0.005	86.5	12.2	0.0006	11.8	0.0012	24.5
deltamethrin	0.005-0.04	0.988	0.005	74.0	17.0	0.0009	18.3	0.0014	28.9
fenhexamid	0.005-0.04	0.995	0.005	84.2	14.3	0.0008	15.9	0.0014	27.7
flonicamid	0.005-0.04	0.993	0.005	93.3	12.5	0.0007	13.1	0.0013	27.0
fluazifop-p-butyl	0.005-0.04	0.995	0.005	82.7	13.1	0.0005	10.9	0.0013	25.1
fludioxonil	0.005-0.04	0.972	0.005	80.8	17.1	0.0008	15.5	0.0016	31.9
flufenacet	0.005-0.04	0.991	0.005	83.4	12.9	0.0010	20.5	0.0012	24.6
fluopicolide	0.005-0.04	0.987	0.005	85.5	13.2	0.0007	13.9	0.0013	26.1
fluopyram	0.005-0.04	0.993	0.005	89.2	11.7	0.0006	12.2	0.0012	24.1
flutolanil	0.005-0.04	0.994	0.005	89.2	11.9	0.0006	12.2	0.0012	24.6
indoxacarb	0.005-0.04	0.980	0.005	73.4	16.9	0.0008	15.1	0.0014	28.6
iprovalicarb	0.005-0.04	0.997	0.005	86.3	13.6	0.0007	13.5	0.0014	27.0
kresoxim-methyl	0.005-0.04	0.990	0.005	82.7	12.0	0.0005	10.7	0.0012	23.0
lambda-cyhalothrin	0.005-0.04	0.991	0.005	82.5	14.1	0.0007	13.8	0.0013	26.9
metazachlor	0.005-0.04	0.980	0.005	85.9	12.5	0.0008	15.6	0.0012	24.6
metrafenone	0.005-0.04	0.982	0.005	79.8	14.1	0.0007	14.7	0.0013	25.9
myclobutanil	0.005-0.04	0.989	0.005	84.5	12.9	0.0006	12.6	0.0013	25.2
penconazole	0.005-0.04	0.999	0.005	89.2	12.4	0.0006	12.9	0.0013	25.4
pendimethalin	0.005-0.04	0.997	0.005	81.1	13.7	0.0007	14.4	0.0013	25.6
pirimicarb	0.005-0.04	0.994	0.005	94.3	12.6	0.0007	13.8	0.0014	27.5
proquinazid	0.005-0.04	0.998	0.005	81.2	13.3	0.0006	12.1	0.0012	25.0
prosulfocarb	0.005-0.04	0.994	0.005	87.4	12.3	0.0006	12.1	0.0012	24.8
pyraclostrobin	0.005-0.04	0.986	0.005	73.5	17.8	0.0009	17.8	0.0015	30.2
pyrimethanil	0.005-0.04	0.988	0.005	91.9	12.4	0.0007	13.5	0.0013	26.4
pyriproxyfen	0.005-0.04	0.995	0.005	80.5	13.7	0.0006	12.4	0.0013	25.4
tebuconazole	0.005-0.04	0.996	0.005	85.0	14.0	0.0007	14.8	0.0014	27.4
tebufenpyrad	0.005-0.04	0.987	0.005	81.1	14.0	0.0007	13.5	0.0013	26.2
tefluthrin	0.005-0.04	0.996	0.005	89.7	12.3	0.0007	13.2	0.0013	25.4
tetraconazole	0.005-0.04	0.998	0.005	89.4	12.1	0.0007	13.2	0.0013	25.0

a RSD was obtained during recovery analyses
b,c Ur = uncertainty of repeatability
d,e UR = uncertainty of reproducibility

Table 4: Validation parameters for potato

Active substance	Linearity range (mg kg^{-1})	R^2	LOQ (mg kg^{-1})	Recovery (%)	RSD (%)[a]	U_r (mg kg^{-1})[b]	U_r (%)[c]	U_R (mg kg^{-1})[d]	U_R (%)[e]
azoxystrobin	0.005-0.04	0.981	0.005	87.9	16.9	0.0009	17.7	0.0017	34.2
benthiavalicarb-isopropyl	0.005-0.04	0.981	0.005	86.4	11.7	0.0007	14.6	0.0012	23.3
boscalid	0.005-0.04	0.984	0.005	86.3	12.4	0.0007	13.9	0.0012	24.8
clomazone	0.005-0.04	0.988	0.005	88.5	14.9	0.0009	18.0	0.0015	30.4
cyflufenamid	0.005-0.04	0.990	0.005	79.2	10.6	0.0006	12.6	0.0010	19.3
cypermethrin	0.005-0.04	0.988	0.005	75.0	17.2	0.0009	17.1	0.0015	29.7
cyprodinil	0.005-0.04	0.997	0.005	82.2	9.3	0.0006	13.0	0.0009	17.4
deltamethrin	0.005-0.04	0.976	0.005	78.1	16.4	0.0009	18.0	0.0015	29.4
fenhexamid	0.005-0.04	0.987	0.005	85.7	12.2	0.0008	16.0	0.0012	24.0
flonicamid	0.005-0.04	0.991	0.005	81.2	9.1	0.0007	14.0	0.0008	16.9
fluazifop-p-butyl	0.005-0.04	0.994	0.005	78.7	11.8	0.0007	13.6	0.0011	21.4
fludioxonil	0.005-0.04	0.980	0.005	82.0	12.2	0.0008	16.4	0.0011	22.9
flufenacet	0.005-0.04	0.992	0.005	82.7	9.3	0.0007	13.2	0.0009	17.5
fluopicolide	0.005-0.04	0.970	0.005	83.6	9.2	0.0007	13.4	0.0009	17.6
fluopyram	0.005-0.04	0.991	0.005	82.6	10.1	0.0006	12.2	0.0010	19.1
flutolanil	0.005-0.04	0.995	0.005	81.5	10.8	0.0006	12.5	0.0010	20.3
indoxacarb	0.005-0.04	0.974	0.005	85.6	18.3	0.0009	18.7	0.0018	36.2
iprovalicarb	0.005-0.04	0.991	0.005	84.4	10.2	0.0006	12.2	0.0010	19.8
kresoxim-methyl	0.005-0.04	0.990	0.005	81.5	9.9	0.0006	12.3	0.0009	18.5
lambda-cyhalothrin	0.005-0.04	0.984	0.005	77.2	13.1	0.0007	14.7	0.0012	23.3
metazachlor	0.005-0.04	0.995	0.005	83.2	9.4	0.0007	13.2	0.0009	17.9
metrafenone	0.005-0.04	0.980	0.005	83.1	12.3	0.0007	14.9	0.0012	23.4
myclobutanil	0.005-0.04	0.988	0.005	83.2	9.7	0.0006	12.9	0.0009	18.5
penconazole	0.005-0.04	0.991	0.005	82.5	9.6	0.0007	13.6	0.0009	18.2
pendimethalin	0.005-0.04	0.992	0.005	75.0	10.9	0.0006	12.4	0.0009	18.7
pirimicarb	0.005-0.04	0.997	0.005	82.1	9.7	0.0007	14.5	0.0009	18.1
proquinazid	0.005-0.04	0.987	0.005	80.9	10.3	0.0007	13.2	0.0010	19.2
prosulfocarb	0.005-0.04	0.996	0.005	79.9	9.2	0.0006	12.8	0.0008	16.8
pyraclostrobin	0.005-0.04	0.977	0.005	89.0	17.7	0.0009	18.4	0.0018	36.4
pyrimethanil	0.005-0.04	0.995	0.005	82.5	8.6	0.0006	12.9	0.0008	16.2
pyriproxyfen	0.005-0.04	0.980	0.005	81.5	11.6	0.0007	14.2	0.0011	21.7
tebuconazole	0.005-0.04	0.984	0.005	84.6	10.2	0.0007	13.9	0.0010	19.9
tebufenpyrad	0.005-0.04	0.980	0.005	81.8	11.4	0.0007	14.2	0.0011	21.4
tefluthrin	0.005-0.04	0.999	0.005	75.0	9.3	0.0006	13.0	0.0008	16.0
tetraconazole	0.005-0.04	0.991	0.005	82.4	10.3	0.0007	14.3	0.0010	19.5

a RSD was obtained during recovery analyses
b,c Ur = uncertainty of repeatability
d,e UR = uncertainty of reproducibility

Table 5: Validation parameters for tomato

Active substance	Linearity range (mg kg^{-1})	R^2	LOQ (mg kg^{-1})	Recovery (%)	RSD (%)[a]	U_r (mg kg^{-1})[b]	U_r (%)[c]	U_R (mg kg^{-1})[d]	U_R (%)[e]
azoxystrobin	0.005-0.04	0.960	0.005	97.1	16.0	0.0009	19.0	0.0018	35.8
benthiavalicarb-isopropyl	0.005-0.04	0.965	0.005	88.7	12.5	0.0010	19.5	0.0013	25.3
boscalid	0.005-0.04	0.963	0.005	90.3	11.2	0.0006	11.7	0.0012	23.4
clomazone	0.005-0.04	0.997	0.005	89.3	16.2	0.0011	21.1	0.0017	33.2
cyflufenamid	0.005-0.04	0.996	0.005	84.1	11.8	0.0009	18.0	0.0011	22.8
cypermethrin	0.005-0.04	0.981	0.005	91.8	14.6	0.0012	23.3	0.0015	30.7
cyprodinil	0.005-0.04	0.995	0.005	84.1	12.6	0.0010	19.6	0.0012	24.2
deltamethrin	0.005-0.04	0.970	0.005	95.1	14.5	0.0008	15.2	0.0016	31.8
fenhexamid	0.005-0.04	0.986	0.005	90.2	12.9	0.0011	22.4	0.0013	26.5
flonicamid	0.005-0.04	0.995	0.005	86.4	13.6	0.0011	22.5	0.0013	26.9
fluazifop-p-butyl	0.005-0.04	0.995	0.005	83.4	12.3	0.0010	20.1	0.0012	23.3
fludioxonil	0.005-0.04	0.991	0.005	87.3	15.2	0.0010	20.1	0.0015	30.6
flufenacet	0.005-0.04	0.996	0.005	89.3	13.7	0.0011	22.2	0.0014	28.0
fluopicolide	0.005-0.04	0.990	0.005	84.7	10.5	0.0009	18.0	0.0010	20.2
fluopyram	0.005-0.04	0.996	0.005	84.4	12.7	0.0010	20.4	0.0012	24.4
flutolanil	0.005-0.04	0.996	0.005	85.1	12.4	0.0010	19.2	0.0012	24.2
indoxacarb	0.005-0.04	0.972	0.005	97.6	15.5	0.0009	17.4	0.0017	34.9
iprovalicarb	0.005-0.04	0.994	0.005	85.4	12.6	0.0010	20.8	0.0012	24.6
kresoxim-methyl	0.005-0.04	0.996	0.005	84.0	12.2	0.0010	19.1	0.0012	23.3
lambda-cyhalothrin	0.005-0.04	0.973	0.005	83.3	11.1	0.0009	17.9	0.0011	21.0
metazachlor	0.005-0.04	0.996	0.005	87.0	13.0	0.0009	18.5	0.0013	26.0
metrafenone	0.005-0.04	0.970	0.005	85.1	10.3	0.0009	17.8	0.0010	19.9
myclobutanil	0.005-0.04	0.994	0.005	84.4	11.2	0.0009	18.3	0.0011	21.6
penconazole	0.005-0.04	0.996	0.005	84.9	12.3	0.0010	19.4	0.0012	23.8
pendimethalin	0.005-0.04	0.993	0.005	83.6	13.5	0.0010	20.3	0.0013	25.9
pirimicarb	0.005-0.04	0.995	0.005	85.7	13.6	0.0011	22.7	0.0013	26.5
proquinazid	0.005-0.04	0.981	0.005	81.8	11.5	0.0009	17.9	0.0011	21.4
prosulfocarb	0.005-0.04	0.996	0.005	84.8	13.6	0.0011	21.6	0.0013	26.2
pyraclostrobin	0.005-0.04	0.977	0.005	100.9	16.7	0.0009	17.1	0.0020	39.0
pyrimethanil	0.005-0.04	0.997	0.005	85.2	13.0	0.0011	21.2	0.0013	25.3
pyriproxyfen	0.005-0.04	0.975	0.005	83.5	9.6	0.0009	17.3	0.0009	18.2
tebuconazole	0.005-0.04	0.981	0.005	85.2	10.2	0.0009	17.6	0.0010	19.8
tebufenpyrad	0.005-0.04	0.980	0.005	84.1	9.8	0.0008	16.3	0.0009	18.7
tefluthrin	0.005-0.04	0.997	0.005	84.8	13.5	0.0011	21.6	0.0013	26.1
tetraconazole	0.005-0.04	0.996	0.005	85.1	12.8	0.0010	19.3	0.0012	25.0

a RSD was obtained during recovery analyses
b,c Ur = uncertainty of repeatability
d,e UR = uncertainty of reproducibility

Table 6: Concentrations and MRLs (mg kg^{-1}) (EC, 2005) of pesticide residues found in 50 vegetable samples

no of sample / active substance	boscalid	flonicamid	fludioxonil	fluopyram	pyraclostrobin	tebuconazole
CARROT						
MRL	2			0.4		
sample no. 4	0.018	-	-	-	-	-
sample no. 9	0.006	-	-	0.006	-	-
sample no. 10	0.005	-	-	0.009	-	-
LAMB`S LETTUCE						
MRL	50		20			
sample no. 21	-	-	0.011	-	-	-
sample no. 23	0.005	-	-	-	-	-
PEPPER						
MRL	3			2	0.5	
sample no. 32	0.060	-	-	0.008	0.027	-
TOMATO						
MRL		0.5		0.5		0.9
sample no. 44	-	0.024	-	0.009	-	0.009

Table 7: Input values for chronic and acute risk assessment

boscalid	carrot		lamb`s lettuce		pepper	
ADI = 0.04 mg kg^{-1} bw/d	STMR (mg kg^{-1})	HR (mg kg^{-1})	STMR (mg kg^{-1})	HR (mg kg^{-1})	STMR (mg kg^{-1})	HR (mg kg^{-1})
ARfD = not applicable	0.006	0.018	0.005	0.005	0.06	0.06
flonicamid	tomato					
ADI = 0.025 mg kg^{-1} bw/d	STMR (mg kg^{-1})	HR (mg kg^{-1})				
ARfD = 0.025 mg kg^{-1} bw	0.024	0.024				
fludioxonil	lamb`s lettuce					
ADI = 0.37 mg kg^{-1} bw/d	STMR (mg kg^{-1})	HR (mg kg^{-1})				
ARfD = not applicable	0.011	0.011				
fluopyram	carrot		pepper		tomato	
ADI = 0.012 mg kg^{-1} bw/d	STMR (mg kg^{-1})	HR (mg kg^{-1})	STMR (mg kg^{-1})	HR (mg kg^{-1})	STMR (mg kg^{-1})	HR (mg kg^{-1})
ARfD = 0.5 mg kg^{-1} bw	0.008	0.009	0.008	0.008	0.009	0.009
pyraclostrobin	pepper					
ADI = 0.03 mg kg^{-1} bw/d	STMR (mg kg^{-1})	HR (mg kg^{-1})				
ARfD = 0.03 mg kg^{-1} bw	0.027	0.027				
tebuconazole	tomato					
ADI = 0.03 mg kg^{-1} bw/d	STMR (mg kg^{-1})	HR (mg kg^{-1})				
ARfD = 0.03 mg kg^{-1} bw	0.009	0.009				

ADI = Acceptable daily intake
ARfD = Acute reference dose
HR = Highest residue
STMR = Supervised trial median residue

The uncertainty of repeatability and uncertainty of reproducibility were determined at contents equal to the LOQs. The results are presented in Tables 3-5. Uncertainty of repeatability ranged for lettuce, potato and tomato from 0.0005 mg kg^{-1} to 0.0014 mg kg^{-1}, which is 10.7 % to 27.1 % of LOQ, from 0.0006 mg kg^{-1} to 0.0009 mg kg^{-1}, which is 12.2 % to 18.7 % of LOQ and from 0.0006 mg kg^{-1} to 0.0012 mg kg^{-1}, which is 11.7 % to 23.3 % of LOQ, respectively. Uncertainty of reproducibility ranged for lettuce, potato and tomato from 0.0011 mg kg^{-1} to 0.0017 mg kg^{-1}, which is 22.7 % to 33.9 % of LOQ, from 0.0008 mg kg^{-1} to 0.0018 mg kg^{-1}, which is 16.0 % to 36.4 % of LOQ and from 0.0009 mg kg^{-1} to 0.0020 mg kg^{-1}, which is 18.2 % to 39.0 % of LOQ, respectively.

3.3 SURVEY OF PESTICIDE RESIDUES IN VEGETABLE SAMPLES

In 50 vegetable samples gathered from stores in Slovenia, 35 active substances were sought. Only 14 % of samples analysed contained pesticide residues. 6 active substances were determined at LOQ (0.005 mg kg^{-1}) and up to 0.06 mg kg^{-1} in carrot, lamb`s lettuce, pepper and tomato. Brussels sprouts, cauliflower, kale, lettuce, potato, spinach and zucchini contained no pesticide residues. Concentrations of all active substances found, were below MRLs. 28 % of samples was of Slovene origin. 21.4 % of samples of Slovene origin and 11.4 % of samples of foreign origin contained pesticide residues. One active substance found is insecticide (flonicamid), the rest 5 are fungicides. Organically produced commodities contained no pesticide residues. 2 active substances (boscalid and fluopiram) were determined in fresh carrot of Slovenian origin. Both of them are authorised for use on carrot in Slovenia. 2 active substances (boscalid and fludioxonil) were determined in fresh lamb`s lettuce of Italian origin. 3 active substances (boscalid, fluopiram and pyraclostrobin) were determined in fresh pepper of Italian origin. 3 active substances (flonicamid, fluopyram and tebuconazole) were determined in fresh tomato of Croatian origin. Results are presented in Table 6.

Table 8: Input values for chronic and acute risk assessment

active substance	% ADI	% ARfD
boscalid	0.1	9.0
flonicamid	0.1	6.0
fludioxonil	0.0003	0.01
fluopyram	0.3	0.1
pyraclostrobin	0.1	5.0
tebuconazole	0.1	2.0

A consumer risk assessment was performed using the EFSA PRIMo model rev. 3.1, in which 36 national diets from EU countries are included. This model was used since Slovenia has not created a model of its own. The same model is used in the process of registration of PPPs in Slovenia. Input values for chronic (STMRs) and acute risk assessment (HRs) are presented in Table 7. Where ARfD was not allocated, ADI value was used instead. Results of risk assessment are presented in Table 9. The highest chronic exposure was < 1% and the highest acute exposure < 10%. Based on these calculations, the conclusion was that the analysed vegetable samples are of no cause for concern for consumers.

Our results were compared with the results from other scientific papers. Santarelli et al. (2018) found in raw green vegetables marketed in Italy boscalid in 22.67 % of samples, cyprodinil in 6.00 % of samples, deltamethrin in 3.33 % of samples, fludioxonil in 2.33 % of samples, azoxystrobin, lambda-cyhalothrin and fenhexamid each in 1.33 % of samples, and fluopicolide in 0.33 % of samples. In comparison to our study, boscalid was found in 10 % of vegetable samples, fluopyram in 8 % of samples, flonicamid, fludioxonil, pyraclostrobin and tebuconazole each in 2 % of samples. In these two studies considering the same active substances sought, only boscalid and fludioxonil were found in both of them.

Fluopyram was found in the Turkey lettuce up to a concentration of 0.03 mg kg^{-1} by Balkan and Yilmaz (2022). Balkan and Yilmaz (2022) also reported that pyraclostrobin was found in Turkey lettuce and spinach at a maximum concentration of 0.24 and 0.01 mg kg^{-1}, respectively. Qin et al. (2021) wrote that tebuconazole was found in 14.63 % of the China vegetable samples analysed, with a maximum concentration of 0.36 mg kg^{-1}. Tebuconazole was also found by Balkan and Yilmaz (2022) in Turkey lettuce at a maximum concentration of 0.01 mg kg^{-1}. In Slovenia, fluopyram, pyraclostrobin and tebuconazole were found up to concentration 0.009, 0.027 and 0.009 mg kg^{-1}, respectively. Maximum concentrations found in

Slovenia are lower than maximum concentrations from literature.

Other active substances analysed in our laboratory, namely cypermethrin, deltamethrin, kresoxim-methyl, metrafenone, pyrimethanil and lambda-cyhalothrin were not detected in Slovenian vegetables, but were found in samples originating from Chile, China, France, Lebanon, Marocco, Mexico, Turkey and Uganda. Concentrations and/or ratio of positive samples are reported in Table 9.

4 CONCLUSIONS

In our research, a method for determining pesticide residues in vegetables was introduced and validated. The limit of quantification was 0.005 mg kg^{-1} for all active substances. The calibration curves gave a linear response with R^2 0.953 to 0.999. The recoveries ranged from 73.4 % to 100.9 % with RSDs from 8.6 % to 18.3 %. The measurement uncertainty of repeatability ranged from 10.7 to 27.1 % and the measurement uncertainty of reproducibility from 16.0 to 39.0 %. The method was found to be fit for purpose of measuring possible breaches of MRL for 35 active substances.

The method was used to analyse 50 vegetable samples gathered from Slovenian stores from organic and conventional production. A total of 35 active substances were sought, but only the insecticide flonicamid and fungicides boscalid, fludioxonil, fluopyram, pyraclostrobin and tebuconazole were found in 7 of these samples (14.0 %). In 86.0 % of the samples analysed, the active substances sought were not determined. A risk assessment revealed that the Slovenian vegetable samples are no cause for concern for consumers.

In national monitoring program, for analyses of pesticide residues in vegetables, requirement is to analyse 1 sample per matrix from organic production. Despite the fact that we did not detect a violation in either conventional or organic vegetables, we recommend increasing the number of taken ecological samples in the

Table 9: Literature results for active substances sought, but not found in our laboratory

active substance	commodity	max content (mg kg $^{-1}$)	ratio of positive samples (%)	country of origin	reference
cypermethrin	cucumber	1.5	not reported	Lebanon	Sahyoun et al., 2022
cypermethrin	cauliflower	0.0034	not reported	Uganda	Ngabirano e tal., 2022
cypermethrin	tomato	0.0034	not reported	Uganda	Ngabirano e tal., 2022
cypermethrin	lettuce	0.166	50	Chile	Calderon et al., 2022
cypermethrin	tomato	0.064	40	Chile	Calderon et al., 2022
cypermethrin	spinach	0.454	33.3	Mexico	Calderon et al., 2022
cypermethrin	tomato	0.061	12.5	Mexico	Calderon et al., 2022
cypermethrin	lettuce	0.2	not reported	Turkey	Balkan and Yilmaz, 2022
deltamethrin	lettuce	0.11	not reported	Turkey	Balkan and Yilmaz, 2022
kresoxim-methyl	tomato	0.0004	not reported	France	Sahyoun et al., 2022
kresoxim-methyl	lettuce	1.43	not reported	Turkey	Balkan and Yilmaz, 2022
metrafenone	lettuce	3.49	not reported	Turkey	Balkan and Yilmaz, 2022
pyrimethanil	vegetables	0.53	6.5	China	Qin et al., 2021
pyrimethanil	lettuce	0.27	not reported	Turkey	Balkan and Yilmaz, 2022
λ-cyhalothrin	cucumber	0.002	not reported	Lebanon	Sahyoun et al., 2022
λ-cyhalothrin	pepper	0.0015	not reported	Marocco	Sahyoun et al., 2022
λ-cyhalothrin	lettuce	0.028	12.5	Mexico	Calderon et al., 2022
λ-cyhalothrin	spinach	0.043	12.5	Mexico	Calderon et al., 2022

national monitoring program from 1 sample per matrix to approximately 30 % of taken samples per matrix.

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Energy saving in shift crops cultivation: An analysis of paddy rice and upland crop production in Hau Giang province, Vietnam

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Energy saving in shift crops cultivation: An analysis of paddy rice and upland crop production in Hau Giang province, Vietnam

Abstract: Agricultural energy analysis and better energy use efficiency will contribute to sustainable development, adaptation to climate change and ensure maintainable production. A case study from Hau Giang province agriculture energy was conducted to compare the cultivation of paddy rice (PR) and upland crops, including corn, mungbean (MB), and black sesame (BS). The life cycle assessment methodology was used to estimate energy consumption and biomass energy production. Based on input-output energy inventoried results, the energy use efficiency, energy productivity, specific energy, and net energy were analyzed. Selected crops require 39,501–59,638 MJ ha^{-1} crop^{-1} that was higher than energy providing for PR. Crop cultivation required a large amount of energy from fossil fuels and electricity (12,946–34,375 MJ ha^{-1} crop^{-1}). Biomass production achieved 779,670 MJ ha^{-1} crop^{-1} through corn cultivation, follow by rice farming (198,723 MJ ha^{-1} crop^{-1}), BS and MB production (103,292 and 63,012 MJ ha^{-1} crop–1, respectively). Corn and PR reached the best energy analysis index because of their high biomass production. This study's results underlined the benefit of net energy from agricultural systems case study in Hau Giang province (19,380–720,032 MJ ha^{-1} crop^{-1}).

Key words: black sesame, corn, energy balance, life cycle assessment, mungbean, paddy rice

Varčevanje energije z menjavo gojenja različnih kultur: Analiza pridelave riža v poplavnih in suhih razmerah v provinci Hau Giang, Vietnam

Izvleček: Analiza porabe energije v kmetijstvu in njena boljša izraba bosta prispevali k trajnostnemu razvoju, prilagoditvam na podnebne spremembe in zagotovili predvidljivo pridelavo. Vzorčna raziskava, izvedena v provinci Hau Giang, je bila izvedena za primerjavo porabe energije pri pridelavi različnih poljščin in njene pretvorbe v biomaso pri gojenju riža v poplavnih razmerah (PR) in na suhem vključno s poljščinami kot so koruza, mungo fižol (MB) in črni sezam (BS). Za določanje porabe energije in njene pretvorbe v biomaso je bila uporabljena metodologija življenskih krogov. Na osnovi vnosa in vezave energije so bili analizirani parametri kot so učinkovitost izrabe energije, produktivnost energije, specifična energija in neto energija. Izbrane poljščine so zahtevale 39,501–59,638 MJ ha^{-1} poljščina^{-1}, kar je več kot je poraba energije pri gojenju poplavnega riža (PR). Pridelava poljščin je zahtevala veliko energije iz fosilnih goriv in elektrike (12,946–34,375 MJ ha^{-1} poljščina^{-1}). Količina energije v biomasi poljščin je znašala 779,670 MJ ha^{-1} poljščina^{-1} pri pridelavi koruze, temu je sledilo pridelovanje riža (198,723 MJ ha^{-1} poljščina^{-1}), mungo fižola (MB) in črnega sezama (BS) (103,292 in 63,012 MJ ha^{-1} poljščina^{-1}). Pridelovanje koruze in poplavnega riža (PR) je doseglo najboljše energetske indekse zaradi velike produkcije biomase. Rezultati te raziskave kažejo tudi prednost v izplenu neto energije pri načinu vzorca kmetovanja v provinci Hau Giang (19,380–720,032 MJ ha^{-1} poljščina^{-1}).

Ključne besede: črni sezam, koruza, energetska bilanca, določanje življenskih krogov, mungo fižol, poplavni riž

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1 INTRODUCTION

Research on agricultural energy has become vast and trendily to achieve sustainability and adaptation to climate change. The energy balance of agriculture is measured as agricultural activities' impact on the environment in the number of inputs and outputs measured in the equivalent of megajoule (MJ). The optimal and efficient energy usage in agriculture production will help succeed in the sustainable development goals (SDGs) under ensure sustainable consumption and production patterns (SDG12) through the sustainable reuse of agricultural residues. In Vietnam, solutions for using energy economically and efficiently in agricultural production have been stipulated in Article 22 of the Law on Economical and Efficient Use of Energy 2010 (The Vietnam National Assembly, 2010). The government, organizations, households, and individuals engaged in agricultural production carry out these energy-saving activities. In 2018, the Ministry of Agriculture and Rural Development of Vietnam also issued a circular guiding measures to use energy economically and efficiently in agricultural production (Vietnamese Ministry of Agriculture and Rural Development, 2018). The Vietnamese government has encouraged farming families to apply 4 leading solutions to use energy economically and efficiently in agrarian production. These groups proposed solutions focusing on the production stage, the processing, preservation, and transportation of farm products, and agricultural sector development. The most prominent of these regulations is encouraging scientific research results to production practices. Besides, using clean energy/renewable energy equipment and technology in production is a priority. Propaganda activities, dissemination of knowledge, and consultation on energy saving and efficiency for farmers are crucial.

The life cycle assessment (LCA) methodology is popularly used for agricultural energy analysis (Camargo et al., 2013; Del Borghi et al., 2022; Iriarte et al., 2010; Kaab et al., 2019; Ruviaro et al., 2012)we compare energy use and greenhouse gas (GHG. Almost all publications on paddy rice (PR) or upland crops have just focused on qualifying the energy requirement of farm inputs and output energy focus on grain or within straw (Elsoragaby et al., 2019a, 2019b; Kaur et al., 2021; Kazemi et al., 2015; Nandan et al., 2021; Soni et al., 2018; Truong et al., 2017) transplanting and broadcast seeding methods to investigate the energy pattern of rice production in Malaysia. The field under transplanting method in the main season showed 8.72% lesser mean total energy input, 6.25% higher mean machinery energy, 55.06% lesser mean seed energy and 23.01% higher mean output energy than the field under broadcasting method. Fertilizer the highest contributor of energy inputs it contributed by 62% in both transplanting and broadcasting methods and fuel was the second-highest contributor. The share of direct and indirect energy in the fields under the transplanting method were 19% and 81% and in the fields under the broadcasting method were 17% and 83% respectively. While the share of renewable and non-renewable energy in the fields under the transplanting method were 7% and 93% and in the fields under the broadcasting method were 14% and 86% respectively. The harvesting operation has the highest mechanization index level (0.99. However, producing energy from main products and by-products is essential to calculate the energy balance and to evaluate energy efficiency.

Agriculture played an essential role in Vietnam's development, contributing 13.97–10.94 % of GDP (General Statistics Office of Viet Nam, 2021, 2022). Rice farming is a vital sector of Vietnam's agriculture, and the Mekong Delta (MD) is the largest cultivated area and the highest production density of paddy rice in the nation. The Vietnamese MD donates to over half of the total rice productivity of Vietnam. The rice area in 2021 was estimated at 7,240,000 ha and reached 43,880,000 tons (General Statistics Office of Viet Nam, 2021). However, PR monocultures would harm the environment long-term through a high source of greenhouse gas emissions and large energy requirement, while rotation could bring several benefits (Elbasiouny & Elbehiry, 2020; Kumar et al., 2022). Although, as mentioned above, energy-efficient usage will help countries achieve SDG12 and sustainable agricultural production, research on a comparison of PR and upland crop cultivation on the aspect of energy analysis in Vietnam's agriculture is a limitation.

PR and upland crop cultivation also require high energy for agricultural activities, from land reparation and crop care to harvest. Energy consumption of PR farming in Vietnam was estimated for product weight or growing area such as 2.8–2.9 MJ kg-rice^{-1} (Ogino et al., 2021), 24.813–32.793 MJ ha^{-1} (Truong et al., 2017), 12.500–15.300 MJ ha^{-1} (Winter-Spring) and 12.600–13.500 MJ ha^{-1} (Summer-Autumn) (Nguyen et al., 2022)two seasons of field trials were conducted to compare different crop establishment practices for rice production in the Mekong River Delta using environmental and economic sustainability performance indicators. The indicators including energy efficiency, agronomic use efficiency, net income, and greenhouse gas emissions (GHGEs. Energy consumption for vegetable cultivation in Vietnam was also limited only leafy vegetables (Napa cabbage, bok choy, and brown mustard), showing the results of energy provided for leafy vegetable cultivation was 44.118 MJ ha^{-1} and 2.68 MJ kg^{-1} (Liem & Phuoc, 2021). Fully understanding the energy balance from crop cultivation would successfully contribute to achieving advanced agriculture planning in VND.

2 MATERIALS AND METHODS

Research site selection: This research will compare the energy efficiency of rice farming and upland crop production. The research was applied several Participatory Rural Appraisal (PRA) activities in three sites (Chau Thanh A District, Long My District, and Vi Thanh City, Hau Giang Province) on farmers about the context of local crop system restructuring and their expectations. This research used a local gorverment recommendation on agricultural restructering process on replacing one rice farming growing season with another upland crop. Based on the discussion results with Department of Agriculture and Rural Development of Hau Giang Province staff members, this study chose Vi Thanh City and Chau Thanh A District to conduct field experiments on upland crops because agricultural restructuring was taking place from a triple rice model to double rice–one cash crop model. Based on energy balance aspects, the study will inform local authorities on which upland crops are suitable for this process. In Long My, our PRA results showed that people were uninterested in crop system restructuring. Therefore, an assessment of rice cultivation in the S–A growing season was necessary. The results of analyzing the cultivation models of corn, MB, and BS in Vi Thanh City and Chau Thanh A District will provide data for farmers' decision-making process in Long My District.

The on-farm experiments on corn (*Zea mays* L.), mung bean (*Vigna radiata* L.), and black sesame (*Vigna cylindrica* L. Skeels) were separately conducted in Chau Thanh A district and Vi Thanh city, which are located in Hau Giang province from March to June 2022. The total experiment area was 3.950 m^2 of corn, 1.800 m^2 of BS, and 2.350 m^2 of MB. We used the surface water from local canal for irrigation. The surface water quality index (WQI) of these canal were classified in "yellow" (Hau Giang Department of Science and Technology, 2022), indicating that it is suitable for agricultural irrigation and other similar purposes (Vietnam Ministry of Resources and Environment, 2019). Our experiments were set up on the classification soil of Gleyic Fluvisols (Hau Giang People Committee, 2022).

For collecting the PR cultivation data, this research randomly selected and interviewed 240 households from February to March 2022 in Long My district, Hau Giang province. The sample size accounted for 1.6 % total rice-farming household in research area.

This study applied a LCA methodology framework with the "cradle-to-farm gate". The research perspective was applied to estimate energy requirements to produce and apply all inputs applied for paddy rice/upland crops cultivation and all the necessary upstream processes. With the "cradle-to-gate" approach, the "cradle" is understood as where raw materials manufacturing place, and the "gate" is at the farm where those agricultural materials are used in the research area in Hau Giang. However, the scope of the study was limited by neglectable the stage of raw materials transportation from the manufacturer site to the farms. Thus, energy used for processing from raw material exploitation to the production of commercial agricultural inputs is estimated in this study.

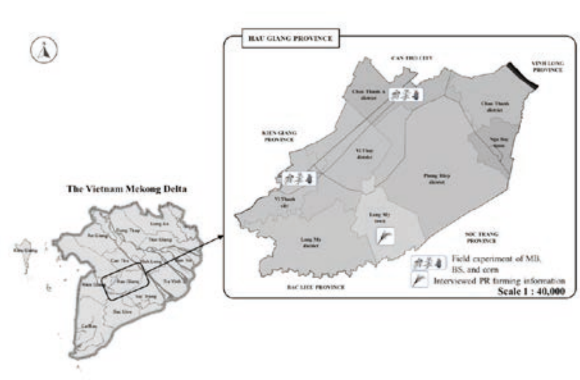

Figure 1: Research area.

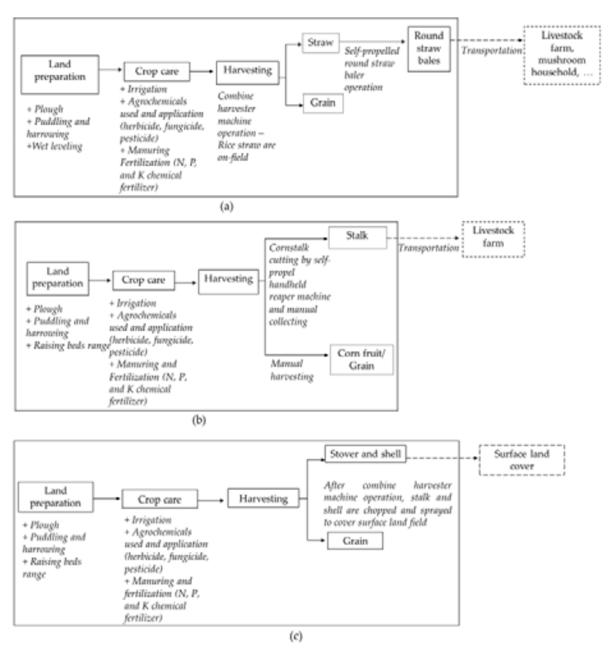

Figure 2: Life cycle assessment system boundary of paddy rice (a), corn (b), mungbean, and black sesame cultivation (c).

During the audit of farming materials, information about raw materials for production is collected, including the amount of fertilizer used (calculated by ingredients including nitrogen, phosphorus, and potassium fertilizers in kg-N, kg-P_2O_5, and kg-K_2O, respectively), amount of agrochemicals used (calculated by active ingredients including herbicide, pesticide, fungicide), amount of fuel/electricity used in tillage, irrigation and spraying agrochemicals. The system boundaries were set for farming activities under land preparation, crop care, and harvest stages presented in Figure 2. The functional unit is net energy in crop production per one hectare of growing area or one biomass tonnage in a growing season.

This study inventoried all agricultural inputs and output products. The mass of by-products was estimated based on the crop-to-residues ratio (CRR). In this study, straw, stover, cob, and shell of rice, corn, bean, and sesame were qualified through their dry grain. The CRRs are presented in Table 1.

This study applied several analysis of input-output energy that were popularly used in the field of agricultural production (Ali et al., 2019; Ghasemi-Mobtaker et al., 2020; Rajaeifar et al., 2014)intensive use of energy sources leads to environmental damages such as global warming and resources depletion. Hence, this study provided energy, environmental and economic overview of wheat cultivation in Hamedan province, Iran. The initial data were collected from 75 wheat farms applying face-to-face interview technique. The prepared data related to the 2017–2018 production cycle. The energy analysis results demonstrated that the total energy consumption and output energy in wheat cultivation were 43054.63 MJ ha^{-1} and 117407.13 MJ ha^{-1}, respectively. Energy productivity, energy use efficiency and net energy gain were computed as 0.12 kg MJ^{-1}, 2.73 and 74352.50 MJ ha^{-1}, respectively. Economic analysis showed that total value and cost of wheat production were 854.86 \$ ha^{-1} and 366.57 \$ ha^{-1}, respectively. Net return was 488.29 \$ ha^{-1} and benefit to cost ratio computed as 2.33 in the investigated region. Wheat environmental impacts were evaluated by applying life cycle assessment methodology. Results of environmental impacts showed the largest emissions were related to marine aquatic ecotoxicity (319757.6377 kg 1,4-DB eq.:

Energy use efficiency = output energy (MJ ha^{-1}) / input energy (MJ ha^{-1})

Energy productivity (kg MJ^{-1}) = grain yield (kg ha^{-1}) / input energy (MJ ha^{-1})

Specific energy (MJ kg^{-1}) = input energy (MJ ha^{-1}) / grain yield (kg ha^{-1})

Net energy (MJ ha^{-1}) = output energy (MJ ha^{-1}) - input energy (MJ ha^{-1})

Output energy (MJ ha^{-1}) = main product energy (MJ ha^{-1}) + by-products energy (MJ ha^{-1}).

The conversion factors of energy equivalent was applied to calculate agricultural inputs and all types of product energy (Table 2).

Table 1: Crop to residues ratio (CRR)

Residue type	CRR	References
Rice straw	1.53	(Purohit, 2009)
Corn stover	2.5	(Soni et al., 2013)
Corn cob	0.15	(Honorato-Salazar & Sadhukhan, 2020)
MB stover	1.35	(Wang et al., 2013)
MB shell	0.323	(Soni et al., 2013)
BS stover	3.8	(Honorato-Salazar & Sadhukhan, 2020)
BS shell	1.86	(S. Ali & Jan, 2014)

Table 2: The energy equivalent of inputs and outputs

Note: *: we used the same data of groundnut shells as in Soni et al. (2013) and Paramesh et al. (2019).

3 RESULTS AND DISCUSSION

Agricultural energy is commonly estimated based on consumption rate and efficiency. All energy requirements will be changed to an ordinary unit (ha^{-1}, t^{-1}) in this research field. The energy required per crop unit or calorie production ratio will be known as energy efficiency (J/MJ per product mass calorie). Researchers usually use energy efficiency for standardized assessments between a diversity of crops. The agricultural biomass used for energy purposes is growing very fast, and because residual and waste biomass sources have largely been depleted, energy harvest from agriculture will be a significant factor in the further growth of biomass use for energy drives (Knápek et al., 2021). The energy analysis results of the crops cultivation are shown in Table 3.

Table 3: Input-Output energy and energy relationship of crops cultivation.

3.1 ENERGY REQUIREMENT

Corn cultivation was the highest energy consumption crop (59,638 MJ ha^{-1}), while rice cultivation was the lowest (31,478 MJ ha^{-1}). MB and BS were the second and third crops that consumed significant energy sources with 43,632 and 39501 MJ ha^{-1}, respectively. All selected crops consumed enormous energy from fuels-electricity

(41.1–57.6 %) and fertilizers (14.6–31.8 %) (Figure 3). Fuels-electricity provided 12,946–34,375 MJ ha^{-1}, while fertilizers supplied 5,753–18,941 MJ ha^{-1}. Corn cultivation required machine energy (3,566 MJ ha^{-1}) lower than other selected crops (5,936–13,104 MJ ha^{-1}). It was a higher agrochemicals energy provided for BS (3,432 MJ ha^{-1}) and MB (3,252 MJ ha^{-1}) than corn (2,160 MJ ha^{-1}) and PR (1,296 MJ ha^{-1}). Seed (0.3–5.6 %) and labor (0.3–1.6 %) were the lowest energy sources for crop cultivation (Figure 3). They provided 104–1,764 and 393–620 MJ ha^{-1}.

Farmers would improve energy input to get a better energy relationship. Fuels-electricity was the largest source of selected crop cultivation, similar to the previ-

Table 2: The energy equivalent of inputs and outputs

A. Inputs	Unit	MJ unit-1	References
1. Human labor	h	1.96	(Aghaalikhani et al., 2013; Heidari & Omid, 2011)
2. Agricultural machines	h	62.7	(Ali et al., 2019)
3. Fossil fuels			
3.1 Gasoline	L	40.9	(Japan Environmental Management Association for Industry - JEMAI, 2014)
3.2 Diesel	L	51.3	(Yousefi et al., 2014)greenhouse gas (GHG
4. Electricity	kWh	3.6	(Ghorbani et al., 2011; Yousefi et al., 2014)
5. Chemical fertilizers			
5.1 Nitrogen	kg N	66.1	(Ozkan et al., 2011)
6. Agrochemicals	kg-active ingredient (kg ai)	120	(Canakci & Akinci, 2006)
7. Seeds			
7.1 PR	kg	14.7	(Yadav et al., 2017)
7.2 Corn	kg	15.7	(Canakci et al., 2005)cotton, maize, sesame
7.3 MB	kg	14.7	(R. Kumar et al., 2021; Lotfi et al., 2021)
7.4 BS	kg	26	(Akpinar et al., 2009)
B. Outputs	Unit	MJ $unit^{-1}$	References
1. Rice product and by-products			
1.1 Grain	kg	14.7	(Yadav et al., 2017)
1.2 Straw	kg	14.87	(Biswas et al., 2017)
2. Corn product and by-products			
2.1 Grain	kg	14.7	(Parihar et al., 2017)
2.2 Stover	kg	18	(Soni et al., 2013)
2.3 Cob	kg	17.16	(Honorato-Salazar & Sadhukhan, 2020)
3. MB product and by-products			
3.1 Grain	kg	15.3	(Paramesh et al., 2019)
3.2 Stover	kg	12.5	(R. Kumar et al., 2021
3.3 Shell*	kg	11.23	(Sajjakulnukit et al., 2005)
4. BS product and by-products			
4.1 Grain	kg	25	(Akpinar et al., 2009)
4.2 Stover	kg	17.47	(Honorato-Salazar & Sadhukhan, 2020)
4.3 Shell*	kg	11.23	(Sajjakulnukit et al., 2005)

Note: *: we used the same data of groundnut shells as in Soni et al. (2013) and Paramesh et al. (2019).

ous study (Elsoragaby et al., 2019a; Kazemi et al., 2015; Yousefi et al., 2014)energy use pattern for rice production was analyzed and compared in different geographical regions, Golestan, Mazandaran and Guilan, northern pro-

Table 3: Input-Output energy and energy relationship of crops cultivation.

	PR	Corn	MB	BS
A. Inputs (MJ ha^{-1})	31,478	59,638	43,632	39,501
1. Human labor	108	393	545	620
2. Agricultural machines	5,936	3,566	7,524	13,104
3. Fossil fuels and electricity	12,946	34,375	24,358	16,488
3.1 Gasoline	0	389	307	0
3.2 Diesel	12,938	33,986	24,024	16,488
3.3 Electricity	8	0	27	0
4. Chemical fertilizers	9,428	18,941	7,585	5,753
4.1 Nitrogen	8,229	15,415	6,280	4,726
4.2 Phosphate	802	1,706	851	621
4.3 Potassium	396	1,820	455	405
5. Agrochemicals	1,296	2,160	3,252	3,432
6. Seeds	1,764	204	368	104
6.1 PR	1,764	-	-	-
6.2 Corn	-	204	-	-
6.3 MB	-	-	368	-
6.4 BS	-	-	-	104
B. Outputs (MJ ha−1)	198,723	779,670	63,012	103,292
1. PR product and by-product	198,723	-	-	-
1.1 Grain	78,001	-	-	-
1.2 Straw	120,722	-	-	-
2. Corn product and by-product	-	779,670	-	-
2.1 Grain	-	184,044	-	-
2.2 Stover	-	563,400	-	-
2.3 Cob	-	32,226	-	-
3. MB product and by-product	-	-	63,012	-
3.1 Grain	-	-	26,928	-
3.2 Stover	-	-	29,700	-
3.3 Shell	-	-	6,384	-
4. BS product and by-product		-	-	103,292
4.1 Grain	-	-	-	23,000
4.2 Stover	-	-	-	61,075
4.3 Shell	-	-	-	19,217
C. Energy relationship				
1. Energy use efficiency	6.31	13.07	1.44	2.61
2. Energy productivity (kg MJ^{-1})	0.17	0.21	0.04	0.02
3. Specific energy (MJ kg^{-1})	5.93	4.76	24.79	42.94
4. Net energy (MJ ha^{-1})	167,245	720,032	19,380	63,791

vinces of Iran. There is a significant difference among the three provinces in respect to input energy and agronomical managements such as crop rotation, transplanting date and land preparation. Data were collected from 50 farmers using a face to face questionnaire-based survey. The data collected belonged to the production period of 2012-2013 with the following results obtained. The energy use efficiency varied from 1.39 for Golestan to 1.67 for Guilan provinces. The research results revealed the main difference between energy consumption in three provinces comes from diesel fuel, chemical fertilizers and electricity. The net energy for paddy production was approximately higher in Guilan (36,927.58MJha-1. Changing from flooded to subsurface drip irrigation helps rice farming reduce power consumption (Coltro et al., 2017)mitigating GHG emissions in agriculture is fundamental to reduce its share of responsibility for the global climate change. Rice (paddy. The drip irrigation method achieved more efficient energy for upland crops than furrow and sprinkler irrigation (Kazemi & Zardari, 2020; Reddy et al., 2015)greenhouse gas (GHG. Specific energy is a significant factor that emphasizes the total energy demand to produce one product unit (Parihar et al., 2017)residue burning, decline in biomass productivity and water tables. In semi-arid regions, the climate-change-induced variability in rainfall and temperature may have an impact on phenological responses of cereals and pulses which in turn would affect biomass production, economic yield and energy and water-use efficiency (WUE. In agricultural production, a product with higher value of specific energy points that means it produce a lower total energy output from input energies (Chaudhary et al., 2017). By changing the current irrigation method to better practices, crop cultivation would save irrigation energy and achieve better total energy consumption and specific energy.

3.2 ENERGY OUTPUTS

Corn produced 779,670 MJ ha^{-1} through total biomass, 3.9 times higher than rice farming (198,723 MJ ha^{-1}), 7.5 times higher than BS cultivation (103,292 MJ ha^{-1}), and 12.4 times higher than MB cultivation (63,012 MJ ha^{-1}). Selected crops' by-products energy was higher than marketable products from 1.3 times (MB stover and shell/grain), 1.5 times (rice straw/grain), 3.2 times (corn stover and cob/grain), and 3.5 times (BS stover and shell/grain).

Government and policy-makers would plan to use the vast potential of by-products. Direct biomass energy source would be provided for surface soil by covering biomass debris from the stover and shell of MB and BS after the combine-harvester machine works. Corn stover and rice straw were used for several purposes, including feedstock, mushroom cultivation, and soil orchard mulching. In addition, by-product biomass sources could be used for gasification, biofuel, ethanol, and biochar production.

3.3 ENERGY RELATIONSHIP

Corn was the most energy efficiency cultivated crop when it produced 13.07 MJ based on one MJ input. One hectare of corn cultivation achieved a net energy of 720,032 MJ. On the other hand, corn also had benefits in energy productivity (the highest value, 0.21 kg MJ^{-1}) and specific energy (the lowest value, 4.76 MJ kg^{-1}). Rice was the second optimum selected crop, reaching 167,245 MJ net energy, and the energy efficiency index was 6.31. Rice cultivation needed 5.93 MJ input to produce one kg of rice grain. Parallelly, a one-hectare growing area had 0.17 kg of rice grain by providing one MJ through agricultural inputs. Although the net and effective energy of MB (19,380 MJ ha^{-1} and 1.44) was lower than BS (63,791 MJ ha^{-1} and 2.61), MB had energy productivity and specific energy value (0.02 kg MJ^{-1} and 42.94 MJ kg^{-1}) better than BS (0.04 kg MJ^{-1} and 24.79 MJ kg^{-1}).

Rice and corn play vital roles in worldwide food security. According to Li and Siddique (2020), MB will be an innovative food crop in the future. BS provides oil as the primary product and extracted oil meal for animals' feedstock as a secondary product. Selected crop cultivation has used high energy through several agricultural inputs. With a whole agricultural ecosystem of crop cultivation, it is possible to conclude that four selected crop cultivations had benefited energy net. Crop by-products provided a huge biomass source for secondary use - recycling activities that adapt to SDG12.

4 CONCLUSIONS

The optimal and efficient energy usage in agriculture production will help succeed in the SDGs under SDG12 through the sustainable reuse of agricultural residues. To explore the impact of farming activities, the LCA methodology was applied. Generally, all studied crops achieved benefits in energy targets. PR is the lowest-consumed energy crop but is the second-largest energy production crop. Upland crops require more energy than PR through labor, power (fossil fuels and electricity), and agrochemicals. Corn and PR reach the best energy analysis index because of their high biomass production, especially in cases of by-products. People must trade off energy to achieve nutrients from the grain. However, this study's results underline the benefit of produced energy from agricultural systems case study in Hau Giang province.

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The effect of nitrogen doses on morpho-physiological traits of safflower at different levels of deficit irrigation

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The effect of nitrogen doses on morpho-physiological traits of safflower at different levels of deficit irrigation

Abstract: This experiment aimed to evaluate the effects of irrigation regimes based on potential evapotranspiration (100 %, 60 %, and 40 % PET) and doses of nitrogenous fertilizers (0, 40, and 80 kg ha-1) on the performance of safflower in Qazvin, Iran. Three fractions of potential evapotranspiration: 100 %, 60 %, and 40 % PET are considered as full irrigation (FI), mild deficit irrigation (MDI) and severe deficit irrigation (SDI), respectively. Results revealed that the effectiveness of nitrogen fertilizers was discernable under FI and MDI conditions, however, under SDI, the application of nitrogen fertilizers did not have any improvement effects on evaluated traits. The highest seed yield was recorded in FI + N80 (1253 kg ha-1) and the seed yield of MDI + N80 (1121 kg ha-1) was in the second place with a difference of 132 kg ha-1. However, the best seed quality in terms of protein percentage was related to plants grown under SDI + N80 (23.44 %), which was 43 % higher than FI + N0 (16.45 %). The plants grown under FI + N80 and SDI + N80 conditions showed the highest seed oil content with 28 % and 27.1 %, respectively. The obtained results showed that MDI can be used as a strategy to improve the efficiency of water consumption under water scarcity conditions in the semi-arid region.

Key words: canopy width, chlorophyll content, potential evapotranspiration, seed oil content, water shortage

Učinek odmerkov dušika na morfološke in fiziološke lastnosti žafranike pri različnih načinih deficitnega namakanja

Izvleček: Namen poskusa je bil ovrednotiti učinke načina namakanja, ki so bili osnovani na potencialni evapotranspiraciji (100 %, 60 %, in 40 %; PET) in odmerkov dušičnih gnojil (0, 40, in 80 kg ha-1) na uspevanje žafranike v Qazvinu, Iran. Tri vrednosti potencialne evapotranspiracije (100 %, 60 %, in 40 %) so bile glede na način namakanja opredeljene kot polno namakanje (FI), blago deficitno namakanje (MDI) in izrazito deficitno namakanje (SDI). Rezultati so pokazali, da je bila učinkovitost dušičnih gnojil opazna v razmerah FI in MDI, medtem, ko gnojenje z dušikom v razmerah SDI ni dalo nobenih učinkov izboljšanja pri obravnavanjih. Največji pridelek semena je bil ugotovljen pri obravnavanju FI + N80 (1253 kg ha^{-1}), na drugem mestu je bil pridelek semena pri obravnavanju MDI + N80 (1121 kg ha^{-1}), z razliko 132 kg ha^{-1}. Najboljša kakovost semen glede na odstotek beljakovin je bila ugotovljena pri rastlinah, ki so rastle v razmerah SDI + N80 (23,44 %), ki je bila za 43 % večja kot pri obravnavanju FI + N0 (16,45 %). Rastline v obravnavanjih FI + N80 in SDI + N80 so imele največjo vsebnost olja, 28 % in 27,1 %. Dobljeni rezultati so pokazali, da bi obravnavanje MDI lahko uporabili kot strategijo za izboljšanje učinkovitosti rabe vode v razmerah njenega pomanjkanja na semiaridnih območjih.

Ključne besede: obseg nadzemnega dela rastline, vsebnost klorofila, potencialna evapotranspiracija, vsebnost olja v semenih, pomanjkanje vode

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1 INTRODUCTION

Optimal management of irrigation is a technique to improve water efficiency and is implemented according to the water requirement of crops (Wu et al., 2022). In semi-arid region precipitation is spatially and temporally diverse and not sufficient to meet crop water requirementsTherefore, it is necessary to use irrigation in some sensitive stages of plant development. However, the available water for irrigation is also severely limited in these areas, and this trend has intensified in recent years due to climate change (Peng et al., 2023). Indiscriminate use of groundwater and failure to recharge the aquifer level will cause many problems in the future, and this process will worsen with the intensification of climate change and global warming (Araya et al., 2017). Therefore, the optimal use of available water resources by using precise or regulated deficit irrigation techniques can play a significant role in sustainable water consumption. However, it must be noted that water deficit stress during the reproductive stage and especially throughout the stages when the primordia of the yield components are forming can decrease the final economic yield (Sah et al., 2020).

Oilseed plants are very important in providing food security. Among oilseed plants, safflower (*Carthamus tinctorius* L.), whose origin seems to be southern Asia, is an underutilized or forgotten oilseed crop, and yields about 32-40 % seed oil (Leus, 2016). Safflower oil is multipurpose and is used in many industries and food preparation. Safflower is relatively tolerant under stressful conditions especially drought stress, because it develops a root system in the soil. Safflower is able to survive under drought stress conditions through its deep root system and the ability to use some protective mechanisms (Hussain et al., 2016).

Most of the precipitation in the semi-arid regions of the middle and northern parts of Iran falls during the cold months of the year and partly at the beginning of the spring season. In such a situation, it seems that the use of regulated irrigation techniques such as deficit irrigation can lead to significant improvement of water use efficiency (WUE) in addition to saving water for the dry months of late spring and early summer. However, water shortage during flowering (BBCH scale 61-69) and fruit development (BBCH scale 75-79) can significantly affect seed yield (Singh et al., 2016; Flemmer et al., 2015). Deficit irrigation is designed based on less water consumption during drought-resistant or drought-insensitive growth stages and water saving for critical and drought-sensitive stages. Water-saving irrigation strategies are applied in various regions of semi-arid areas in which water used in irrigation systems is less than the amount of water needed for evapotranspiration in the plant, therefore can lead to an increase in WUE (Sidhu et al., 2021). In this strategy, the irrigation schedule is done in such a way that sufficient water provides *during* the specific *crop* development periods (drought-sensitive stages) to improve the WUE, by exposing the crops to some degree of water shortage and an acceptable yield reduction (Geerts & Raes, 2009). Deficit irrigation methods will be considered based on the amount of precipitation, water storage in the soil, and plant needs in different growth periods. It seems that the moisture condition of the soil and the amount of irrigation can affect the availability of some nutrients such as nitrogen in the soil (He et al., 2023). The application of nitrogen by developing vegetative structures and increasing leaf surface can affect water loss by increasing the transpiration and *stomatal conductance* (Mu & Chen, 2021). Despite some investigations regarding the effect of water stress on safflower in these areas, the safflower performance under deficit irrigation and nitrogen fertilizers application has not been well studied. This research aimed to evaluate the effect of deficit irrigation and nitrogen fertilizer on the growth characteristics and seed yield of safflower.

2 MATERIAL AND METHODS

2.1 CLIMATE OF THE SITE AND SOIL CHARACTERISTICS

A field experiment was designed to scrutinize the impacts of various irrigation levels and nitrogen doses on safflower growth and yield components during 2021-2022 growing seasons in the Qazvin, middle and northwest, Iran (1270 m height above sea level. between 36°15′ N latitude and 50°03′ E longitude). The climate of the studied area is cold semi-arid (Type BSk) based on the Köppen Climate Classification System. The soil texture of the studied location was clay loam. Soil characteristics were: pH = 7.62, electrical conductivity = 0.716 dsm-1, organic matter = 1.69 g kg^{-1}, nitrogen (N) = 0.081%, available phosphorus = 8.21 mg kg^{-1} and available potassium (K) = 286 mg kg^{-1}. The amount of annual potential evaporation and transpiration in the studied area was 1415 mm. The monthly evapotranspiration, temperature, and precipitation during the growing season are shown in Table 1.

2.2 PREPARATION OF THE SEED BED

The mentioned farm was under wheat cultivation during the previous years. The field was *plowed* in the

Table 1: Monthly evapotranspiration, temperature and precipitation in the field experiment area during the growing season of safflower (2022).

	Mar	Apr	May	Jun	July
Average of minimum temperature (˚C)	8.9	13.2	20.4	25.2	24.3
Average of maximum temperature (˚C)	16.2	23.4	31.9	36.0	34.4
Average of temperature (˚C)	12.55	18.30	26.15	30.6	29.3
Total evapotranspiration (mm)	89.2	129.5	208.4	282.3	299.4
Total rainfall (mm)	40.36	26.38	5.2	3.1	1.0

autumn by a moldboard plow to a depth of 35 cm deep and then a disc harrow was used to crush the lumps and mix the residues and animal manure. Rotten farmyard manure was added to the land at the rate of 20 t ha^{-1} and mixed with the topsoil with secondary tillage. In the early spring, before planting, the soil was re-tilled by a rotary cultivator to achieve a fine tilth seedbed. The experimental field was divided into plots (4 × 3 m). The distance between the blocks was chosen to be 1 meter to avoid merging the effects of fertilizer and irrigation treatments. The seeds were sown manually in experimental plots that included 8 rows by 50 cm distance between adjacent rows and 15 cm plant to plant distance and 3 m row length. The experimental field included 27 plots. The planting dates were 13 May 2022. The seed of safflower (*Carthamus tinctorius*'Saffeh' was selected by taking into account features such as spring type, drought resistance, acceptable seed yield, and relatively high adaptability to the semi-arid conditions of Iran. The seeds were obtained from Pakan Bazr of Isfahan, Iran.

2.3 EXPERIMENTAL DESIGN

The experiment was conducted as a split-plot design (SPD) over randomized complete block design (RCBD) with three replications. Different irrigation treatments including full irrigation (FI) or irrigation to 100 % potential evapotranspiration (ETP), 60 % ETP as mild deficit-irrigation (MDI), and 40 % EPT as severe deficit-irrigation (SDI) assigned to the main plots. The subplots were assigned to different doses of nitrogen fertilizer, which consisted of N_0: no use of nitrogen fertilizer, N_{40}: application of 40 kg ha^{-1} of nitrogen through urea fertilizer, and N_{80}: utilization of 80 kg ha^{-1} of nitrogen through urea fertilizer. The level setting of nitrogen fertilizer is based on the soil background value.

2.4 FIELD IRRIGATION

In this research, the irrigation method was chosen as a surface drip irrigation system with a cycle of 6-10 days (according to plant requirement and soil water depletion), and irrigation was measured by volumetric water meters. In this study a time domain reflectometry (TDR) with two parallel probes of 7.5 cm length have used to calculate soil volumetric water content. The water requirement at each time was determined based on potential evapotranspiration, irrigation cycle, and the relevant plant coefficient. Potential evapotranspiration was estimated through the relationship $ETP = E_{pan} \times K_{pan}$. Where K_{pan} was the coefficient of evaporation pan and E_{pan} was the amount of evaporation from the pan in millimeters per day, which was considered a cumulative amount during the growth period (Doorenbos and Pruitt 1975). For K_{pan} value of 0.70 was used as the 50-year average of the experimental site. The irrigation depth was calculated through the Vermeiren and Jobling (1980) suggested method. The irrigation depth in the growing season was 242 mm (SDI), 326 mm (MDI), and 465 mm (FI). Total rainfall was 76 mm during the growing season (April– August). To prevent the possible reducing effects of deficit irrigation on the yield components, the amount of irrigation during the critical stages, i.e. beginning of flowering to fruit development, was applied as full irrigation.

2.5 DATA RECORDING

During the filling stage, chlorophyll was measured in the upper leaves using a portable chlorophyll meter (Chlorophyll Meter SPAD-502- Japan). Canopy width was measured by measuring the amount of canopy expansion from the right to the left of the plant through a ruler. The height of the plant and lateral branches was measured at the maturity stage. In the physiological maturity stage, the mass of 1000 seeds and the yield after harvesting 2 m^2 from the central parts of the plots were randomly weighed. The biological yield was determined after oven-drying of total aboveground biomass at 75 °C until constant mass. The electrical balance was used to take the fresh and dry mass. The American Association of Cereal Chemists AACC-30-10 (2003) was used for the assessment of the seed oil content. Seed protein content was determined using a near-infrared seed analyzer (ZX-50SRT, Zeltex, USA).

2.6 STATISTICAL ANALYSIS

Before analysis of variance, the homogeneity of variances was done with Anderson–Darling homogeneity test. All figures (box plots) were created using SOSS 19. Analyses of variance ANOVA) was fitted using the general linear model procedure in software SAS. Means comparison was done using the least significance difference test at $p < 0.05$.

3 RESULTS

The results of the meteorological data indicated that most of the spring rains occur during March and April, and with the increase in temperature and decrease in rainfall during the following months, the amount of evaporation and transpiration in the field increased sharply (Table 1). The highest water requirement was recorded during July, which coincided with the stages of seed filling, and the use of continuous deficit irrigation can cause a sharp decrease in yield under the mentioned conditions.

3.1 PLANT AND CAPITULUM HEIGHT

The results of the analysis of variance indicated the presence of significant interaction effects of irrigation × fertilizer on the plant height component (Table 2). So, the longest plants were observed in full irrigation conditions and the improving effect of nitrogen in the mentioned conditions on the height was quite evident and obvious. Consumption of 80 kg ha^{-1} of nitrogen in full irrigation conditions increased the height by 18 % compared to the control (no fertilizer consumption). However, in SDI conditions, the effect of nitrogen fertilizer application on height was not very noticeable and caused a slight increase in plant height. Evaluation of FCH (distance between *ground level* and the *first* capitulum) showed that its response to nitrogen was largely similar to the plant height. High use of nitrogen under FI conditions increased the FCH, however, the lowest FCH was recorded under the MDI condition (Table 2).

3.2 CHLOROPHYLL CONTENT AND CANOPY WIDTH

Table 2: Effect of deficit irrigation and nitrogen fertilizer doses on growth parameters of safflower (*Carthamus tinctorius* L.).

	PH	FCH	CHL	CNP	CD	TSM	BYM	HI	OIL	
FI	N0	71.99d	41.00b	46.98de	9.17c	14.48bc	31.23c	7097.88c	14.55e	25.58bc
	N40	80.57b	39.33c	55.39c	10.99b	15.29b	31.81bc	7620.72b	14.71e	26.25b
	N80	85.62a	44.22a	66.42a	12.21a	16.75a	32.72a	8388.56a	14.98e	28.19a
MDI	N0	61.72ef	37.00c	47.10de	8.61c	14.02cd	30.11d	5746.07f	15.75d	25.18bc
	N40	75.10c	38.00bc	50.14d	10.17b	14.67bc	31.23c	6147.86e	16.41c	26.28b
	N80	81.37b	39.66bc	59.43b	12.38a	15.47b	32.04b	6490.77d	17.27b	27.61a
SDI	N0	56.93g	40.66b	41.02fg	6.91d	12.04e	28.72e	4559.81h	18.99a	23.99d
	N40	60.66f	39.66bc	39.79g	7.30d	13.25d	29.10e	4774.23g	18.62a	25.01cd
	N80	63.86e	41.00b	43.81ef	8.79c	33.88cd	28.15f	4897.99g	17.70b	24.88cd
					statistical significance					
I	**	**	**	**	**	**	**	**	**	
N	**	*	**	**	**	**	**	*NS*	**	
I×N	**	*NS*	**	*	*NS*	*	**	**	*NS*	

FI: full irrigation (100 % PET), MDI: mild deficit irrigation (60 % PET), SDI: sever deficit irrigation (40 % PET), N0: no nitrogen application (control), N40: application of nitrogen at doses 40 kg ha-1, N80: application of nitrogen at doses 80 kg ha-1, PH: plant height (cm), FCH: the height of first capitulum from the ground level (cm), CHL: leaf chlorophyll content (SPAD unit), CNP: capitulum number per plants, CD: capitulum diameter (mm), TSM: thousand seed mass (g), BYM: biological yield (kg ha-1), HI: harvest index (%), OIL: seed oil content (%). In each column rows with different letters have statistically significant differences at the 5% level ($p \leq 0.05$). **: significant at 0.01 level, *: significant at 0.05 level, NS: not statistically significant.

Chlorophyll content as one of the important photosynthetic pigments was investigated under the influence of the treatments. The highest amount of chlorophyll SPAD value in upper young leaves was recorded in the flowering stage under FI and N80 conditions. However, low irrigation treatments significantly reduced the amount of chlorophyll. The use of MDI and SDI reduced the amount of leaf chlorophyll by about 8 and 26 %, respectively, compared to FA conditions (Table 2). The application of both levels of nitrogen under full irrigation conditions (FI+N_{40}, FI+N_{80}) significantly increased the chlorophyll content of leaves. Under MDI conditions, only using 80 kg ha^{-1} of nitrogen improved the chlorophyll content. Under SDI conditions, nitrogen application had no significant effect on leaf chlorophyll content. It did no influence the content of this article. In addition, canopy width is considered an important component describing vegetative growth. Results showed that the maximum canopy width was recorded under IF and the application of 40 and 80 kg ha^{-1} nitrogen in the mentioned conditions increased the canopy width by 10 % and 25 %, respectively, compared to the condition of no nitrogen fertilizer application (Figure 1). However, the use of N_{40} under MDI conditions and the use of N_{40} and N_{80} under SDI conditions could not positively affect the canopy width. Although the use of low levels of nitrogen under MDI conditions did not improve the lateral growth of the canopy, the use of N_{80} was able to improve the canopy width by about 13 % compared to the condition of not using fertilizer under the aforementioned irrigation regime.

3.3 SEED YIELD COMPONENTS

Assessment of the number of secondary branches (NSB) showed that irrigation treatments and application of nitrogen fertilizer strongly affected this component and reducing the amount of irrigation causes a significant decrease in the number of lateral branches. However, the application of N_{80} under MDI conditions was able to stimulate lateral growth to some extent and improved the number of lateral branches by 47 % compared to SDI+N_0 conditions (the lowest NSB). The highest number of NSB was recorded in the condition of applying large amounts of nitrogen and providing a large volume of water in irrigation with 10.5 branches. The lowest number of NSB was recorded under SDI + N_{80}, which was 60 % less than plants grown with the same nitrogen level under FI condition (Figure 2).

Number of the capitula per plant (CNP) significantly affected by the interaction effects of irrigation and nitrogen ($p < 0.05$). The highest number of capitulum was obtained under IF and MDI along with the application of high levels of nitrogen. However, the use of nitrogen in SDI conditions did not have much effect on the improve-

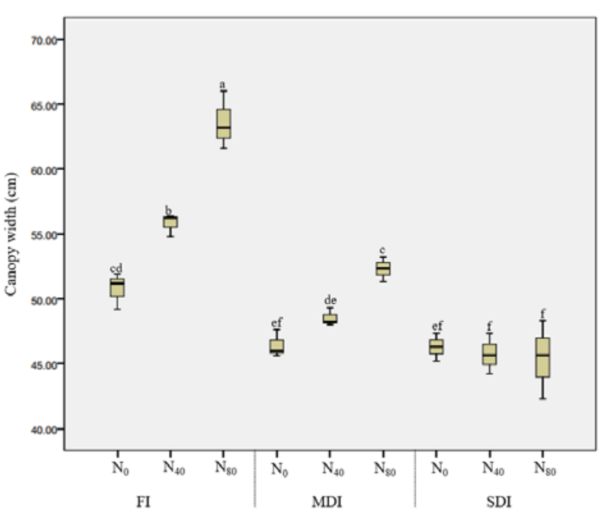

Figure 1: Mean comparison of the canopy width of safflower plants grown under different levels of deficit irrigation along with the application of different dose of nitrogen fertilizer. FI: full irrigation (100 % PET), MDI: mild deficit irrigation (60 % PET), SDI: sever deficit irrigation (40 % PET), N0: no nitrogen application (control), N40: application of nitrogen at doses 40 kg ha-1, N80: application of nitrogen at doses 80 kg ha-1. Boxes with different letters are statistically significant (p ≤ 0.05).

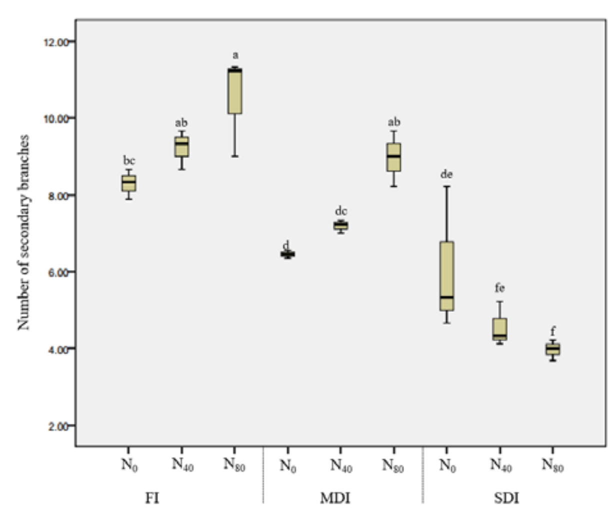

Figure 2: The effects of irrigation levels and nitrogen fertilizer doses on number of secondary branches of safflower. FI: full irrigation (100 % PET), MDI: mild deficit irrigation (60 % PET), SDI: sever deficit irrigation (40 % PET), N0: no nitrogen application (control), N40: application of nitrogen at doses 40 kg ha-1, N80: application of nitrogen at doses 80 kg ha-1. Boxes with different letters are statistically significant (p ≤ 0.05).

ment of this important yield component. The lowest CNP recorded for plants grown under SDI+N_0 and the use of N_{80} under FI and MDI increased this component by 44 %. Consumption of N_{80} under IF and MDI conditions increased CNP by about 15 %, while under SDI conditions it did not have a significant effect. Evaluation of capitulum diameter (CD) showed that application of N_{40} and N_{80} increased this trait by 6 % and 13 %, respectively. On the other hand, the mean comparison of CD among irrigation levels indicated that the use of SDI and MDI caused a decrease of 16 % and 6 % respectively in CD (Table 2). The examination of the mass of one thousand seeds showed that this component was very sensitive to irrigation treatments, so the use of MDI and SDI caused a significant decrease in the mass of seeds (4 % and 11 %). Nitrogen application was able to partially compensate adverse effects of water deficiency on seed weight under MDI conditions, however, the effect of nitrogen application under SDI conditions was not significant. Biological performance was strongly affected by irrigation and nitrogen treatments and their mutual effects. Although the application of N_{80} under IF conditions could improve biological yield by 18 %, the application of the same amount of fertilizer under MDI and SDI conditions led to an increase of 12 % and 7 %. In addition, the reduction of water consumption in MDI and SDI caused a reduction of 21 % and 39 % in biomass. The evaluation of seed yield indicated that the application of fertilizer under IF conditions significantly improve this trait (N_0: 1033 kg ha^{-1}, N_{40}: 1221 kg ha^{-1}, N_{80}: 1253 kg ha^{-1}). The increasing trend of fertilizer application on yield under MDA conditions was also visible (N_0: 905 kg ha^{-1}, N_{40}: 1049 kg ha^{-1}, N_{80}: 1161 kg ha^{-1}). However, fertilizer application could not have a positive effect under SDI conditions (Figure 3). Interestingly, the highest harvest index (HI) was recorded under SDI+ N_0 or N_{40} conditions, and the lowest harvest index was observed under full irrigation conditions. However, the application of N_{80} under MDI conditions significantly improves the HI.

3.4 SEED OIL AND PROTEIN CONTENT

Nitrogen application increased the seed oil content. The seed oil content of plants grown under the application of N_0, N_{40}, and N_{80} was 24.92 %, 25.84 %, and 26.89 % respectively. Applying SDI reduced the seed oil content by 2 % compared to full irrigation. The evaluation of seed protein content showed that this trait was significantly affected by nitrogen and the application of nitrogen at all irrigation levels increased the percentage of seed protein. However, the highest protein percentage was recorded under SDI+N_{80} (23.6 %), and the lowest protein percentage was related to the plants grown under FI+N_0 (Figure 4).

The clustering of traits classified them into three

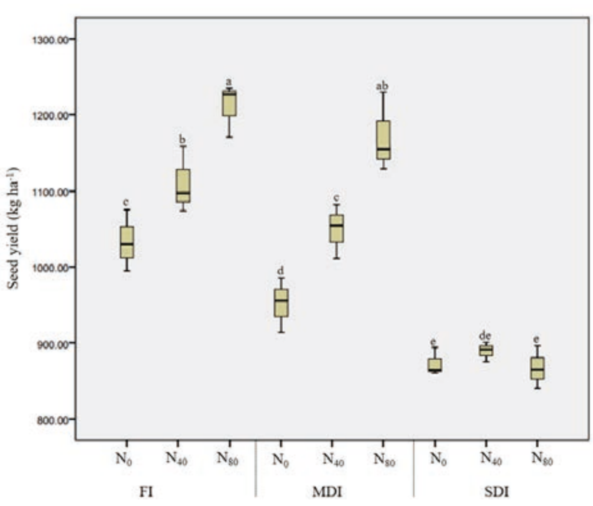

Figure 3: The effects of irrigation levels and nitrogen fertilizer doses on seed yield of safflower. FI: full irrigation (100 % PET), MDI: mild deficit irrigation (60 % PET), SDI: sever deficit irrigation (40 % PET), N0: no nitrogen application (control), N40: application of nitrogen at doses 40 kg ha-1, N80: application of nitrogen at doses 80 kg ha-1. Boxes with different letters are statistically significant (p ≤ 0.05).

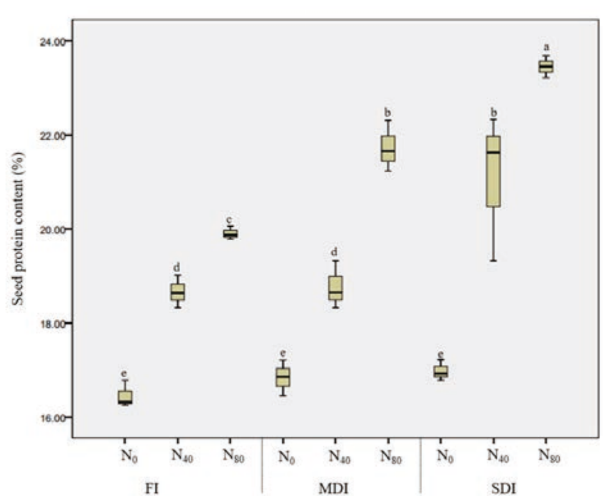

Figure 4: Mean comparison of the seed protein content of safflower plants grown under different levels of deficit irrigation along with the application of different dose of nitrogen fertilizer. FI: full irrigation (100 % PET), MDI: mild deficit irrigation (60 % PET), SDI: sever deficit irrigation (40 % PET), N0: no nitrogen application (control), N40: application of nitrogen at doses 40 kg ha-1, N80: application of nitrogen at doses 80 kg ha-1. Boxes with different letters are statistically significant (p ≤ 0.05).

groups in terms of similarity based on response to the investigated treatments. Cluster I include traits that showed their best performance under full irrigation conditions and with the application of high levels of nitrogen fertilizer. Cluster II included FCH, which was significantly reduced under MDI conditions. Cluster III included seed protein percentage and harvest index, which were increasingly stimulated by decreasing the amount of irrigation (Figure 5).

4 DISCUSSION

The results obtained in this research indicated that during the first months of the growing season, the rate of evapotranspiration was relatively low, but with the increase in temperature during the months of late spring and early summer, the rate of evapotranspiration increased significantly. The results of meteorological data showed that the amount of rainfall during the months of March to June was about 10 % of the amount of evaporation and transpiration from the evaporation pan. This indicates that the region is facing a severe lack of rainfall and the use of irrigation or the presence of water storage in the soil before cultivation is necessary. The evaluation of traits related to vegetative growth indicated that irrigation and nitrogen fertilizer treatments significantly affected these components. Plant growth is based on the accumulation of water in the vacuole and also due to the interaction of phytohormones (Pashkovskiy et al., 2022). On the other hand, due to the role of nitrogen-containing functional groups in key structures such as proteins and functional structures of enzymes, nitrogen is considered a key constituent in cells and its optimum supply is necessary for ideal plant growth (Govindasamy et al., 2023). However, consuming large amounts of nitrogen through increasing the level of evapotranspiration can accelerate the occurrence of drought stress in semi-arid areas. It appears that water shortage, such as the one observed under SDI conditions, causes the closing of the stomata, the reduction of photosynthesis, and finally the growth reduction through the rapid increase of abscisic acid (ABA) biosynthesis or increasing the release of previously made hormones from organelles such as chloroplasts (Car-

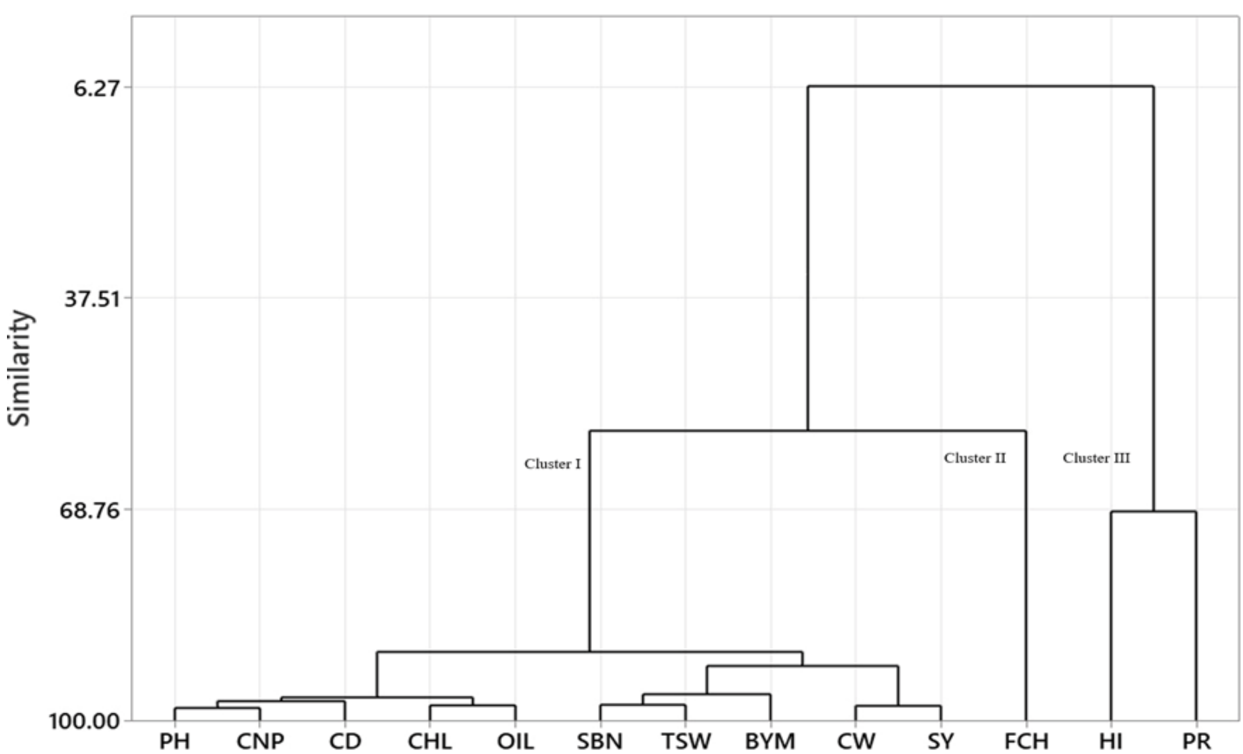

Figure 5: Clustering of different growth traits and yield components of safflower based on the response to different levels of irrigation and doses of nitrogen fertilizer. PH: plant height (cm), SBN: number of secondary branches, SY: seed yield, PR: seed protein content, FCH: the height of first capitulum from the ground level, CHL: leaf chlorophyll content, CW: canopy width, CNP: capitulum number per plants, CD: capitulum diameter, TSM: thousand seed mass, BYM: biological yield, HI: harvest index, OIL: seed oil content.

doso et al., 2020). However, nitrogen application under MDI conditions improved growth-related characteristics compared to MDI + N_0. The above results point out that water shortage under MDI conditions has not reached the limiting and extreme level. Probably water reduction in the mentioned conditions activated some adaptation mechanisms such as semi-*closed* stomata, preferential uptake of CO_2 and reduction of water loss through transpiration, increase of water absorption through the development of root growth and increased hydraulic conductivity has increased the efficiency of available water. Nitrogen utilization under MDI conditions was able to improve the efficiency of nitrogen and water consumption by stimulating growth and yield components and compensate the reduction caused by water shortage to a significant extent. The external application of nitrogen, as the most effective nutrient and agricultural input, can improve growth even in conditions of low water supply (Xing et al., 2019).

Another important result of the current experiment was that despite the tremendous effect of water in improving growth and yield characteristics, in optimal agricultural management, water supply alone is not enough to improve growth and achieve high yield, and other nutritional needs and ecological factors of the plant must be provided. These results further support the idea of Hu et al. (2023) who reported that the highest biomass of corn was obtained under the conditions of simultaneous application of nitrogen and water supply through supplementary irrigation during the stress-sensitive stages. These researchers found that the supply of moisture and nitrogen through increasing the activity of nitrate reductase, glutamine synthase, and glutamate dehydrogenase leads to more nitrogen supply to different parts of the plant and can produce a higher seed yield. The obtained results showed that the longitudinal growth of the plant as well as the canopy width and the size and the number of side branches increased with the supply of moisture (FI and MDI) and high level of nitrogen (N_{80}). Supplying sufficient nitrogen and moisture to the plant can increase the levels of auxin and cytokinin and intensify their positive interactions on vegetative growth (Abualia et al., 2023; Deepika et al., 2023). The application of nitrogen at different levels of irrigation had a different effect on chlorophyll. Under severe irrigation conditions, nitrogen application could not improve the chlorophyll content. This was predictable considering the key structural role of nitrogen in chlorophyll (Hirel et al., 2023). These results indicate that the nitrogen use efficiency is strongly influenced by the available moisture in the soil. The obtained results showed that despite less water consumption under MDI conditions, the utilization of N_{80} was able to increase the amount of this pigment up to the values recorded for plants grown under FI. This may refer to the compensatory effect of nitrogen in the mentioned conditions. In other words, under MDI conditions, the consumption of large amounts of nitrogen could compensate the diminishing effects of water deficiency to a significant extent. Chlorophyll content can be considered as an indicator of resource activity and ability to produce photo-assimilates. In the present experiment, nitrogen consumption under SID conditions could not have positive effects. The high correlation between yield and canopy width (closeness in clustering) indicates that higher canopy growth during the vegetative phase through the production of more photo-assimilates (larger source size) can better support the filling sinks during the reproductive stage. Since deficit irrigation was applied during the vegetative period and the end of reproductive stages, it seems that the differences between the yield components (CNP, CD, TSM, and SY) in different conditions of irrigation and nitrogen utilization were caused by the difference in the amount of photo-assimilates synthesized during vegetative growth and their remobilization from vegetative organ to the filling fruits. Koutroubas et al. (2021) stated that the remobilization of stored materials from the stem to the seed has a significant contribution to determine the yield of safflower, and management treatments such as irrigation and fertilization affect the amount of remobilization. Higher seed protein percentage under SDI conditions also confirm their findings. Our results showed that severe water shortage created by SDI increased the amount of protein in the seed. It has been determined that the protein content of the seed is largely dependent on the amino acids and nitrogen compounds that have reached to seed through remobilization and drought stress accelerates leaves *senescence* and the degradation of photosynthetic enzymes to amino acids and improves their remobilization to the filling seed (Hajibarat & Saidi, 2022). However, the response of oil content to the investigated treatments was largely similar to that of chlorophyll (Figure 5). These findings suggest that the seed oil content is largely dependent on the photo-assimilates produced in current photosynthesis, and its amount was significantly reduced under SDI and no nitrogen consumption conditions. However, nitrogen application under MDI condition leads to tuned growth and acceptable oil content. The current results showed that not necessarily all the water used under the FI is needed by the plant and sometimes it lacks improving effects on the yield, and low irrigation is regulated and mild along with the use of N_{80} while maintaining the yield at an acceptable level, the harvest index and some improves quality components.

5 CONCLUSION

The obtained results revealed that safflower production in the investigated climatic zone is not economical under rainfed or severe deficit irrigation condition (PET 40 %). Results showed regular supply of water in amounts less than full irrigation (PET 60 %) along with the application of nitrogen fertilizer (80 kg ha^{-1}) produced the acceptable seed yield and seed oil content. The results revealed that nitrogen application under mild deficit irrigation conditions (PET 60%), improved the growth and yield components to some extent, thereby slightly diminished the negative effects of water deficiency. Therefore, the policies should be in such a way that by supplying nitrogenous fertilizers to small-scale farmers and encourage the use of MDI increase the water productivity. The supply of cheap, subsidized nitrogenous fertilizers and the promotion of precise irrigation are considered an important step in the direction of preserving underground water resources.

6 ACKNOWLEDGEMENTS

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Evaluation of amino acid composition in different types of meat and plant-based burger patties

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Evaluation of amino acid composition in different types of meat and plant-based burger patties

Abstract: The study examined the protein content and amino acid composition of various commercially available plant-based and meat-based burger patties. The aim of this study was to determine whether plant-based burger patties meet the requirement for essential amino acid content in the human diet. Amino acid profiles were determined using the Amino Acid Analyzer and FAO/WHO guidelines were considered for essential amino acid requirements. In this study, the protein content and amino acid composition of various meat-based burger patties (ABB), including chicken, pork and beef, and plant-based burgers (PBB) were analysed. The results showed that among the plant-based samples, PBB 4 had the highest protein content (24.81 g / 100 g), which was almost equal to that of ABB 1 (26.48 g / 100 g). The most abundant amino acids detected were Glu, Asp, Leu, Lys, Arg, Ser, Pro and Gly, with samples PBB 6, PBB 3 and ABB 1 having the highest concentrations. PBB 1 stood out as a valuable protein source with the highest content of essential amino acids (400.08 mg/g protein) among the plant-based burger patties. Some plant-based burger patties were deficient in essential amino acids, with PBB 3 and PBB 4 having the highest deficiency. The practical value of this study is that it helps people to make informed dietary choices.

Key words: human nutrition, essential amino acids, proteins, meat substitutes, plant-based burger patties, nutrition value

Določitev aminokislinske sestave mesnih in rastlinskih burgerjev

Izvleček: V študiji smo preučevali vsebnost beljakovin in aminokislinsko sestavo različnih komercialno dostopnih mesnih in rastlinskih burgerjev. Namen študije je bil ugotoviti, ali rastlinski burgerji izpolnjujejo zahteve po vsebnosti esencialnih aminokislin v humani prehrani. Aminokislinski profili so bili določeni z analizatorjem aminokislin, kemijski indeksi posameznih esencialnih aminokislin za odrasle pa izračunani na podlagi smernic FAO/WHO. V študiji smo analizirali vsebnost beljakovin in aminokislinsko sestavo različnih mesnih burgerjev (ABB) iz piščančjega, svinjskega in govejega mesa ter burgerjev iz sestavin rastlinskega izvora (PBB). Rezultati so pokazali, da je imel med rastlinskimi vzorci PBB 4 najvišjo vsebnost beljakovin (24,81 g/100 g), ki je bila skoraj primerljiva z ABB 1 (26,48 g/100 g). Najbolj zastopane aminokisline v vzorcih so bile Glu, Asp, Leu, Lys, Arg, Ser, Pro in Gly, pri čemer so bile najvišje koncentracije le-teh izmerjene v vzorcih PBB 6, PBB 3 in ABB 1. PBB 1 se je pokazal kot dober vir beljakovin z najvišjo vsebnostjo esencialnih aminokislin (400,08 mg/g beljakovin) med rastlinskimi burgerji. Pri nekaterih rastlinskih burgerjih je bilo ugotovljeno pomanjkanje esencialnih aminokislin, največje pri vzorcih PBB 3 in PBB 4. Praktična vrednost te študije je v tem, da ljudem pomaga sprejemati premišljene prehranske odločitve.

Ključne besede: humana prehrana, esencialne aminokisline, beljakovine, mesni nadomestki, rastlinski burgerji, prehranska vrednost

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1 INTRODUCTION

The traditional meat industry is reaching production limits and is associated with environmental problems (*e.g.* impact on natural resources, greenhouse gas emissions, and use of land mass) (Godfray et al., 2018). Therefore, alternative protein sources, particularly plant-based meat analogues, are becoming increasingly popular as a potential solution to reduce the supply gap and environmental impact of conventional meat production. The popularity of plant-based meat substitutes valued for its ability to mimic traditional meat products reflects a broader trend among consumers seeking sustainable and environmentally friendly food options. The study addresses the nutritional aspects of meat and meat substitutes, aligning with the current imperative to address the environmental consequences of dietary choices. This study contributes to informing people about the quality of alternative protein sources.

A balanced diet is a key factor in human life and well-being. It consists of various foods from which humans obtain the nutrients necessary for health and the performance of vital functions (Ahmad et al., 2018). As an important food group that contributes to a balanced and healthy diet, meat and its products are a rich source of nutrients (Pereira & Vicente, 2022). However, meat consumption is considered unfavorable by some due to its presumed impact on the environment, ethics, and certain religious traditions. Customer awareness of sustainability and environmentally-friendly food production practices is growing and there are trends towards adopting vegetarian or vegan diets or limiting meat consumption (Graça et al., 2019).

An alternative could be plant-based meat analogues, which are plant-derived foods that have the sensory and chemical properties of traditional meat products (Ismail et al., 2020). While not all meat analogues fall under the classification of ultra-processed foods, a significant number of modern meat analogues available in the food market today meet this criterion. Ultra-processed foods are food products that contain minimal or no whole foods and are instead produced with processed ingredients or substances that are obtained from whole foods. These processed ingredients can include protein isolates, oils, hydrogenated oils and fats, flours and starches, sugar variants, refined carbohydrates, and other added ingredients that increase the value of the product (Bohrer, 2019; Monteiro et al., 2019). Ultra-processed foods are associated with an increased risk of obesity, cardiovascular diseases, and metabolic disorders due to high quantity of added sugars, harmful fats, and low nutritional value (Monteiro et al., 2013). Some consumers prefer modern meat analogues because they fulfill their expectations by imitating meat in terms of appearance, quality, and taste.

According to the forecast of the International Institute for Sustainable Development, the number of people on our planet will reach 9.9 billion by 2050 (Population Reference Bureau, 2020). Feeding such a mass population with meat could have a detrimental effect on the environment. The benefits of reducing meat consumption are therefore manifold. Environmental studies have been conducted on protein-rich products, including plant-based meat analogues (soybeans, green peas, lupines, rice, etc.), animal proteins (milk, meat, insects, lab-produced proteins), and mycoproteins. Most studies have shown that plant-based meat analogues have less environmental impact than meat analogues that include a broader range of substitutes, including plant-based, fungi-based, and cultured meat products that appeal to a wider audience (Kyriakopoulou et al., 2019).

Meat and its products are primary sources of protein and provide complete proteins containing all essential amino acids such as phenylalanine, valine, threonine, tryptophan, isoleucine, methionine, histidine, leucine and lysine. Complete proteins, which are found in animal foods such as eggs, milk, fish and meat, have the correct ratio of essential amino acids, making them sufficient sources of protein. Incomplete proteins lack some essential amino acids (Arentson-Lantz et al., 2021), but can be complemented by combining different incomplete or complete protein sources. Secondary proteins, commonly found in plants, may be deficient in some essential amino acids. Adequate dietary proteins are crucial at every stage of life and ensure the uptake of essential amino acids (Brestenský et al., 2019). For example, cereal proteins with low lysine content and legumes with a lack of sulphur-containing amino acids (methionine and cysteine) pose a challenge for the utilisation of proteins in the human body. The amino acid composition and digestibility of proteins from different plant sources are therefore of crucial importance. Studies by Day et al. (2022) and Foschia et al. (2017) on plant proteins as meat analogues emphasize the optimal mixture of certain grains and legumes to achieve a meat-like amino acid profile.

This study aimed to detect amino acids in different burger patties (plant- and animal-based). In addition, this study investigated whether plant-based meat substitutes (vegetable-based meats) meet the daily requirements for the intake of proteins and essential amino acids in the human diet when consuming these products.

2 MATERIAL AND METHODS

2.1 BURGER SAMPLES

Burger samples were collected from ten fast-food restaurants in Budapest, Hungary, during the study period of March–September 2023. A total of 50 burger samples of meat-based burgers (ABB) and plant-based burgers (PBB) were analyzed, with five samples for each type of burger. The patties of animal origin included chicken, pork, and beef, coded as ABB 1, ABB 2, ABB 3 and ABB 4. For the plant-based burgers, six different types were taken for analysis: PBB 1, PBB 2, PBB 3, PBB 4, PBB 5, and PBB 6 (Table 1). Samples were independently collected to ensure no cross-contamination and stored at −20 °C. The quality standards used for testing were based on the Crown Food Group – CFG (2023).

2.2 AMINO ACID DETERMINATION

Protein-building amino acids were detected as described by Berisha et al. (2023). Briefly, 500 mg portions of burger patties were subjected to hydrolysis in a vessel containing 10 mL of 6 mol L-1 hydrochloric acid, while being exposed to a nitrogen atmosphere at a temperature of 110 °C for a duration of 24 hours in a controlled thermostat. The process of neutralization was carried out in a 25 ml volumetric flask by adding 10 ml of a 4 M L-1 NaOH solution to the hydrolyzed sample. The flask was then filled with buffer solution at a pH of 2.2. The samples that had been neutralized were passed through a membrane filter with a pore size of 0.25 µm. The determination of amino acids was conducted using a AAA400 Automatic Amino Acid Analyzer (Ingos Ltd., Prague, Czech Republic), which was equipped with a cation-exchange column. The separation was carried out using a stepwise gradient elution technique with lithium buffer systems. Following post-column derivatization using a ninhydrin reagent, colorimetric detection was performed at wavelengths of 570 nm and 440 nm specifically for Pro. Three samples were produced in parallel for analysis (Berisha et al., 2023).

2.3 AMINO ACID SCORE CALCULATION

The calculation of the essential amino acids scores or the so-called chemical score is done using the formula given by WHO (1991), equation 1, expressed either as a ratio of unity (recommended) or on a percentage scale (Food and Agriculture Organization Expert Working Group, 2018).

$$Amino\ Acid\ Score = \frac{\text{mg of amino acid in 1 g of the tested protein}}{\text{mg of amino acid in 1 g of the reference protein}} \qquad (1)$$

Reference protein amounts are shown in three

Table 1: Ingredients for plant-based burger patties

Burger type	Composition
PBB 1	Water, 18% pea protein, rapeseed oil, refined coconut fat, rice protein, natural flavour, dry yeast, butter, emulsifier: methylcellulose, less than 1% potato starch; table salt, potassium chloride, beetroot concentrate, apple extract, pomegranate concentrate, sunflower lecithin, food vinegar, lemon concentrate, vitamins and minerals (zinc sulfate, niacin [vitamin B3], pyridoxine [vitamin B6], cobalamin [B12]) pantothenic acid [vitamin B5].
PBB 2	Water, 20% pea protein, diced onion, sunflower oil, diced turnip, stabilizer: methylcellulose, pea fiber, potato starch, vinegar, maple syrup, spices, beet syrup, edible salt, smoked lemon concentrate.
PBB 3	Water, 14% soybean meal, carrot, refined coconut fat, wheat flour, 4% wheat protein, rapeseed oil, acidity regulator: potassium lactate, potassium acetate; emulsifier: methylcellulose, yeast extract, flavouring, green pea starch, soy protein, table salt, spices, beetroot with colouring effect, mushroom powder, spice extracts, smoke flavour.
PBB 4	Water, 11.8% soy protein, 9.9% wheat protein, carrot, refined coconut fat, rapeseed oil, emulsifier: methylcellulose, spices, yeast extract, flavouring, starch, natural flavour, acidity regulator: potassium lactate, acetate; table salt, vinegar, beet concentrate with colouring effect, bamboo fiber, spice extracts, smoke flavour, food acid: citric acid, antioxidant: ascorbic acid.
PBB 5, PBB 6	ABB4, PBB5, PBB6 the proportions of the ingredients in the patty and their full list are secret. However, the ABB4 is known to be made from beef, the PBB5 is made primarily from potatoes, carrots, green peas and corn, while the PBB6 is made from soy and wheat from sustainable farms.

Source: adjusted from food product label groups: ages 3–14 years, 15–18 years, and more than 18 years, approved by FAO (2013). In this research, the ratio of essential amino acids for adults (over 18 years) was evaluated.

2.4 ESSENTIAL AMINO ACID DEFICIENCY INDEX CALCULATION

The essential amino acid deficiency index is determined by calculating the differences in the essential amino acid value compared to the reference protein (Table 2).

Table 2: Procedure for calculating the essential amino acid (EAA) deficiency index

Essential amino acids (EAA)	Reference protein (mg/g)	ABB4 (mg/g protein)	EAA score	Calculation 1 – EAA score	Index of EAA deficiency
Histidine	16	31.29	1.96	1–1.96	−0.96
Isoleucine	30	22.36	0.75	1–0.75	0.25
Leucin	61	79.80	1.31	1–1.31	−0.31
Lysine	48	82.06	1.71	1–1.71	−0.71
Methionine and Cysteine	23	18.66	0.81	1–0.81	0.19
Phenylalanine and Tyrosine	41	64.75	1.58	1–1.58	−0.58
Threonine	25	46.02	1.84	1–1.84	−0.84
Valin	40	37.78	0.94	1–0.94	0.06

If the EAA deficiency index is positive, this means that the amino acid is present in the sample in a lower proportion than the amino acid of the reference protein. If it is negative, there is a surplus. The deficit is therefore indicated by positive values. The sum of these positive values gives the essential amino acid deficiency index. The calculation procedure is shown in Table 2 for the samples of ABB4 burgers; this procedure was used for all analyzed burger types.

3 RESULTS

3.1 PROTEIN CONTENT ON MEAT PATTY AND PLANT-BASED MEAT ANALOGUES

The total protein content of the samples was calculated using the results of the amino acid analysis (Table 3). Tryptophan is not included in the amino acid values obtained by chromatography, as its indole group is degraded by the acidic sample preparation, so that this amino acid cannot be detected by this method. Due to the variety of ingredients in burger patties, there is no information in the literature on their tryptophan content. The main components of the analyzed plant products can be classified as legumes, which are characterized by the limiting amino acid in the group of sulfur-containing amino acids (Met + Cys), so that the lack of tryptophan values does not pose a problem in the evaluation. This essential amino acid is rather limiting in maize.

Table 3 shows the total protein content of the samples per 100 g of product and the standard deviation of the analyzed samples. The protein content of the ABB1 is 26.48 g / 100 g. The highest value of veggie patties is the PBB 3 (24.81 g / 100 g), approaching the protein content of the ABB 1 and surpassing all three meat samples in terms of protein. This burger contains 14 % soy flour, 4 % wheat protein, and mushrooms also contribute to the development of favorable protein content, so the product can be considered a good substitute for meat in this regard. The lowest protein, only 4.34 g / 100 g, is found in the PBB 5.

Table 3: Total protein content of samples per 100 g of product

Type of burger patty	Protin Content (g/100 g) ± standard deviation (n = 3)
PBB 1	16.96 ± 0.54
PBB 2	15.76 ± 0.42
PBB 3	24.81 ± 2.23
PBB 4	12.90 ± 2.81
PBB 5	4.34 ± 0.44
PBB 6	24.72 ± 2.15
ABB 1	26.48 ± 2.24
ABB 2	22.74 ± 0.72
ABB 3	18.26 ± 0.22
ABB 4	19.14 ± 0.18

3.2 PROTEIN-BUILDING AMINO ACIDS

The results for the composition of protein-forming amino acids in plant-based burger patties are presented in Table 4. The main amino acids detected in the analyzed samples include Glu, Asp, Leu, Lys, Arg, Ser, Pro, and Gly which account for more than 50 % of the total amount of amino acids detected. By comparing the total amount of amino acids detected in plant-based burger patties, it was found that the PBB 6 (247.21 mg/g) had the highest amount of the amino acids, followed by PBB 3 (248.1 mg/g). The lowest amount of amino acids was detected in PBB 5 (43.42 mg/g).

Table 6 shows that PBB 1 with 400.08 mg/g protein has the highest total content of essential amino acids among the plant-based samples. PBB 2 and PBB 6 contain 381.57 mg/g and 351.96 mg/g, respectively, while PBB 3 has the lowest total essential amino acid content at 304.01 mg/g protein. PBB 2, containing highest proportion of pea protein, has the highest content of leucine and isoleucine among the plant-based variants and is therefore a good source of these essential amino acids. PBB 3 has the lowest proportion of branched-chain amino acids, which make up around a third of the essential amino acids in muscle tissue. PBB 3, PBB 4, PBB 5 and PBB 6, which contain wheat protein, have a lower lysine content than the other two plant-based meat analogues. This is because lysine is the limiting amino acid in whole grains. According to the product label (Table 1), the soy flour content in the PBB 3 patty was higher (14 %) than in the PBB 4 patty (11.8 %). This is due to the fact that soy supplements the missing lysine content in wheat flour, which could explain the higher lysine content (42.35 mg/g and 39.29 mg/g respectively). The methionine content was relatively low in all plant-based products, as they contained a high proportion of pulses, which are low in sulphur-containing amino acids such as methionine and cysteine. The PBB 3 patties contained the highest level of sulphur-

Table 4: Amino acid composition of plant-based burger patties (mg/g)

Amino acids	PBB 1	PBB 2	PBB 3	PBB 4	PBB 5	PBB 6
Glutamic acid	38.36	35.98	86.72	42.17	10.34	67.59
Aspartic acid	19.46	18.53	18.53	9.85	5.52	24.33
Leucine	15.24	14.26	14.33	10.16	2.84	21.02
Lysine	13.1	11.75	10.49	5.04	2.29	13.94
Arginine	12.61	14.15	10.64	6.85	2.39	17.4
Serine	10.09	9.43	13.6	6.83	2.37	13.11
Proline	8.3	5.37	23.33	13.33	2.59	15.19
Glycine	8.24	7.38	10.86	5.4	2.12	12.49
Phenylalanine	7.52	7.39	9.99	5.47	1.59	10.95
Threonine	7.48	6.65	8.14	5.18	2.14	9.78
Valina	6.91	6.04	7.64	4.3	1.83	9.33
Alanine	6.87	6.63	9.25	4.24	2.39	9.97
Tyrosine	5.73	5.41	11.6	3.95	2.43	8.15
Histidine	4.26	3.95	7.24	2.82	1.64	6.35
Isoleucine	4.24	3.99	3.87	2.68	0.7	6.21
Methionine	1.17	0.69	1.84	0.74	0.23	1.4
Sum mg/g	169.6	157.6	248.1	129	43.42	247.21

Table 5 shows the results for the amino acid composition of meat-based burger patties (ABB). Amino acids such as Glu, Gly, Asp, Lys, Leu, Arg, Ala, Thr, and Ser were detected in the highest amounts in these burgers. ABB 1 (264.82 mg/g), and ABB 2 (227.4 mg/g) had the highest amount of amino acids, followed by beef burgers (191.41 mg/g), while the lowest amount of amino acids was detected in pork burgers (182.64 mg/g).

The total essential amino acid content is presented in Tables 6 and 7. All types of analyzed burgers contained all essential amino acids. Essential amino acids are present in plant products in amounts between 304.01 to 400.08 mg/g of protein.

Table 5: Amino acid composition of meat-based burger patties (mg/g)

Amino acids	ABB 1	ABB 2	ABB 3	ABB 4
Glutamic acid	53.23	46.65	36.59	34.47
Glycine	26.30	9.11	7.71	11.62
Aspartic acid	24.09	20.68	16.14	18.72
Lysine	21.71	22.07	17.42	15.91
Leucine	21.16	22.85	18.43	16.26
Arginine	19.54	17.68	14.27	12.46
Alanine	17.47	11.23	8.59	10.68
Threonine	12.20	12.16	9.30	9.26
Serine	12.03	10.16	7.71	8.08
Proline	10.80	6.38	4.35	9.49
Valine	10.00	10.97	8.20	10.77
Phenylalanine	9.23	10.00	8.04	6.45
Histidine	8.27	4.44	7.83	6.75
Tyrosine	7.92	8.28	6.60	5.68
Isoleucine	5.93	7.62	6.04	7.84
Methionine	4.93	7.14	5.42	6.98
Sum mg/g	264.82	227.40	182.64	191.41

containing amino acids (7.52 mg/g) and the lowest level in PBB 2 (4.36 mg/g). PBB 2 has the highest content of legume protein (20 % pea protein) and some other vegetables (turnips and onions), which explains the low value of the limiting amino acid.

Table 7 shows the content of essential amino acids in meat-based burgers. The ABB 1, made from raw animal material, contains 382.72 mg/g of protein, while the ABB 2, ABB 3, and ABB 4 contain higher amounts, ranging from 409.36 to 477.70 mg/g of protein.

In general, plant-based products do not fall significantly short of the total amino acid content of a traditional animal-based burger.

3.3 ESSENTIAL AMINO ACIDS SCORE AND LIMITING AMINO ACIDS

The amount of each essential amino acid in mg/g protein in each sample was divided by the amount of mg/g protein of each of the essential amino acids of the FAO/WHO reference protein to give the score for each essential amino acid (EAAS- essential amino acid score). The closer this score is to 1 for each essential amino acid, the more the food corresponds to the FAO/WHO reference protein composition. If the essential amino acid has a ratio less than l then it is counted as the limiting amino acid.

Table 6: Essential amino acid composition of plant-based samples (mg/g protein)

Essential amino acids	PBB 1	PBB 2	PBB 3	PBB 4	PBB 5	PBB 6
Histidine	25.15	25.05	29.00	22.12	37.23	25.68
Isoleucine	25.03	25.30	15.75	20.60	16.46	25.10
Leucine	89.94	90.49	58.89	79.07	66.40	84.96
Lysine	89.94	74.61	42.35	39.29	53.08	56.27
Methionine	6.90	4.36	7.52	6.02	5.40	5.50
Phenylalanine and Tyrosine	78.16	81.23	87.04	73.06	92.65	77.27
Threonine	44.16	42.21	32.67	40.40	49.09	39.51
Valine	40.80	38.32	30.79	33.68	42.17	37.67
Sum	400.08	381.57	304.01	314.24	362.48	351.96

Table 7: Essential amino acid composition of animal-based samples (mg/g protein)

Essential amino acids	ABB 1	ABB 2	ABB 3	ABB 4
Histidine	31.29	19.67	42.86	35.29
Isoleucine	22.36	33.46	33.03	40.96
Leucine	79.80	100.44	100.87	84.95
Lysine	82.06	97.04	95.37	83.11
Methionine	18.66	31.37	29.66	36.51
Phenylalanine and Tyrosine	64.75	80.35	80.13	63.33
Threonine	46.02	53.40	50.90	48.38
Valine	37.78	48.19	44.90	56.40
Sum	382.72	463.92	477.72	431.05

The results for the essential amino acid score are shown in Table 8 for plant-based burger patties and in Table 9 for animal-based burger patties, in which the limiting amino acids are marked.

The amino acids methionine and cysteine, which are normally found in larger quantities in meat, are the limiting amino acids in all plant-based burger patties, which contain significant amounts of legumes. The lowest amount is found in PBB 2, which contains 20 % pea protein. The ABB 1 patty contains three essential amino acids (isoleucine, methionine and valine) with a value of less than 1 compared to the FAO/WHO reference proteins. It can be assumed that a vegetable protein-based additive was added to the minced meat as an enhancer, binder or fat substitute, which can be measured as protein together with the meat content of the sample, resulting in values for essential amino acids below 1.

Among meat-free products, PBB 3 has the highest protein content per 100 g, but qualitatively it is considered the most unfavorable source of protein, as it has

Table 8: Essential amino acids score and limiting amino acids of plant-based burger samples

Essential amino acids	PBB 1	PBB 2	PBB 3	PBB 4	PBB 5	PBB 6
Histidine	1.57	1.57	1.81	1.38	2.33	1.60
Isoleucine	0.83	0.84	0.52	0.69	0.55	0.84
Leucine	1.47	1.48	0.97	1.30	1.09	1.39
Lysine	1.87	1.55	0.88	0.82	1.11	1.17
Methionine and Cysteine	0.30*	0.19*	0.33*	0.26*	0.23*	0.24*
Phenylalanine and Tyrosine	1.91	1.98	2.12	1.78	2.26	1.88
Threonine	1.77	1.69	1.31	1.62	1.96	1.58
Valine	1.02	0.96	0.77	0.84	1.05	0.94

Table 9: Essential amino acids score and limiting amino acids of meat-based burger samples

Essential amino acids	ABB 1	ABB 2	ABB 3	ABB 4
Histidine	1.96	1.23	2.68	2.21
Isoleucine	0.75*	1.12	1.10	1.37
Leucine	1.31	1.65	1.65	1.39
Lysine	1.71	2.02	1.99	1.73
Methionine and Cysteine	0.81	1.36	1.29	1.59
Phenylalanine and Tyrosine	1.58	1.96	1.95	1.54
Threonine	1.84	2.14	2.04	1.94
Valine	0.94	1.20	1.12	1.41

low levels of essential amino acids (isoleucine, leucine, lysine, methionine, valine), compared to the reference protein. PBB 1 and PBB 5 are deficient only in methionine and isoleucine. The amount of histidine, phenylalanine, tyrosine, as well as threonine, is above the minimum values determined by FAO/WHO experts.

3.4 ESSENTIAL AMINO ACID DEFICIENCY INDEX

The greatest deficiencies in essential amino acids were found in PBB 3 (1.53) and PBB 4 (1.39) (Table 10). PBB 5, which probably contains mainly potatoes, was not the worst product due to the added egg content, but still showed a large deficit of 1.22. PBB 2 and PBB 6 have almost identical values of 1.01 and 0.98, respectively. Although PBB 1 is deficient in only 2 essential amino acids, their sum amounts to 0.87. Since the total ratio of essential amino acids in most meat-based burgers is above 1 (Table 9), their essential amino acid deficiency index is 0. However, ABB 1 has a deficiency of 0.50, which may be due to the addition of vegetable protein-based additives. The ratios of the essential amino acids threonine (2.14), lysine (2.02), phenylalanine and tyrosine (1.96), leucine (1.65) in ABB 2, histidine (2.68), threonine (2.04), lysine (1.9), phenylalanine and tyrosine (1.95), leucine (1.65) in ABB 3, histidine (2.21), threonine (1.94), lysine (1.73), methionine (1.59), phenylalanine and tyrosine (1.54) in ABB 4 are much higher than those of the reference protein. Most of the plant-based samples contain less methionine, isoleucine and valine. In PBB 4, the amount of lysine is also below the recommended value, while in PBB 3 the leucine content is suboptimal compared to the reference protein.

Table 10: Index of the deficiency of essential amino acids of the samples compared to the reference protein

Type of burger	Index of the deficiency of essential amino acids
PBB 1	0.87
PBB 2	1.01
PBB 3	1.53
PBB 4	1.39
PBB 5	1.22
PBB 6	0.98
ABB 1	0.50
ABB 2	0.00
ABB 3	0.00
ABB 4	0.00

4 DISCUSSION

The amino acid composition of meat-based burger patties and plant-based burger patties can be very different, primarily depending on the protein source. Meat-based burger patties typically provide more complete and balanced protein nutrition, including essential vitamins and minerals naturally present in meat, while plant-based meat analogues vary based on the ingredients used in their formulation, as in the study conducted by Day et al. (2022). Meat and its products are thought to have been part of the human diet from 2–6 million years ago and increased over time as a result of increases in income and population number. However, the trend towards the use of plant-based meat substitutes in the human diet is increasing (Godfray et al., 2018; Filin et al., 2023).

When comparing meat-based burger patties and plant-based burger patties, consumers should consider their dietary goals, preferences, and ethical concerns. If someone relies heavily on meat analogues, they may need to supplement their diet to ensure they are getting all the essential amino acids. It is also important to know the specific amino acid composition of the two types of burger patties and how it meets nutritional needs and individual health status. A study conducted by Bryant *et al.* (2019) examined the consumer acceptance of plant-based and clean (cultured) meat products in the United States, China, and India. The findings indicate that urban, well-educated, and high-income consumers in India and China demonstrate a higher propensity to purchase clean meat and plant-based meat compared to consumers in the USA. The research conducted by Ismail et al. (2020) also highlights a significant bias towards urban, educated, and wealthy groups in China and India, as opposed to the overall population. The study revealed that disgust plays a crucial role in determining the adoption of plant-based and clean meat, a distinctive observation limited to the United States. In China, there is a notable deviation from the generally observed demographic pattern in the Western countries regarding the acceptance of clean meat, particularly in relation to gender. The attitudinal factors influencing the acceptance of both plant-based and clean meat in China include perceptions of healthiness, nutritional value, excitement, goodness, and necessity. Given the recommended dietary allowance (RDA) for protein intake in adults is 0.8 grams per kilogram of body weight per day (Nishimura et al., 2023) and incorporating these plant-based options can help meet protein needs without the associated health risks of excessive red meat consumption. High intake of red meat, particularly processed varieties, has been linked to increased risks of chronic diseases such as cardiovascular disease, type 2 diabetes, and certain cancers due to its saturated fat, cholesterol content, and potential carcinogens formed during cooking (Fogelholm et al., 2015; Larsson and Orsin., 2014; Zheng et al., 2019).

The analysis of various burger patties used in popular fast-food offerings reveals that these meat substitutes can be made from a variety of plant-based ingredients, such as soy, peas, beans, or mushrooms, and may serve as satisfactory protein substitute in the human diet, as they contain all essential amino acids. However, it should be noted that while meat contains a balanced profile of essential amino acids, plant-based burger patties (*i.e.* legume-based meat analogues) are often deficient in amino acids, as methionine, lysine, and cysteine. PBB 1 stands out as a valuable protein source among these options due to its high content of essential amino acids. A review paper by Kyriakopoulou et al. (2021) in functionality of ingredients in plant-based meat analogues showed that the main ingredients in commercial meat analogues are soy, pea, and gluten, which are widely available, and by-products of established food production lines.

This provides additional opportunities to enhance functionality and optimize resource utilization. For example, pea protein and rice protein can be combined to create a complete amino acid profile, making the product more nutritionally comparable to animal protein. Similarly, combining soy protein with wheat gluten can improve the texture and binding properties of meat substitutes, as noted by Asgar et al. (2010). Additionally, the incorporation of algae or seaweed extracts can enhance the nutritional value and provide unique flavours and textures (Abdel-Moatamed et al., 2024; Schuler et al., 2020). These combinations not only enhance functionality but also optimize resource utilization, opening new possibilities for sustainable and nutritious food products.

The shift towards individual dietary patterns such as vegetarian or vegan diets is now being driven by a variety of factors, including health concerns about saturated fats in animal products, environmental impact, greenhouse gas emissions, climate change and ethical concerns about animal husbandry and slaughter practices. However, consumers may need to pay more attention to their overall diet to ensure they are getting all the essential amino acids. In addition, a combination of plant and animal proteins can be explored to produce healthy burger patties that meet essential amino acid requirements while reducing saturated fat content from animal products. The evaluation of amino acid composition highlights the importance of considering the overall quality of the protein and the completeness of the nutritional content when choosing between meat-based and plant-based burger patties. It is crucial to educate consumers about the differences in amino acid composition and the nutritional impact of choosing between meat and meat substitutes to help them make dietary choices in line with their needs and preferences. Ongoing research to improve the amino acid composition of meat substitutes and fortify them with essential nutrients could bring products closer to meat products and have a siginificant impact on the food industry.

5 CONCLUSIONS

This study provides a comprehensive analysis of the protein content and amino acid composition of a wide variety of burger patties, including both plant-based and meat-based options. The results of the amino acid composition studies showed a wide range of amino acids in the products, with the major amino acids Glu, Asp, Leu, Lys, Arg, Ser, Pro, and Gly accounting for more than half of all amino acids found. The analysis of the protein composition revealed that plant-based substitutes *(e.g.* PBB 3) can have competitive protein levels comparable to those of traditional meat products. Certain differences in the composition of essential amino acids were found, especially in the plant-based patties with lower amounts of methionine and lysine. Plant-based patties with a high proportion of legumes had limiting values for cysteine and methionine, confirming their status as limiting amino acids. However, it is also important to consider the bioavailability of proteins. Factors that affect the bioavailability of plant-based proteins include the presence of antinutritive factors such as phytates, which can inhibit mineral absorption, and the protein matrix itself, which can affect digestion and absorption. Although the amino acid profiles and protein content of plant-based burgers are promising, further bioavailability studies are needed to fully understand their nutritional impact. The results of the study point to the potential of mixing animal and plant protein sources to address the lack of essential amino acids in plant-based burger patties. This approach could reduce meat consumption while ensuring adequate nutrient intake. This study aims to provide information to make better dietary decisions.

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Influence of different substrates on fruiting bodies yield and antioxidant properties of oyster mushroom

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Influence of different substrates on fruiting bodies yield and antioxidant properties of oyster mushroom

Abstract: The present study was designed to evaluate the influence of different substrates on the yield and antioxidant properties of oyster mushroom (*Pleurotus ostreatus* (Jacq.) P. Kumm.) cultivated in grow bags. The substrates used in this study were: (1) maize straw, (2) beech sawdust supplemented with wheat bran at a rate of 20 %, (3) mixture of maize straw and spent coffee grounds in a ratio of 70 : 30, and (4) mixture of maize straw and spent coffee grounds in a ratio of 50 : 50. Total phenolics, total flavonoids and total antioxidant activity of oyster mushroom were determined by the Folin-Ciocalteu method, Aluminum chloride method and Ferric Reducing Antioxidant Power assay respectively. The highest fruiting bodies yield of oyster mushroom was obtained from substrate 4 while the least was from substrate 1. Total phenolic contents ranged from 3.80 mg in oyster mushroom grown on substrate 4 to 4.85 mg of gallic acid equivalents g^{-1} dry mass in oyster mushroom from substrate 2. Total flavonoid contents were very low in all analysed mushroom extracts. There was no significant difference between total antioxidant activities of oyster mushroom grown on different substrates.

Key words: coffee grounds, *Pleurotus ostreatus*, sawdust, straw, total phenolic and flavonoid contents

Vpliv različnih gojišč na pridelek trosnjakov in antioksidacijske lastnosti bukovega ostrigarja

Izvleček: Namen raziskave je bil ovrednotiti vpliv različnih gojišč na pridelek trosnjakov in antioksidacijske lastnosti bukovega ostrigarja (*Pleurotus ostreatus* (Jacq.) P. Kumm.) gojenega na substratu v vrečah. V raziskavi so bila uporabljena naslednja gojišča: (1) koruznica, (2) bukova žagovina dopolnjena z 20 % pšeničnih otrobov, (3) mešanica koruznice in usedline kave v razmerju 70 : 30, (4) mešanica koruznice in usedline kave v razmerju 50 : 50. Vsebnost celokupnih fenolov, celokupnih flavonoidiov in antioksidacijska aktivnost bukovega ostrigarja so bile določene s Folin-Ciocalteujevo metodo, metodo aluminijevega klorida in preiskusom antioksidacijske moči z redukcijo železa. Največji pridelek trosnjakov je bil pri uporabi gojišča 4, najmanjši pri gojišču 1. Vsebnost celokupnih fenolov je bila v območju od 3,80 mg, kadar je ostrigar rastel v gojišču 4 do 4,85 mg, izraženo kot ekvivalent galne kisline na gram suhe mase ostrigarja, ko je ta rasel v gojišču 2. Celokupna vsebnost flavonoidov je bila pri vseh analiziranih vzorcih zelo majhna, pravtako ni bilo značilne razlike v antioksidacijski aktivnosti ostigarja rastočega na različnih gojiščih.

Ključne besede: usedlina kave, *Pleurotus ostreatus*, žagovina, slama, celokupna vsebnost fenolov in flavonoidov

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1 INTRODUCTION

Oyster mushroom cultivation attracts attention of scientists, traders and consumers due to low production costs and high biological efficiency. The interest of oyster mushrooms is also increasing due to its taste, high nutritional value and health benefits (Guragain et al., 2024). Therefore, it is not surprising that oyster mushrooms, scientifically known as *Pleurotus* species are one of the most cultivated edible mushrooms in the world (Obodai et al., 2003; Shnyreva et al., 2017).

In comparison to other cultivated edible mushrooms, oyster mushroom (*Pleurotus ostreatus* (Jacq.) P. Kumm.) requires little growth time, approximately 60 days from media cultivation inoculated with fungi until the fruiting body in bag is ready to be harvested. It is also well known that fruiting bodies of oyster mushroom have good resistance to pests and bacterial and fungal diseases (Bhatti et al., 2024).

Pleurotus ostreatus can grow on a wide range of substrates, including grass clippings, sawdust, straw, coffee grounds, coco-coir, cottonseed hulls and even office paper (Hoa et al., 2015). *Pleurotus ostreatus*, as well as other mushrooms, utilize their mycelium to penetrate into organic substrates in which they grow. As they grow within the substrate, they exude enzymes that convert complex organic molecules into simpler substances which can then be absorbed and utilized (Girmay et al., 2016). In this light, it is obvious that the yield of *Pleurotus ostreatus* largely depends on the structure and chemical composition of the growing media, and therefore, the choice of substrate is of vital importance for the growth of both mycelium and fruiting body of *Pleurotus ostreatus*. Besides, the mineral composition, nutritional value as well as the antioxidant properties of mushrooms also depends on the type of substrate (Oyetayo and Ariyo, 2013).

The aim of this study was to evaluate the influence of different substrates on the yield and antioxidant properties of *Pleurotus ostreatus* cultivated in grow bags. We hypothesized that changes in the structure and chemical composition of the growing media would affect the yield and antioxidant properties of *Pleurotus ostreatus*.

2 MATERIALS AND METHODS

2.1 LOCATION OF STUDY SITE

The experiment was conducted from November 2023 to March 2024 at the experimental station of the Faculty of Agriculture and Food Science in Sarajevo (43°49'34.41'' N and 18°19'18.47'' E).

2.2 SUBSTRATE COLLECTION

Maize straw was collected from a farmer's field from around the Sarajevo town and transported to the experimental station. After transportation, the maize straw was sun-dried for 2 to 3 days and then stored in a dry place. Beech sawdust was purchased from the wood market, and coffee grounds from the coffee bar located in Sarajevo. Coffee grounds consisted of the waste created by brewing coffee during its final preparation stages. Freshly brewed coffee grounds were used for substrate preparation.

2.3 SUBSTRATE PREPARATION

The maize straw-based substrate was prepared as follows: dry maize straw was chopped into small pieces approximately 2–5 cm long. Exactly 10 kg was weighed and put into a barrel made with high-density polyethylene. The barrel was filled with 20 l of hot water (80 °C). It was soaked for 24 h, and during this time, the barrel containing the maize straw was covered in order to extend the heat treatment. The purpose of this treatment was to destroy all pathogenic organisms that might be present in substrate and that could compete with the cultivated mushrooms. Following hot treatment, maize straw was removed, and strained using raffia baskets. It was allowed to settle for about 1 h to drain excess water.

The maize straw was also utilized for making substrates 3 and 4. These substrates were created by mixing maize straw with spent coffee grounds in a ratio of 70 : 30 and 50 : 50.

Substrate containing beech sawdust and wheat bran was prepared as follows: 10 kg of beech sawdust was weighed and placed in a styrofoam container, followed by 2 kg of wheat bran. The substrate components were mixed thoroughly before adding 25 l of water. Thereafter, the substrate components were mixed again until there was no more water at the bottom of the container.

Final moisture contents of each substrate were determined with an Infrared Moisture Determination Balance (Kett Electric Laboratory, Model FD-770) and ranged from 69 % in substrate 2: beech sawdust supplemented with wheat bran to 73 % in substrate 1: maize straw.

1.5 kg of each homogenized substrates (wet mass) was then placed in polypropylene bags (20.32 × 30.48 cm) and pasteurized at 65 °C for 8 h.

2.4 SUBSTRATE INOCULATION AND INCUBATION

Following pasteurization, filled bags were cooled at room temperature, and then were inoculated with Pleurotus ostreatus spawn at the rate of 10 % (150 g per bag on a w/w wet-weight basis). The spawn was evenly distributed throughout the substrate and then mixed by hand into it. Pleurotus ostreatus spawn was generously donated by Urban Farm Mikić (Orašje, Bosnia and Herzegovina), and was prepared using the method of spawn preparation outlined by Stamets and Chilton (1983). Grain spawns with the mycelium of Pleurotus ostreatus (strain number P10001 acquired from Mushrooms & Equipment Shop, Münster Germany, type of grain: sorghum) were used for inoculation.

After inoculation, the bags were incubated in a climate chamber at 22 °C in darkness at 80 % relative humidity for 20 days, until the mycelium was grown through the whole substrate. The bags were then exposed to 16 °C for an additional three days to encourage fructification after five to six holes were drilled along the length of each bag. Thereafter, the growth conditions in climate chamber were modified to 18 ± 2 °C, 85 % relative humidity and incandescent light (400 lux) for 12 h on/off cycle.

This study was carried out with four substrate treatments and three replications, so that a total of 12 grow bags (bags with inoculated substrate) were used. Matured *Pleurotus ostreatus* fruiting bodies (white in colour, with up curved pileus) were harvested to determine biological efficiency and yield. A total of three flushes was harvested and data were collected in mass (grams) for each flush. The biological efficiency (BE) of *Pleurotus ostreatus* was calculated using the following formula:

BE (%) = fresh mass of havested ,mushroom (g)/ substrated dry mass (g) x 100 (1)

2.5 COLLECTION AND PREPARATION OF *PLEUROTUS OSTREATUS* FRUITING BODIES FOR ANALYSIS

Samples of complete fruiting bodies (100 g) from each substrate were dried in an oven at 40 °C to a constant mass. Then dried fruiting bodies were ground into a fine powder using an electric blender and stored in paper bags in dark cool place until analysis.

Preparation and extraction of mushroom samples for determination total phenolics and flavonoids and total antioxidant capacity was performed as follows: 1 g of mushroom powder was placed in an Erlenmeyer flask (100 ml), and then 25 ml of 30 % ethanol was added. Flasks were left to stand for 1 h with frequent shaking and then kept in the dark for 16 h. After that, the mixture was filtered through coarse filter paper into a 25 ml volumetric flask and diluted to the mark with extract solution (30 % ethanol). The extracts thus obtained were used to evaluate total phenolic and flavonoid contents and total antioxidant activities.

2.6 TOTAL PHENOLIC CONTENT ESTIMATION

The total phenolic content of mushroom extracts was determined by spectrophotometric method based on Folin-Ciocalteu reagent (Ough and Amerine, 1998). In brief, 0.1 ml of extract, 6 ml of distilled water, and 0.5 ml of Folin-Ciocalteu reagent (previously diluted with 1:2 distilled water in a ratio 1:2) were transferred into a 10 ml flask. After 5 min, 1.5 ml of saturated solution of Na_2CO_3 was added and mixed thoroughly. The flask was then filled to the mark with 30% ethanol solution and left at room temperature for 1 h, after which the absorbance was read at 750 nm. The gallic acid standard curve ranging from 0 to 500 mg l^{-1} was used to determine the total phenolic content of each sample, and then the obtained values were recalculated to dry mass of mushroom fruiting bodies (mg eq. GAE g^{-1} DM).

2.7 TOTAL FLAVONOID CONTENT ESTIMATION

The total flavonoid content of mushroom extracts was determined spectrophotometrically using the Aluminium chloride colorimetric assay (Zhishen et al., 1999). In brief, 1 ml of extract, 4 ml of distilled water, 0.3 ml of 5 % $NaNO_2$ and 0.3 ml of 10 % $AlCl_3$ were transferred into a 10 ml flask and mixed thoroughly. After 5 min, 2 ml of 1 mol l^{-1} NaOH was added and the flask was diluted to the mark with distilled water. The flask was then left at room temperature for 15 min, after which the absorbance was read at 510 nm. The catechin standard curve ranging from 0 to 100 mg l^{-1} was used to determine the total flavonoid content of each sample, and then the obtained values were recalculated to dry mass of mushroom fruiting bodies (mg eq. C g^{-1} DM).

2.8 TOTAL ANTIOXIDANT ACTIVITY ESTIMATION

The total antioxidant activity of mushroom extracts was determined spectrophotometrically using the ferric reducing antioxidant power (FRAP) assay (Benzie and Strain, 1996). This method is based on the ability of the antioxidants to reduce the brown-coloured Fe^{3+} complex to a blue-coloured Fe^{2+} complex at low pH. In brief, 100 µl of extract and 2000 µl of FRAP reagent (0.3 mol l^{-1} acetate buffer (pH = 3.6), 10 mmol l^{-1} TPTZ (2, 4, 6-tripyridyl-s-triazine) and 20 mmol l^{-1} $FeCl_3$ x 6 H_2O in a ratio 10:1:1) were transferred into a 10 ml flask and mixed thoroughly. After 15 min, the absorbance was read at 510 nm. The $FeSO_4 \times 7H_2O$ standard curve ranging from 0 to 2000 µmol l^{-1} was used to determine the total antioxidant activity of each sample and then the obtained values were recalculated to dry mass of mushroom fruiting bodies (µmol Fe^{2+} g^{-1} DM).

2.9 STATISTICAL ANALYSIS

All data collected was subjected to Analysis of Variance using a Microsoft Excel software program. When Fisher's F values were significant, the analysis was continued by comparing the means using the least significant difference (LSD) test at the threshold of $p < 0.05$.

3 RESULTS AND DISCUSSION

As shown in Table 1, the maximum fruiting bodies yield of *Pleurotus ostreatus* was obtained in substrate 4: maize straw mixed with spent coffee grounds in a ratio of 50:50 (414 g per bag), followed by substrate 3: maize straw mixed with spent coffee grounds in a ratio of 70:30 and substrate 2: beech sawdust mixed with wheat bran in a ratio of 80:20. The lowest yield was obtained in substrate 1: maize straw (368.6 g per bag).

In this study, *Pleurotus ostreatus* cultivated on substrates obtained by mixing maize straw with spent coffee grounds showed higher yield compared to other substrates, indicating that the spent coffee grounds can be used as an effective supplementary material for *Pleurotus ostreatus* growing media. Numerous studies have also shown a positive correlation between *Pleurotus ostreatus* yield parameters and substrates containing spent coffee grounds (Chai et al., 2021; Gemechu, 2023; Tambaru et al., 2023). Elsisura and Figueroa (2022) reported that spent coffee grounds contain large amounts of organic compounds (lignin (23.90 g 100 g^{-1} dry product), non-starch polysaccharides (60.40 g 100 g^{-1} dry product) as well as nutrients such as potassium and phosphorus, which are essential for the mushroom growth. In addition, spent coffee grounds have a high water-holding capacity (Turek et al., 2019), and that is also of vital importance for mushroom growth. So, it is not surprising that mushroom producers are increasingly using spent coffee grounds as a supplementary substrate for cultivating oyster mushrooms and other edible mushrooms.

However, on the other hand, growing mushrooms on substrates containing spent coffee grounds can be a limiting factor for mushroom growth. Namely, substance with a high water-holding capacity such as spent coffee grounds can decrease mycelium spreads through the substrate, increasing its incubation times (Bellettini et al., 2013). For the above reasons, spent coffee grounds can be used as a substrate for growing mushrooms but only if mixed with other organic substrates such as maize or wheat straw.

In this study, substrate 2: beech sawdust mixed with wheat bran in a ratio of 80:20 exhibited a higher fruiting bodies yield compared to maize straw (substrate 1), indicating that maize straw or other crop residues without nutritional additives such as wheat bran or coffee grounds are not the most suitable growing media for *Pleurotus ostreatus*. These findings are in line with those from previous studies (Nyochembeng et al., 2008; Carrasco et al., 2018).

In this study, substrates that gave higher mushroom fruiting bodies yield are also given a higher biological efficiency (BE) value. The highest percentage of BE in this

Table 1: Yield and biological efficiency of Pleurotus ostreatus grown on different substrates

Substrate*	Fruiting bodies yield per bag				
	I flush (g)	II flush (g)	III flush (g)	Total yield (g)	Biological efficiency (%)
Substrate 1	221.6 ± 28.7	120.0 ± 23.1	27.0 ± 11.3	368.6 ± 21.1c**	73.7 ± 6.9c
Substrate 2	282.2 ± 27.1	108.5 ± 24.3	-	390.7 ± 25.2b	78.1 ± 5.0b
Substrate3	247.4 ± 24.1	123.2 ± 14.1	30.5 ± 14.5	401.1 ± 15.4a	80.2 ± 3.6ab
Substrate 4	250.7 ± 25.2	150.5 ± 26.3	12.8 ± 4.3	414.0 ± 24.6a	82.8 ± 4.9a
$LSD_{0.05}$				20.4	0.4

* Substrate 1: maize straw, Substrate 2: beech sawdust + wheat bran in a ratio of 80:20, Substrate 3: maize straw + spent coffee grounds in a ratio of 70:30, Substrate 4: maize straw + spent coffee grounds in a ratio of 50:50
**Averages denoted by the same letter indicate no significant difference ($p \leq 0.05$)

study was obtained from substrate 4: maize straw mixed with spent coffee grounds in a ratio of 50:50, followed by substrate 3: maize straw mixed with spent coffee grounds in a ratio of 70:30. These results are consistent with other studies in which coffee grounds have been identified as an important additive for improving substrate chemical composition and thus oyster mushroom yield and biological efficiency (Alsanad et al, 2021; Dissasa, 2022). The possible justification put forward is that coffee grounds provide nutrients, especially nitrogen and potassium, needed for mushrooms to grow. In addition, coffee grounds have a fast decomposition rate (Ballesteros et al., 2014).

Total phenolic and flavonoid contents and total antioxidant activity (FRAP) values of oyster mushrooms cultivated on different substrates are presented in Table 2.

The highest total phenolic contents were found in *Pleurotus ostreatus* cultivated on substrate 2: beech sawdust mixed with wheat bran in a ratio of 80:20, followed by substrate 1: maize straw and substrate 3: maize straw mixed with spent coffee grounds in a ratio of 70:30. The lowest total phenolic contents were obtained from *Pleurotus ostreatus* cultivated on substrate 4: maize straw mixed with spent coffee grounds in a ratio of 50:50. The results of this study also showed that *Pleurotus ostreatus* is an excellent source of phenolic compounds that have been shown to have strong anti-inflammatory, antiproliferative, and antioxidant properties.

Many scientists agree that the accumulation and synthesis of phenolic compounds in mushroom fruiting bodies strongly depend on growth conditions, including primarily substrate structure and chemical composition (Paz et al. 2012; Diamantopoulou et al., 2023). In this light, it is very important to choose a substrate that could increase the phenolic content in mushroom fruiting bodies without negatively affecting the yield. However, all the types of substrates used in this study did not have the desired effect of increasing total phenolic content in mushroom fruiting bodies without decreasing the oyster mushroom yield. For example, *Pleurotus ostreatus* cultivated on maize straw had a higher total phenolic content as compared to mushrooms grown on some other substrates, but at the same time the *Pleurotus ostreatus* yield on maize straw was the lowest. These findings strongly suggest that substrate composition has a significant impact on both mushroom growth and metabolism.

In this study, total flavonoid contents were very low in all analysed fruiting bodies and ranged from 0.11 in substrate 3 to 0.17 mg of catechin equivalents (CAE) g^{-1} dry mass in substrate 2. Numerous studies have also shown that flavonoids are present in mushrooms in very small quantities (Gan et al., 2013; Izham et al., 2022). In this light, Gil-Ramirez et al. (2012) point out that flavonoids cannot be synthesized by mushrooms. In view of this, the presence of flavonoids in *Pleurotus ostreatus* in this study could be due to the ability of *Pleurotus ostreatus* to absorb them from their substrate. However, the results of this study cannot confirm this hypothesis but neither the hypothesis that the flavonoids are metabolites synthesized by the mushroom body.

In this study, there was no significant difference in total antioxidant activities between *Pleurotus ostreatus* grown on different substrates. These findings are somewhat surprising given the fact that there was a significant difference in total phenolics and flavonoids among *Pleurotus ostreatus* grown on different substrates. In view of the above, it is obvious that phenolics are not the only large group of compounds that strongly contribute to the antioxidant capacity of mushrooms. Giavasis (2014) reported that polysaccharides in mushrooms also possess strong antioxidant activity and that among different types of mushroom polysaccharides with antioxidant

Table 2: Antioxidant properties of *Pleurotus ostreatus* grown on different substrates

Substrate*	Total phenolics (mm) (mg g^{-1} dry mass)	Total flavonoids (mg g^{-1} dry mass)	FRAP value (µmol Fe^{2+} g^{-1} dry mass)
Substrate 1	$4.65 \pm 0.73^{ab**}$	0.15 ± 0.02^{ab}	8.23 ± 1.92
Substrate 2	4.85 ± 0.42^{a}	0.17 ± 0.02^{a}	6.96 ± 2.28
Substrate 3	4.08 ± 0.58^{bc}	0.11 ± 0.03^{c}	6.50 ± 0.76
Substrate 4	3.80 ± 0.61^{c}	0.13 ± 0.03^{bc}	9.43 ± 3.32
$LSD_{0.05}$	0.63	0.03	-

* Substrate 1: maize straw, Substrate 2: beech sawdust + wheat bran in a ratio of 80:20, Substrate 3: maize straw + spent coffee grounds in a ratio of 70:30, Substrate 4: maize straw + spent coffee grounds in a ratio of 50:50
**Averages denoted by the same letter indicate no significant difference ($p \leq 0.05$)

activity, β-D-glucans are considered to be the most important. However, further research is required to confirm this hypothesis.

4 CONCLUSIONS

Generally, the present study confirmed that *Pleurotus ostreatus* can grow on different substrates. In this study, substrates obtained by mixing maize straw and spent coffee grounds produced a significantly higher yield and biological efficiency of *Pleurotus ostreatus* compared to the other substrates. Therefore, spent coffee grounds can be recommended as a suitable supplementary substrate to increase *Pleurotus ostreatus* yield. The study results also revealed that changes in the structure and chemical composition of the substrates used in this experiment did not significantly affect the total antioxidant activity of the oyster mushroom.

The authors declare that they have no conflict of interest related to the manuscript.

The data that support the findings of this study are available on request from the corresponding author (SM).

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Carthamus tinctorius L. response to nano-silicon foliar treatment under organic and inorganic fertilizer application

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Carthamus tinctorius **L. response to nano-silicon foliar treatment under organic and inorganic fertilizer application**

Abstract: This research was studied the impacts of usage of nano-silicon in combination with various fertilizer treatments on the yield and its components of safflower. The trial evaluated the application of 0.0- and 20-mM nano-silicon in conjunction with different fertilizer treatments, including a control, 90 kg ha^{-1} NPK, and organic fertilizer at rates of 15 and 30 t ha^{-1}. Principal components approach revealed that the first two components of the treatment by trait biplot explained 66 % and 22 % of the variability, respectively. Positive correlations were observed among straw yield, achene yield, and harvest index, as well as among capitula number per plant, seed per subsidiary capitulum, and the number of the highest capitula. The polygon indicated that the NPK with 20 mM nano-silicon resulted in superior yield, whereas using 30 t ha^{-1} organic fertilizer with 20 mM nano-silicon exhibited enhanced yield components. Plant height emerged as the most representative trait, with high discrimination potential. Treatment with 30 t ha^{-1} organic fertilizer, combined with both 0.0- and 20-mM nano-silicon, was identified as the optimal treatments for discriminating among traits. The NPK plus 20 Mm nano-silicon and 30 t ha^{-1} organic manure was the best treatments.

Key words: fertilizer, organic manure, NPK, nano-silicon

Odziv žafranike (*Carthamus tinctorius* **L.) na folioarno dodajanje nano-silicija pri različni uporabi organskih in anorganskih gnojil**

Izvleček: Namen raziskave je bil preučiti vpliv uporabe nano silicija v kombinaciji z različnimi načini gnojenja na pridelek in njegove komponenete pri žafraniki. V poskusu so bili ovrednoteni uporaba nano silicija v odmerkih 0,0- in 20-mM v povezavi z različnimi režimi gnojenja, ki so vsebovali kontrolo, 90 kg ha^{-1} NPK in organska gnojila v odmerkih 15 in 30 t ha^{-1}. Ovrednotenje z glavnimi komponentami je odkrilo, da sta prvi komponeneti pri obravnavi lastnosti v biplotu razložili 66 % in 22 % variabilnosti. Pozitivne korelacije so bile ugotovljene med pridelkom slame, pridelkom rožk in žetvenim indeksom kot tudi med številom stranskih poganjkov na rastlino, semen na stranskih koških in številom najvišjih koškov. Obravnave so pokazale, da je gnojenje z NPK in 20 mM nano-silicija dalo najboljši pridelek medtem, ko je uporaba 30 t ha^{-1} organskega gnojila z 20 mM nano-silicija pospešila komponente pridelka. Višina rastlin se je izkazala kot najbolj reprezentativna lastnost z velikim potencialom razločevanja. Obravnava s 30 t ha^{-1} organskega gnojila, v kombinaciji z 0,0- ali 20-mM nano-silicija je bila prepoznana kot optimalno obravnavanje za prepoznavanje razlik med latnostmi. Obravnavanje z NPK in 20 Mm nano-silicijaon ter 30 t ha^{-1} organskega gnojila se je izkazalo kot najboljše.

Ključne besede: gnojilo, organsko gnojilo, NPK, nano-silicij

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1 INTRODUCTION

Safflower (*Carthamus tinctorius* L.) is a member of the Asteraceae and has its origins in southwest Asia. It is cultivated for multiple purposes such as vegetable oil production, forage, and medicinal applications. Safflower demonstrates the potential for acceptable yields, particularly in regions conducive to the growth of winter cereals, highlighting its versatility and underexplored possibilities (Shahrokhnia and Sepaskhah, 2017). Currently, global production estimates stand at approximately one million metric tons of safflower achenes, harvested from an area covering 1,200,000 ha, with a mean yield performance of around 830 kg ha^{-1} (FAOSTAT, 2022). In semi-arid regions, water availability is the primary restricting factor for crop cultivation, restricting the range of crops that can be grown successfully. Safflower, however, exhibits resilience to water scarcity due to its efficient deep root system and numerous fine lateral roots. This characteristic enables safflower to endure periods of moisture deficiency, a trait that sets it apart from many other crops whose performance are severely impacted by abiotic stresses like drought (Hussain et al., 2016). Safflower holds promise not only for seed production but also as a valuable forage crop in dryland cropping systems with limited water resources. Optimal forage quality is attained during the vegetative growth period when the plant has low pricks, rendering it palatable to farm animals. In such areas, the green plant of safflower can be used for feeding, contributing to livestock nutrition and overall farm productivity.

Nutrients application has a pivotal role in addressing the global imperative to enhance production and fulfill the dietary needs of an expanding population. The use of fertilizers in agriculture has a substantial effect on crop productivity as it influences traits such as phenological characteristics and root properties, which subsequently impacts physiological processes like water absorption and transpiration. (Farooq et al., 2019). However, the rates of nutrients are used vary across various environmental conditions, a variability driven by climax changes, crop types, and cropping systems. In rainfed agriculture, nutrients application is notably shaped by precipitation levels and the availability of soil moisture. In semi-arid regions, controlling the negative effects of terminal drought needs optimizing soil's capacity to capture and retain precipitation, thus it maximizes water storage for next utilization, and facilitate root penetration and proliferation (Zia et al., 2021). The widespread adoption of intensive chemical fertilizer application traces back to the Green Revolution, primarily aimed at meeting the nutrient demands of high-yielding crop varieties. Despite their advantages, chemical fertilizers are not without drawbacks. These include a heightened risk of leaching, substantial energy consumption during production, potential exposure to toxic chemicals, promotion of excessive growth, and depletion of soil moisture reserves.

Organic manure represents a viable option for field crop fertilization (Das and Avasthe, 2018). Its application can substantially enhance soil properties, leading to reduced reliance on mineral fertilizers, improved organically equilibrium, and enhanced soil moisture retention and efficiency of water usage. The application of organic fertilizers is particularly crucial in semi-arid regions of Iran, where soils are frequently subjected to intensive tillage, resulting in low organic matter content and weak structural stability (Sabaghnia and Janmohammadi, 2024). Moreover, the common practice of removing straw for animal feed further underscores the significance of organic fertilizer application (Lal et al., 2020). Organic manure has been shown to provide essential macronutrients and micronutrients necessary for plant growth, while also maintaining nutrients and promoting different aspects of fertility characteristic of soil.

Nanoparticles represent a burgeoning field of research with promising applications, particularly in agriculture, as materials for the new millennium. They interchange with corps, inducing various changes contingent upon their unique characteristics. Among these nanoparticles, nano-silicon has garnered significant attention in recent years which is abundant element in soils (Souri et al., 2021). However, the effectiveness of nano-silicon may vary among different crops or under diverse environment and climatic conditions. Despite its potential, very little research has been done to evaluate the effects of nano silicon application on safflower in semi-arid regions. Thus, this research aimed to create new insights into the effectiveness of nano-silicon on the safflower under different fertilizer management treatments.

2 MATERIALS AND METHODS

For this research, a field trial was conducted at Maragheh, Iran (37°23'N 46°14', situated in an upland semi-arid region. The soil is characterized as sandy loam, with a particle size distribution showing a decreasing order of sand, silt, and clay. Soil pH was measured at 7.5, with EC of 0.51 dS m^{-1}. Organic matter content was approximately 2 g kg^{-1} and nitrogen at 0.06 % while phosphorus was at 5.7 mg kg^{-1} and potassium was at 34 mg kg^{-1}. The experimental design employed a factorial arrangement with split-plot layout, following a randomized block scheme with three replications. Fertilizer treatments were applied to the main plots: control (Con), 90 kg ha^{-1} conventional fertilizer (nitrogen, phosphorus, and potassium, NPK), 15 t ha^{-1} organic manure (OM15), and 30 t ha^{-1} organic manure (OM30). Nano-silicon (SiO_2) treatments were applied in foliar form at 0.0 Mm (N0) and 20 Mm (N20). Field preparation included plowing and disking in the autumn season, followed by manual sowing of the Esfahan variety on April 14th. Each plot measured 4.0 m in length and 3.0 m in width, comprising 12 rows spaced 0.25 m apart. Plots were rainfed and supported with supplementary irrigated practices during the seed-filling step. Organic fertilizer as cow manure was mixed in a uniform position to a depth of 15 cm, while NPK fertilizer was surface-applied after field preparation.

For the measurement of yield components and morphological traits, 10 random samples were chosen from each unit, and the following parameters were assessed: plant height (PH), the highest branch (HB), the highest capitula (HC), capitula per subsidiary branch (CSB), capitula number per plant (CNP), seed per primary capitulum (SPC), seed per subsidiary capitulum (SSC), and capitulum seed mass (CSM). Central rows of plots were manually harvested from late June to early July, with straw yield (SY) and achene yield (AY) measured, and harvest index (HI) calculated. Thousand-achene mass (TAM) was measured from three random subsamples. Principal component analysis using a treatment by trait (TT) interaction layout was conducted, and the biplot model was generated via the GGEbiplot application (Yan, 2019).

3 RESULTS AND DISCUSSION

The TT biplot elucidated 66 % to 22 % of the variation within the standardized data two-way dataset (Fig. 1), showing a relatively substantial proportion that underscores the directness of associations among the traits. However, to glean the basic structures among the traits visually, vectors are generated from the graph origin to the traits, facilitating the visualization of trait associations. Given that the TT biplot model captured a considerable magnitude of variability (about 88 %), the associations between two traits are estimated by the cosine of their vectors, with cos0°= +1, cos90°= 0, and cos180°= $^{-1}$ (Sabaghnia and Janmohammadi, 2023). The extensive variability depicted by the TT biplot model emanated from all measured traits, evident from the elongated vectors (Fig. 1). Prominent associations unveiled include: (i) positive associations among straw yield (SY), achene yield (AY), and harvest index (HI); positive associations among capitula number per plant (CNP), seed per subsidiary capitulum (SSC), and the highest capitula (HC); and positive associations among capitulum's seed mass (CSM), thousand-achene mass (TAM), capitula per subsidiary branch (CSB), and seed per primary capitulum (SPC), as depicted by acute angles. Additionally, relatively near-zero associations were observed among SY, AY, and HI with CSM, TAW, CSB, and SPC, as evidenced by the near-perpendicular vectors (Fig. 1). Thus, the TT biplot model visually delineated trait associations in safflower, aligning with findings from other researchers such as Janmohammadi et al. (2016), who reported a positively association between straw yield, achene yield, and harvest index of safflower. Similarly, Fattahi et al. (2023) noted high positive associations between capitulum's seed mass and thousand-achene mass, as well as between capitula per subsidiary branch and seed per primary capitulum. However, exact parallels should not be expected between these results and correlation coefficients because the TT biplot model explicates associations among traits based on the general structure of the data, whereas Pearson's linear simple coefficients solely elucidate the association between two traits.

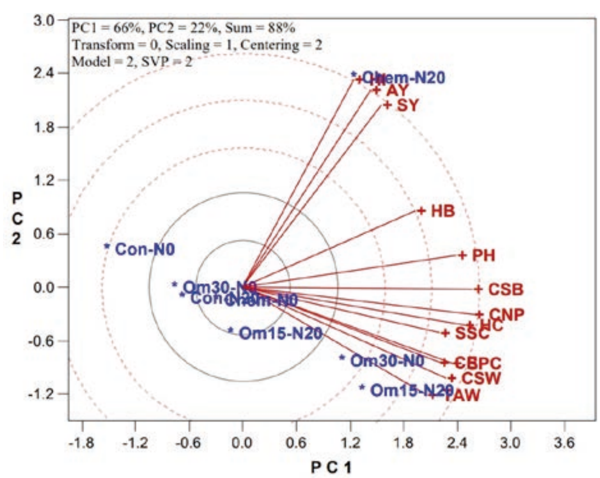

Fig. 1: Ranking entries (treatment combinations) based on testers (traits).
Treatment combinations are: control plus 0.0 Mm nano-silicon (Con-N0), control plus 20 Mm nano-silicon (Con-N20), 90 kg ha^{-1} NPK fertilizer plus 0.0 Mm nano-silicon (Chem-N0), 90 kg ha^{-1} NPK fertilizer plus 20 Mm nano-silicon (Chem-N20), 15 t ha^{-1} organic manure plus 0.0 Mm nano-silicon (OM15-N0), 15 t ha^{-1} organic manure plus 20 Mm nano-silicon (OM15-N20), 30 t ha^{-1} organic manure plus 0.0 Mm nano-silicon (OM30-N0), and 30 t ha^{-1} organic manure plus 20 Mm nano-silicon (OM30-N20).
Traits are: plant height (PH), the highest branch (HB), the highest capitula (HC), capitula per subsidiary branch (CSB), capitula number per plant (CNP), seed per primary capitulum (SPC), seed per subsidiary capitulum (SSC), capitulum's seed mass (CSW), straw yield (SY), achene yield (AY), harvest index (HI), and thousand achene mass (TAW).

Fig. 2 demonstrates how the TT biplot model can facilitate the comparison of treatment combinations based on the measured traits and identify those combinations that excel in specific aspects, thus serving as candidates for ideal fertilization practices recommended to safflower farmers. For instance, comparing Chem-N20 (NPK chemical fertilizer plus 20 Mm nano-silicon) and OM30-N20 (30 t ha^{-1} organic manure plus 20 Mm nano-silicon) revealed that Chem-N20 exhibited superior yield performance (AY), whereas OM30-N20 excelled in yield components such as CSB, CNP, SPC, SSC, and TAM (Fig. 2). Additionally, Chem-N20 showed the highest values for plant height (PH) and highest branch (HB), while OM30-N20, followed by OM30-N0 (30 t ha^{-1} organic manure plus 0 Mm nano-silicon), demonstrated high levels of the highest capitula (HC) and capitulum's seed mass (CSM).

Although, the TT biplot model may not precisely depict the averages of traits for treatment combinations, as it does not encompass all variance of the dataset, it provides insights into the underlying properties of the data structure. The polygon of the TT biplot model delineated four sections, with the remaining treatment combinations, such as Con-N0 (control plus 0 Mm nano-silicon) and OM15-N20 (15 t ha^{-1} organic manure plus 20 Mm nano-silicon), not performing optimally for any of the measured traits. Likewise, Con-N20 (control plus 20 Mm nano-silicon), Chem-N0 (NPK chemical fertilizer plus 0 Mm nano-silicon), and OM15-N0 (15 t ha^{-1} organic manure plus 0 Mm nano-silicon) were situated in unfavorable sectors or undesirable positions within favorable sectors, rendering them unsuitable candidates for advising to safflower farmers as proper fertilization practices. According to the TT biplot model (88 % in current case), if it adequately estimates the dataset, treatment combinations falling on the same section of the vertical line as AY should perform above the mean, whereas those on the opposite side should perform below the mean.

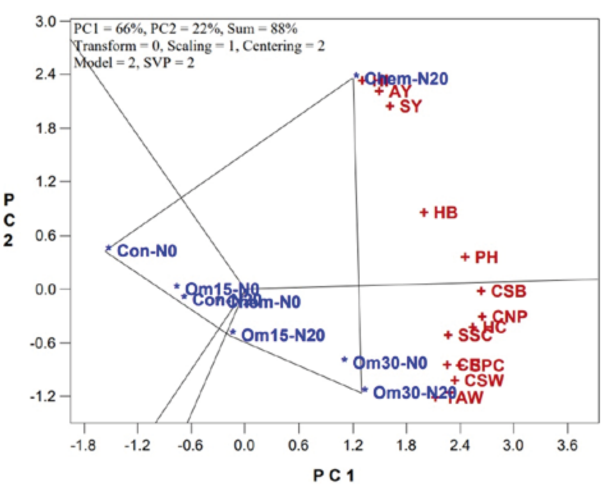

Fig. 2: Which entry (treatment combinations) wins which tester (trait).
Treatment combinations are: control plus 0.0 Mm nano-silicon (Con-N0), control plus 20 Mm nano-silicon (Con-N20), 90 kg ha^{-1} NPK fertilizer plus 0.0 Mm nano-silicon (Chem-N0), 90 kg ha^{-1} NPK fertilizer plus 20 Mm nano-silicon (Chem-N20), 15 t ha^{-1} organic manure plus 0.0 Mm nano-silicon (OM15-N0), 15 t ha^{-1} organic manure plus 20 Mm nano-silicon (OM15-N20), 30 t ha^{-1} organic manure plus 0.0 Mm nano-silicon (OM30-N0), and 30 t ha^{-1} organic manure plus 20 Mm nano-silicon (OM30-N20).
Traits are: plant height (PH), the highest branch (HB), the highest capitula (HC), capitula per subsidiary branch (CSB), capitula number per plant (CNP), seed per primary capitulum (SPC), seed per subsidiary capitulum (SSC), capitulum's seed mass (CSW), straw yield (SY), achene yield (AY), harvest index (HI), and thousand achene mass (TAM).

Fig. 3 illustrates the representative and discrimination potential of the traits, with vector length serving as a scale of discrimination potential, where a longer vector indicates a greater potential for discriminating a trait. Additionally, the stretch of a trait's projection onto the mean trait coordinate signifies its representative potential, with a shorter distance indicating a higher potential for representation of a trait. Notably, plant height (PH) exhibited the highest potential for both representative

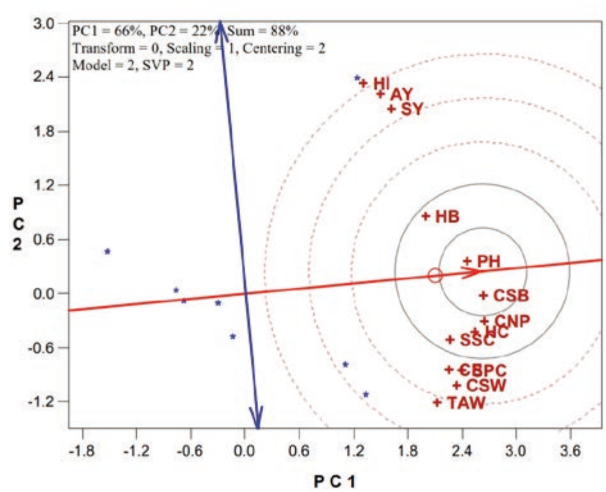

Fig. 3: Ranking testers (traits) based on discriminative and representativeness potentials.
Traits are: plant height (PH), the highest branch (HB), the highest capitula (HC), capitula per subsidiary branch (CSB), capitula number per plant (CNP), seed per primary capitulum (SPC), seed per subsidiary capitulum (SSC), capitulum's seed mass (CSW), straw yield (SY), achene yield (AY), harvest index (HI), and thousand achene mass (TAW).

and discrimination capabilities, positioned at the ideal trait location (Fig. 3).

Following PH, traits such as CSB, CNP, HC, SSC, and HB also demonstrated strong representative and discrimination potentials. Traits CSM, TAM, CSB, and SPC, as well as SY, AY, and HI, exhibited good discrimination potential, as they were positioned far from the plot center, but their representative capabilities of the trait's mean were limited due to their long projection onto the mean trait coordinate. In the future studies of safflower, using these identified traits as good indices will be useful for detection differences among treatments. In Fig. 4, the center of the circles denotes the location of an ideal treatment combination, with its projection on the vertical axis of the mean trait coordinate set to be equivalent to the largest vector among all treatment combinations. This projection on the horizontal axis of the mean trait coordinate is zero, indicating low variability and higher reliability. Thus, the closer a treatment combination's interval to this hypothetical treatment, the more optimal the treatment is. Consequently, OM30-N0 and OM30-N20, followed by Chem-N20, were the closest to the position of the ideal treatment combination. Conversely, the remaining treatment combinations, including OM15-N0, OM15-N20, Chem-N0, and Chem-N0, did not exhibit significant differences, while other treatment combinations were inferior (Fig. 4). Consequently, OM30-N0, OM30-N20, and Chem-N20 treatment combinations were capable of discriminating the differences among the measured traits of safflower.

Inspecting the performance of Chem-N20 for the measured traits of safflower (Fig. 5) revealed that straw yield (SY), achene yield (AY), and harvest index (HI) were proximate to this treatment combination. Therefore, for achieving high achene yield in safflower, the recommendation is to utilize 90 kg ha^{-1} conventional chemical fertilizer (nitrogen, phosphorus, and potassium or NPK) along with foliar application of 20 Mm nano-silicon. The observed enhancement in safflower yield performance with the use of NPK aligns with the reports of Sampaio et al. (2016), who found favorable yield outcomes with nitrogen, phosphorus, and potassium application in safflower cultivation.

However, the specific NPK requirements for safflower may vary depending on soil conditions, farming practices, cultivar selection, crop growth stage, and environmental factors. Additionally, the potential for high safflower yields is closely linked to the soil's phosphorus and potassium levels, Although, drought conditions can hinder their uptake and translocation in the crop due to reduced transpiration rates (Silva et al., 2022). Usage of nano-silicon in foliar form has shown to increase crop growth and performance by boosting the reaction of antioxidants and improving the yield of the photosynthetic system. In the research of Seyed-Sharifi et al. (2024), the application of nano-silicon under water-limited conditions led to increased safflower seed yield by augmenting total chlorophyll content and enhancing the antioxidant

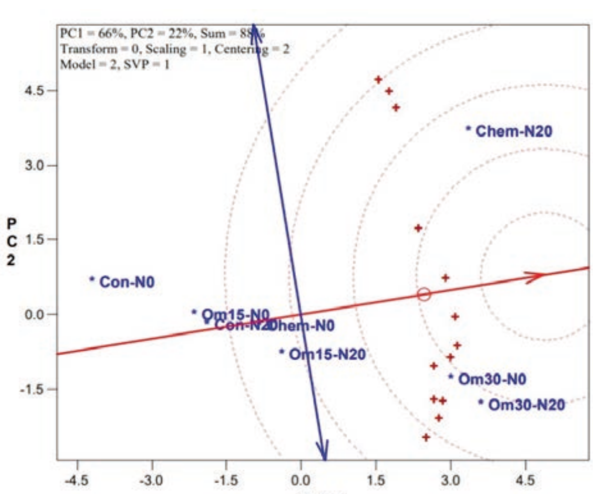

Fig. 4: Ranking entries (treatment combinations) based on testers (traits).

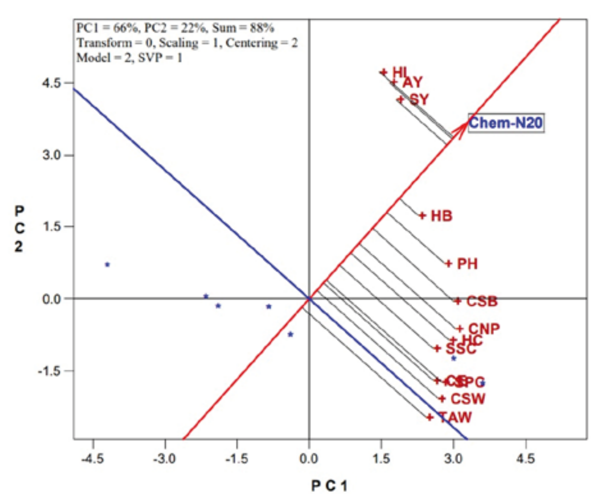

Fig. 5: Ranking traits based on the target entry (Chem-N20); 90 kg ha^{-1} NPK fertilizer plus 20 Mm nano-silicon.
Traits are: plant height (PH), the highest branch (HB), the highest capitula (HC), capitula per subsidiary branch (CSB), capitula number per plant (CNP), seed per primary capitulum (SPC), seed per subsidiary capitulum (SSC), capitulum's seed mass (CSW), straw yield (SY), achene yield (AY), harvest index (HI), and thousand achene mass (TAW).

enzymes functions and compatible osmolytes like proline and soluble sugars.

Similarly, analyzing the performance of OM30-N20 for the measured traits of safflower (Fig. 6) revealed that most yield components (CNP, SPC, SSC, CSM, and TAM), as well as the highest capitula (HC), were in close proximity to this treatment combination. Therefore, for achieving a high number of seeds and heavier seeds in safflower, the recommendation is to apply 30 t ha^{-1} organic fertilizer along with foliar usage of 20 Mm nano-silicon. The utilization of organic fertilizer has shown to enhance the yield components of safflower, is in accordance with the findings of Sudhakar et al. (2020), who found a remarkable enhance in yield performance of safflower due to the positive impacts of organic fertilizer. These impacts are attributed to the delivery of nutrients by organic fertilizer, which provides the required energy for microorganisms and aids in the degradation of organic matter, serving as an additional energy source for field microflora. Furthermore, Karchedu et al. (2023) demonstrated that usage of nano-silicon in foliar form in rice resulted in increased yield performance and zinc content. Silicon plays a significant role in improving rice quality by providing essential macronutrients, such as nitrogen, thereby contributing to enhanced yield outcomes.

Upon examining the achene yield (AY) across the studied treatment combinations, it was evident that only Chem-N20 (NPK chemical fertilizer plus 20 Mm nano-silicon) demonstrated high levels of achene yield, while all other treatment combinations fell below average for this trait (Fig. 7). Moreover, utilizing AY as the reference for evaluating the other measured traits (Fig. 8) reaffirmed the significance of straw yield and harvest index. Among the other traits, the order of importance for AY was as following list: the highest branch (HB) > plant height (PH) > capitula per subsidiary branch (CSB) > capitula number per plant (CNP) > the highest capitula (HC) > seed per subsidiary capitulum (SSC) > seed per primary capitulum (SPC) > capitulum's seed mass (CSM) > thousand-achene mass (TAM), indicating the relatively low importance of thousand-achene mass for safflower. Nanomaterials represent a novel approach to improving productivity by improving the efficiency of nutrient use and facilitating slow release of nutrients, thereby reducing the risk of overuse of growth stimulants and fertilizers (Kumar et al. 2023). Such nano-materials not only improve yield performance but also decrease farming costs, thereby playing a significant role in sustainable agriculture (Usman et al., 2020). Consistent with previous research, our findings demonstrate that nano-silicon application enhances crop production, with foliar ap-

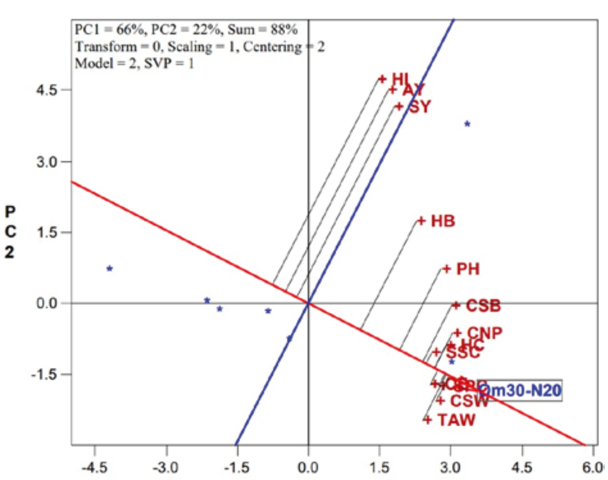

Fig. 6: Ranking traits based on the target entry (OM30-N20); 30 t ha^{-1} organic manure plus 20 Mm nano-silicon.
Traits are: plant height (PH), the highest branch (HB), the highest capitula (HC), capitula per subsidiary branch (CSB), capitula number per plant (CNP), seed per primary capitulum (SPC), seed per subsidiary capitulum (SSC), capitulum's seed mass (CSW, straw yield (SY), achene yield (AY), harvest index (HI), and thousand achene mass (TAW).

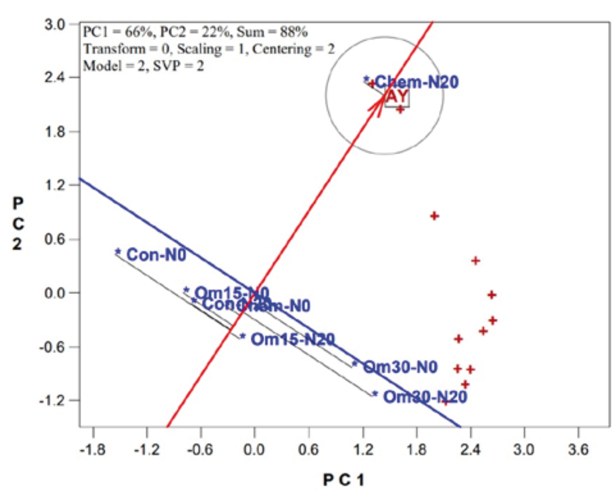

Fig. 7: Ranking treatment combinations based on the target tester AY (achene yield).
Treatment combinations are: control plus 0.0 Mm nano-silicon (Con-N0), control plus 20 Mm nano-silicon (Con-N20), 90 kg ha^{-1} NPK fertilizer plus 0.0 Mm nano-silicon (Chem-N0), 90 kg ha^{-1} NPK fertilizer plus 20 Mm nano-silicon (Chem-N20), 15 t ha^{-1} organic manure plus 0.0 Mm nano-silicon (OM15-N0), 15 t ha^{-1} organic manure plus 20 Mm nano-silicon (OM15-N20), 30 t ha^{-1} organic manure plus 0.0 Mm nano-silicon (OM30-N0), and 30 t ha^{-1} organic manure plus 20 Mm nano-silicon (OM30-N20).

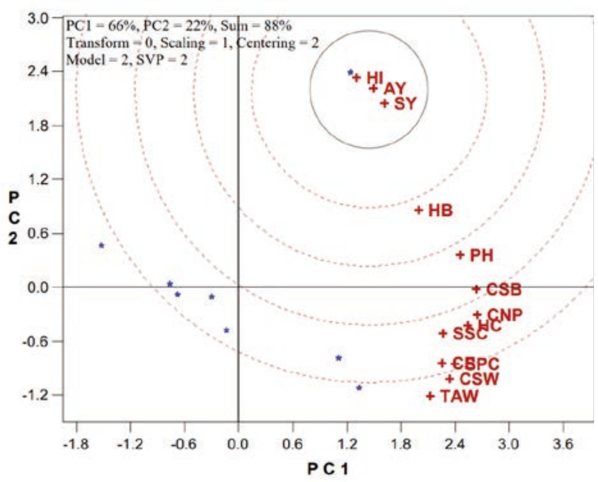

Fig. 8: Ranking traits based on the target tester AY (achene yield).
Traits are: plant height (PH), the highest branch (HB), the highest capitula (HC), capitula per subsidiary branch (CSB), capitula number per plant (CNP), seed per primary capitulum (SPC), seed per subsidiary capitulum (SSC), capitulum's seed mass (CSW), straw yield (SY), achene yield (AY), harvest index (HI), and thousand achene mass (TAW).
N0), 15 t ha^{-1} organic manure plus 20 Mm nano-silicon (OM15-N20), 30 t ha^{-1} organic manure plus 0.0 Mm nano-silicon (OM30-N0), and 30 t ha^{-1} organic manure plus 20 Mm nano-silicon (OM30-N20).

plication of nano-silicon in conjunction with chemical NPK fertilizer or organic fertilizer leading to increased safflower productivity. However, using nano-silicon and chemical fertilizers in minimal amounts presents an environmentally friendly option, promoting low-cost production within a sustainable agriculture system, and may be advisable for many farmers.

The release of nano-silicon into crops should be carefully managed to ensure effectiveness. Simultaneous use of nano-silicon and chemical NPK fertilizers has been shown to enhance yield performance in potato (Ha et al., 2019) and rice (Elekhtyar and Al-Huqail, 2023). Nanotechnology is increasingly becoming commonplace in crop production, as the use of nano-materials not only reduces fertilizer usage but also decreases production costs. Application of nano-silicon has demonstrated positive effects in crops under environmental stresses (Namjoyan et al., 2020; Hajihashemi and Kazemi, 2022). Despite the benefits of nanotechnology, it has major importance to acknowledge the potential risks related with its use in crop production. Some scientists are actively working to evaluate these risks to ensure safe and responsible usage in agriculture. However, challenges such as high evaluation costs, public environmental concerns, and human health risks remain major obstacles in this field. In other word, it is important to recognize the potential risks associated with the use of nanotechnology in agriculture, despite its many benefits, so some agronomists are assessing these risks to guarantee the biologic safety of these technologies in crop sciences. The agricultural field still faces significant barriers, including expensive evaluation and public worries about the environment, and potential risks to human health. Therefore, it is imperative to establish international standards to monitor this field before widespread public release and commercial usage.

4 CONCLUSION

The optimal fertilizer treatment for maximizing safflower yield was found to be the usage of 90 kg ha^{-1} NPK (nitrogen, phosphorus, and potassium) fertilizer, coupled with foliar application of 20 Mm nano-silicon. Conversely, for achieving high yields of safflower components, the most effective fertilizer treatment was the usage of 30 t ha^{-1} organic manure combined with 20 Mm nano-silicon foliar application. Understanding these nuances will contribute to optimizing crop yield and quality across various agricultural settings.

5 STATEMENTS

5.1 CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Naser Sabaghnia: Writing – review & editing, Supervision, Conceptualization. Mohsen Janmohammadi: Investigation, Formal analysis, Data curation.

5.2 DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

5.3 DATA AVAILABILITY

Data will be made available on request.

5.4 ACKNOWLEDGEMENT

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Drought-induced expression of *PvDERB1F* and *PvDREB5A* with promoted antioxidant activities possibly enhanced drought stress tolerance in Common bean (*Phaseolus vulgaris* L.)

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Drought-induced expression of *PvDERB1F* and *PvDREB5A* with promoted antioxidant activities possibly enhanced drought stress tolerance in Common bean (*Phaseolus vulgaris* L.)

Abstract: The common bean (*Phaseolus vulgaris* L.) is an important source of protein, fiber, vitamins, and minerals, making it essential for food programs in Botswana. Prioritizing its integration into diversified farming is crucial for achieving social, environmental, and economic benefits. Previous studies primarily focused on performance under rainfed, while the effect of drought stress remains unclear. The study aimed at evaluating the effect of drought stress on four (4) genotypes: DAB541, DAB515, CAL96, and GK011, while Tepary serves as the control. The study identifies CAL96 and DAB541 genotypes as the most promising genotypes for drought tolerance as demonstrated by their increased biomass production. The increase in biomass production may be due to the overexpression of the *Phaseolus vulgaris* dehydration-responsive element binding (*PvDREB*) genes, namely *PvDREB1F* and *PvDREB5A*. Higher proline and lower malondialdehyde (MDA) levels correlated with increased catalase (CAT) and ascorbate peroxidase (APX), which are linked to hydrogen peroxide (H_2O_2) scavenging activity. Conversely, GK011 and DAB514 exhibited decreased dry biomass, downregulated *PvDREB1F*, *PvDREB5A*, and *PvDREB6B*, along with greater levels of MDA and H_2O_2 and a steady activity of APX and CAT. This suggested an enhanced membrane lipid peroxidation and a loss of membrane integrity.

Key words: *Phaseolus vulgaris*, drought stress, *DREB* genes, lipid peroxidation, antioxidants, ROS scavenging

S sušo vzpodbujenim izražanjem genov *PvDERB1F* in PvDREB5A morda lahko s povečano antioksidacijsko aktivnostjo povečamo toleranco navadnega fižola (*Phaseolus vulgaris* L.) na sušni stres

Izvleček: Navadni fižol (*Phaseolus vulgaris* L.) je pomemben vir beljakovin, vlaknin, vitaminov in mineralov, zaradi česar je bistven v programih prehane v Botswani. Njegovo prednostno vključevanje pri povečavanju raznolikosti kmetijske pridelave je bistveno za doseganje socialnih, okoljskih in ekonomskih ciljev. Predhodne raziskave so se prvenstveno usmerjale na njegovo uspevanje v razmerah namakanja z dežjem med tem, ko je ostajal učinek sušnega stresa nepojasnjen. V tej raziskavi so bili ovrenoteni učinki sušnega stresa na štiri genotipe in sicer DAB541, DAB515, CAL96 in GK011, pri čemer je 'Tepary' služil kot kontrola. V raziskavi sta bila genotipa CAL96 in DAB541 prepoznana kot najbolj obetajoča glede tolerance na sušo, kar se je pokazalo v njuni povečani izgradnji biomase. Povečana tvorba biomase bi lahko bila zaradi močno povečanega izražanja genov v fižolu, odzivnih na dehidracijo (*PvDREB*), kot sta gena *PvDREB1F* in *PvDREB5A*. Večja vsebnost prolina in manjša vsebnost malondialdehida (MDA) sta soupadali v povečanjem aktivnosti katalaze (CAT) in askorbat peroksidaze (APX), kar je povezano z odstranjevanje vodikovega peroksida (H_2O_2). Nasprotno sta genotipa GK011 in DAB514 pokazala zmanjšanje suhe biomase, zmanjšano izražanje *PvDREB1F*, *PvDREB5A*, in *PvDREB6B* genov s hkratnim povečanjem vsebnosti MDA in H_2O_2 in enakomerno aktivnostjo APX in CAT. To nakazuje povečano peroksidacijo membranskih lipidov in izgubo delovanja membran.

Ključne besede: *Phaseolus vulgaris*, sušni stres, DREB geni, peroksidacija lipidov, antioksidati, ROS odstranjevanje

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1 INTRODUCTION

The common bean (*Phaseolus vulgaris* L.) is an important grain legume that represents a valuable source of protein in the diet (Broughton et al., 2003). *P. vulgaris* has been hailed as one of the priority crops for integration into diversified agricultural systems in Botswana (Ministry of Agriculture, 2023). This policy has been motivated by common bean being considered the most important food legume crop in the human diet, mainly for its rich protein, carbohydrates, vitamins, dietary fibre and minerals (Beebe et al., 2013; Mangole et al., 2022). It was further stated that the common bean is consumed in hospitals and school feeding schemes, and it is also offered as part of the government's supplementary feeding scheme for underage children at clinics nationwide (Mangole et al., 2022). Although it is important, production has been reported to be low, resulting in a high import bill. In that respect, various common bean genotypes from neighbou countries have been introduced.

Previous research on the introduced genotypes has been focused on planting date, nutritional value and yield stability under rainfed conditions (Moatshe-Mashiqa et al., 2021; Molosiwa et al., 2019). In addition, drought stress is a factor of economic importance in common bean production in Botswana. Southern Africa Drought Resilience Initiative (SADRI) (2021) indicated that the 2018/19 drought season resulted in two-thirds of crop failure. Botswana is a southern African country with an arid to semi-arid climate, resulting in desert-like conditions and very variable rainfall patterns across about a third of the country. This is caused by drastic and rapid changes in the global climate that can occur during the common bean's life cycle, such as initial seedling establishment, vegetative growth, flowering and/or grain filling are exacerbated (Rao et al., 2013). This, therefore, calls for screening of the introduced genotypes for drought stress tolerance.

The response to drought stress is mediated by subtle changes in gene expression that lead to changes in the composition of the plant transcriptome, proteome and metabolome and ultimately the phenotype (Ansari et al., 2017, 2018; Deeba et al., 2012; Lin et al., 2016). The adaptive strategies used by drought-tolerant plants are of major importance for the selecting and adopting crops with improved performance under erratic water deficit conditions, such as Botswana's. Pholo-Tait et al. (2022), reported the role of *Phaseolus vulgaris ALLANTONAISE (PvALN)* in drought stress in common bean genotypes. Meanwhile, transcriptional factors, especially the dehydration-responsive element binding (DREB) factor family members comprise critical targets for selection of crops that confer tolerance to abiotic stress. *DREBs* regulates gene expression through a mechanism that involves recognizing the dehydration responsive element (DRE), which consists of the conserved motif A/GCCGAC (Sakuma et al., 2002). Amongst the six subgroups (A-1- A-6) of DREB genes, the expression of *DREB2 (A-2), DREB5 (A-5; RAP2.1)* and *DREB6* genes (A-6; *RAP2.4*) showed to be highly induced under drought stress in *Arabidopsis* (ChunJuan & JinYuan, 2010; Nakashima, Ito, & Yamaguchi-Shinozaki, 2009). In another study, overexpression of *GmDREB1* increased the drought resistance of transgenic soybeans while increasing yield (Chen et al., 2022). Similarly, transgenic tobacco lines expressing the transcription factor gene of the DREB 5-A subgroup of *Ricinus communis* L. *(RcDREB1)* showed improved growth, drought tolerance and higher pollen viability (do Rego et al., 2021). Subsequent research showed that under drought stress conditions, the expression level of *DREB1* rose more in wheat genotypes that were drought tolerant than in those that were drought sensitive (Rustamova et al., 2021). Previous studies reported that drought stress tolerance in the common bean is due to the overexpression of *Phaseolus vulgaris* DREB genes such as *PvDREB1F, PvDREB5A* and *PvDREB6B* (Konzen et al., 2019). Therefore, these DREB genes offer the possibility of serving as a basis for screening different genotypes of common beans for drought tolerance.

Interestingly, the differential expression of DREB genes has been shown to also alter the functional expression of nonenzymatic antioxidant defense (Ghaffari et al., 2019; Moloi & van der Merwe, 2021; Wei et al., 2016). The overexpression of DREB genes has been reported to induce a decrease in malondialdehyde (MDA) content, and such an inverse correlation plays a vital role in drought stress tolerance. MDA is one of the final products of polyunsaturated fatty acid peroxidation in cells. It is widely used as a reliable marker for determining the degree of membrane damage in tissues under stress and the ability of plants to tolerate drought stress (Blokhina et al., 2003; Morales & Munné-Bosch, 2019; Vendruscolo et al., 2007). The production of reactive oxygen species (ROS) under drought stress correlated positively with an increase in MDA content, resulting in increased permeability of the plasma membrane and extravasation of the content of cells. This inevitably impairs the production of biomolecules, such as lipids, proteins, and nucleic acids (Kong et al., 2016; Ripullone et al., 2021). On the other hand, lower concentrations of MDA have been reported to be associated with lower production of ROS. In soybean, tomato and wheat (Amoah & Seo, 2021; Raja et al., 2020; Saruhan Guler & Pehlivan, 2016), low levels of MDA suggested lower production of ROS and ultimately a reduction in membrane damage under drought stress. The overexpression of DREB genes suppressed the production of MDA, resulting in an improved reactive oxidative species (ROS) scavenging capability. This was demonstrated in transgenic *Arabidopsis*, where overexpression of *AtDREB1A* resulted in a lower MDA content (Morales & Munné-Bosch, 2019). Similarly, it was observed that the regulation of antioxidant mechanisms by *Arabidopsis thaliana DREB1A* (*AtDREB1A)* was associated with a reduction in MDA levels in peanut (*Arachis hypogaea* L.) under water deficiency (Bhalani et al., 2019). In that respect, the production of MDA has been used as a robust reliable marker for determining the degree of injury to a drought-stressed plant (Alché, 2019; Kong et al., 2016; Morales & Munné-Bosch, 2019).

In addition to MDA, osmolytes such as proline play an important role in plants under drought stress. Proline protects plants through cellular osmotic adjustment, ROS detoxification, protection of membrane integrity, and enzyme/protein stabilization (Ghaffari et al., 2019). Drought stress significantly enhanced the accumulation of proline in the leaves of canola at the flower initiation stage and pod filling stage under drought stress. This response justified that proline accumulation under drought stress is an adaptive response that enhances survival and tissue water status (Morsi et al., 2023). This response is ascribed to enhanced osmotic modifications to acclimatize to recompense for plant survival and, accordingly, assist in tolerating drought stress (Morsi et al., 2023). Conversely, a negative correlation between proline levels and plant growth has been reported in common beans. Higher levels of proline induced by stress inhibited growth in highly drought-sensitive cultivars and as such proline has been proposed as a suitable large-scale screening biochemical marker for common bean under water deficit and salt stress (Arteaga et al., 2020). Likewise, overexpression of the *DREB* gene has been shown to enhance proline accumulation (Nguyen et al., 2019). Transgenic *Arabidopsis* plants overexpressing *DREB1A* showed a positive correlation of increased accumulation of proline. Transgenic rice overexpressing *Oryza sativa DREB1A* (*OsDREB1A)* also accumulated proline in stressed and controlled conditions (Dubouzet et al., 2003). Similar results were also demonstrated in soybean, in which overexpression of the *Glycine max DREB6* and *DREB2 (GmDREB6; GmDREB2)* genes increased proline accumulation and tolerance to drought stress (Nguyen et al., 2019; Pham et al., 2020). Furthermore, peroxidase and catalases are among the versatile enzymatic hydrogen peroxide (H_2O_2) scavenging systems (Caverzan et al., 2012; Foyer & Shigeoka, 2011; Shigeoka, 2002), hence contributing to the regulation of redox homeostasis and signaling pathways (Sohag et al., 2020; Tyagi et al., 2021). Given that specific DREB genes induce tolerance to drought stress in *P. vulgaris* (Konzen et al., 2019), it is worth noting that genetic variability exists among genotypes. The study aimed to evaluate the physiological and molecular responses of introduced common bean genotypes for drought tolerance. The objectives were to evaluate both enzymatic and nonenzymatic antioxidant responses, as well as conducting transcriptional screening for drought stress tolerance using DREB genes.

2 MATERIALS AND METHODS

2.1 PLANT MATERIALS AND TREATMENTS

The study was conducted at Sebele Research Station (24° 34’25”S and 25° 58’0”E) under the growth cabinet growth conditions. The growth cabinet was set at a long-day photoperiod of 16 h light and 8 h dark with the temperature set at 30 °C (day) and 25 °C (night). The light intensity was set at 300 μE m^{-2} s^{-1} light, while the humidity was maintained at 60 %. The study was conducted on four (4) common bean varieties, namely, DAB541, DAB514, CAL96, and GK011 The choice of common genotypes was based on their performance in the previous studies, demonstrating their potential in terms of promising previous results on production and productivity stability under rainfed, as well as nitrogen fixation capability under water deficit conditions (Molosiwa et al., 2019; Pholo-Tait et al., 2022). Tepary bean, which is the commonly grown bean landrace in Botswana was used at the control. Seeds were directly sown on a mixture (1:2) of sterilized sandy soil and loose jiffy growth media. The experiment was subjected to a factorial complete randomized design with six (6). replications. The two treatments consisted of a maximum water holding capacity (control) and a drought stress treatment in which water was withhold for fourteen days (Nakashima et al., 2009).

2.2 DRY LEAF BIOMASS DETERMINATION

Six (6) replicates of leaf plant material were harvested at the end of the drought stress period. Samples were oven-dried at 65 °C) for three (3) consecutive days. Thereafter, the samples were weighed for total leaf dry biomass production.

2.3 PROLINE AND MALONDIALDEHYDE (MDA) CONTENTS

Leaf tissue material (0.1 g) was ground in liquid nitrogen and homogenized in 3 % (w/v) aqueous sulfosalicylic acid. The homogenate was centrifuged at 4000 × g for 10 minutes, while the resulting supernatant was added to a mixture of equal volumes of acid-ninhydrin and acetic acid. The mixture was incubated at 98 °C for 30 minutes and cooled to 25 °C, followed by the addition of toluene. The upper layer of the solution was aliquoted and used for proline determination. The proline content was quantified by spectrophotometer at an absorbance rate of 520 nm and calculated based on the standard curve using proline as a standard (Bates et al., 1973). Malondialdehyde (MDA) content was measured to determine the degree of membrane lipid peroxidation (Zhang and Huang, 2013). The total ground leaf samples (0.1 g) were homogenized in 0.1 % (w/v) trichloroacetic acid (TCA). The homogenate was centrifugated at 10,000 × g for 10 min. The supernatant was mixed with 20 % TCA consisting of 0.5 % thiobarbituric acid (TBA). The mixture was then heated at 95 °C for 15 min, followed by cooling on ice. Cooled samples were centrifuged at 4800 rpm for 10 min, after which the absorbance was measured at 450, 532 and 600 nm.

2.4 ACTIVITIES OF ANTIOXIDANT ENZYMES

Leaf tissues (250 mg) were homogenized in 0.1 % (w/v) TCA, centrifuged at 12 000 × g for 15 min before the addition of 10 mM potassium phosphate buffer (pH 7.0) and 1 M potassium iodide (KI) to the supernatant. The enzyme activity was read at an absorbance rate of 390 nm, while the H_2O_2 concentration was calculated using a standard curve (Velikova et al., 2000). Ascorbate peroxidase (APX) and catalase (CAT) were extracted from ground leaf tissue (0.2 g) in liquid nitrogen. The materials were homogenized in a mixture of 0.2 M sodium phosphate buffer (pH 7.8) and 0.1 mM EDTA. Consecutively, the homogenate was centrifuged at 15000 × g for 20 minutes at 4 °C followed by APX and CAT enzymatic activity assays. APX enzymatic activity was assessed in a reaction mixture consisting of an extract, 1 M potassium phosphate buffer (pH 7.8), 10 mM hydrogen peroxide, and 10 mM ascorbate. The reaction mixture without an extract was used as a blank. The reaction was initiated with the addition of H_2O_2 at 25 °C room temperature. The oxidation rate of ascorbate was determined by the decrease in absorbance at 290 nm for 3 min. CAT enzymatic activity (Aebi, 1974) was performed in a reaction mixture containing 0.01 M H_2O_2 and 0.05 M potassium phosphate buffer (pH 7.0). The CAT enzyme was added to initiate the reaction, while the decrease in absorbance at 240 nm during the initial 3 min was used to measure H_2O_2 activity.

2.5 REAL-TIME QUANTITATIVE ANALYSIS OF DREB GENES

CTAB protocol (Hu et al., 2002) was followed to isolate total RNA from three biological replicates of leaf tissue material (250 mg). RNA was purified using a Qiagen RNase-free DNase Kit (Cat #79254) and eluted in RNase-free water according to the manufacturer's instructions. RNA concentrations were checked using a NanoDrop ND-1000 UV–Vis Spectrophotometer (Thermo Fisher Scientific), while RNA integrity was validated by visualizing the RNA on a 1 % agarose gel. RNase-DNase free RNA was reverse transcribed to complementary DNA (cDNA) using an oligo (dT^{18}) primer and M-MLV (H-) reverse transcriptase (Promega, Anatech, South Africa) following the manufacturer's instructions. cDNA template was checked by RT-PCR using reference genes, namely, the *SKP1/ASK-INTERACTING PROTEIN 16 (PvSKIP16), ACTIN 11 (PvACT11),* and *TUBULIN BETA-8 (Pvβ-TUB8)* genes (Borges et al., 2012), which serve as internal control genes (Table 1).

Real-time PCR was performed to test for the relative expression of the drought stress-related marker genes *PvDREB1F, PvDREB5A* and *PvDREB6B* (Konzen et al., 2019) on four bean genotypes to evaluate their tolerance to drought stress (Table 1). RNA templates were replicated thrice (3) and diluted to a concentration of 10 ng μl^{-1}. The PCR experiment was conducted using a Luna Universal One-step RT-qPCR Kit (New England Biolabs, USA) consisting of 1X Luna Universal One-Step Reaction Mix (10 µl), 1 x Luna WarmStart® RT Enzyme Mix (1 µl), 0.4 µM of each primer (0.8 µl), 1 µg of diluted RNA and RNase-free water. Triple-replicate RT-qPCRs were performed in 96-well plates using a LineGene 9600 Bioer PCR machine (Hangzhou Bioer Technology) following SYBR Green/FAM detection. The RNA template was reverse transcribed at 55 °C for 60 s, followed by initial denaturation at 95°C for 60 s, 40 cycles of denaturation at 95°C for 10 s and extension at 60 °C for 35 s, and a melting step at 95 °C. PCR efficiency (E) was calculated using LinRegPCR (version 2014.5), while threshold (Ct) values were used to determine the relative expression level of a given gene using the $2^{-\Delta\Delta}$Ct method (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008). The relative expression of genes common bean genotypes was then compared to that of Tepary bean as the control.

2.6 STATISTICAL DATA ANALYSIS

The data were subjected to analysis of variance

Table 1: Reference and target genes for the real-time PCR-based relative expression of genes.

Reference genes (Borges et al., 2012)				
NCBI ID	Gene	Forward primer (5'- 3')	Reverse Primer (5'- 3')	Amplicon size (bp)
62703083	*ACTIN-11* *(PvACT11)*	TGCATACGTTGGT-GATGAGG	AGCCTTGGGGTTAA-GAGGAG	190
171656465	*TUBULIN BETA-8* *(PvB-TUB8)*	AATGTGAAGTC-CAGCGTGTG	CTTCCCCAGTGTAC-CAATGC	163
187434529	*SKP1/ASK-INTER-ACTING PROTEIN 16 (PvSKIP16)*	CACCAGGATG-CAAAAGTGG	ATCCGCTTGTCCCTT-GAAC	163
Biotic stress genes (Konzen et al., 2019)				
Phytozome accession ID	Gene	Forward primer (5'- 3')	Reverse Primer (5'- 3')	Amplicon size (bp)
Phvulv091025959m.g	*PvDREB1F*	TGCGTCGAGCAATTA-GAGAA	TCCTGATGCGTCTG-GTATTG	153
Phvulv091010162m.g	*PvDREB5A*	TTGGGTACTTTTCC-CACTGC	GCCTTCCATGTCAT-CATCCT	177
Phvulv091016691m.g	*PvDREB6B*	AATTCTGCATCTCC-CTCACG	GCTGGGCTTGATTTA-GACGA	167

(ANOVA) using the statistical software SPSS (version 22 of Windows; SPSS). One-way analyses of variance followed by Tukey's HSD test comparison at $p \leq 0.05$ were performed to determine the relevant differences between the control and drought-stressed variants of the respective genotype.

3 RESULTS

3.1 DRY LEAF BIOMASS IN RESPONSE TO DROUGHT

Similarly, compared with that of the control plants, the growth rate of the drought-stressed plants in terms of dry biomass was not affected in the CAL96 or DAB541 genotype. Interestingly, drought stress significantly reduced dry biomass production in the GK011 and DAB514 genotypes. This response translated to significant dry biomass production inhibition of 0.68 g (28 %) and 0.94 g (33 %) in GK011 and DAB514, respectively (Table 2).

3.2 LIPID PEROXIDATION LEVELS AND PROLINE PRODUCTION

The lipid peroxidation in leaves as determined by the MDA content, varied significantly between stressed and control plants for the GK011 tepary bean and DAB514 common bean. The most significant drought-induced increase in the accumulation of MDA of 5.06 µmol g^{-1} fresh mass (117.3 %) was demonstrated in tepary bean, followed by 2.75 µmol g^{-1} fresh mass (42.04 %) in the DAB514 genotype. However, compared with those in the control plants, the MDA content in the stressed plants was not significantly greater for CAL96 and DAB541 (Table 2).

A significant increase in the biosynthesis of proline of 1.09 (23.6 %) and 1.70 (39.6 %) mg^{-1} fresh mass was induced in the CAL96 and DAB541 drought-treated plants, respectively, in comparison to their corresponding control plants. However, drought stress significantly inhibited the production of proline by 0.94 mg^{-1} fresh mass (22 %) in GK011 tepary beans (Table 2).

3.3 ANTIOXIDANT ENZYMATIC ACTIVITIES

The enzymatic activities varied between the stressed plants and the control plants in terms of H_2O_2 concentration were observed for all the genotypes except for the DAB514 genotype. Drought stress significantly promoted this enzyme activity by 5.43 µmol g^{-1} fresh mass (68.6 %) in GK011 tepary bean and 2.82 µmol g^{-1} fresh mass (47.1 %) in DAB514 common bean. The reverse was observed in CAL96, which exhibited a significant 2.5 µmol g^{-1} fresh mass (27 %) reduction in H_2O_2 enzymatic

Table 2. Dry biomass production and accumulation of metabolites in response to drought stress. Values are represented as the mean ± SEM (n = 6) of independent biological replications. Values followed by the same letter do not differ from each other by Tukey's test ($p \leq 0.05$).

Genotype	Dry biomass (g)		MDA content (mg^{-1} fresh mass)		Proline (mg^{-1} fresh mass)	
	Control	Drought stress	Control	Drought stress	Control	Drought stress
GK011	2.40 a	1.73 b	5.67 a	9.29 b	4.19 a	3.25 bc
CAL96	2.77 a	2.76 c	10.99 b	10.06 b	4.60 a	5.69 e
DAB514	2.86 a	1.92 b	6.95 ad	11.23 bc	3.82 b	3.48 bc
DAB541	3.67 d	3.60 d	9.67 bcd	5.35 a	4.29 b	5.98 e

activity in response to drought stress (Fig. 2A). While drought stress increased APX activity in DAB514 (3.37 μmol mg^{-1} FM protein min^{-1} 19,6 %), drought stress did not affect APX activity in the GK011 and CAL96 genotypes (Fig. 2B). Drought stress also induced variations in catalase activity between the stressed plants and the control plants. Although plants exposed to drought stress presented significant 2.71 μmol mg^{-1} FM protein min^{-1} (14.5 %) and 3.30 μmol mg-1 FM protein min^{-1} (15.4 %) increases in catalase activity in CAL96 and DAB541, respectively, such increases in enzyme activity in GK011 and DAB514 were not significant (Fig. 2C).

3.3 RELATIVE EXPRESSION OF DEHYDRATION-RESPONSIVE ELEMENT BINDING (DREB) GENES

The qPCR analysis was conducted on a highly intact RNA template that showed clear gel bands corresponding to 18S and 28S rRNA and the absence of a smear (Fig. 2A). The first qPCR experiment on the GK011 tepary bean and CAL96 common bean genotypes demonstrated an increase in the relative expression of the *PvDREB1F*, *PvDREB5A*, and *PvDREB6B* genes in GK011 tepary bean plants. Drought stress induced at least a 1-fold decrease in the relative expression of *PvDREB1F* and *PvDREB6B* as well as a 2-fold decrease in *PvDREB5A* in GK011 tepary bean plants. Similar results were observed for the CAL96 genotype, in which the relative expression of *PvDREB5B* and *PvDREB6B* were inhibited. However, there was a significant 1.2-fold increase in the expression of *DREB1F* in drought-stressed plants compared to that in the corresponding control plants. (Fig. 2B). A study between GK011 tepary bean and DAB514 revealed the distinct suppression of the differential expression of all three DREB genes in both genotypes. The highest average levels of 1.7-fold and 1.3-fold inhibition of *PvDREB5A* relative expression were revealed in the GK011 and DAB514 genotypes, respectively. Taken together, these findings indicated that drought stress induced significant and marked downregulation of the differential expression of *PvDREB5A* compared with that of the other two DREB genes in both the GK011 and DAB514 genotypes (Fig. 2C). A similar trend of a downregulated expression of *PvDREB1F, PvDREB5A,* and *PvDREB6B* in the which DREB gene were analyzed in GK011 and DAB541. Amongst the three genes, *PvDREB5A* was highly differentially expressed (2.9-fold). Drought stress downregulated the relative expression of PvDREB1F and *PvDREB6B* in the DAB541 genotype. In contrast, a marked increase in the expression of *PvDREB5A* (1.9-fold) was detected in DAB541 drought-stressed plants compared with control plants (Fig. 2D).

4 DISCUSSIONS

Climate-adaptive strategies such as the use of drought-tolerant plants in Botswana are highly important for the selection and introduction of crops with improved performance under fluctuating water deficit conditions. This study adopted the considerable effort that is devoted to the selection of crops using plant morphophysiological parameters coupled with molecular and biochemical selection approaches. Intriguingly, drought stress upregulated the differential expression of *PvDREB1F*, which might have contributed to the maintenance of dry biomass production in the CAL96 common bean genotypes. This finding was in agreement with that of a previous study on rice, which demonstrated the overexpression of *OsDREB1F* under salt, drought, and low-temperature tolerance (Wang et al., 2008). The induced overexpression of *PvDREB1F* was accompanied by an increase in proline and antioxidant enzymes in the present study. Interestingly, an increase in proline level positively correlated with the maintained levels of MDA, hence suggesting the prevention of lipid peroxidation. In addition, an increase in CAT activity suggested an enhanced CAT enzymatic antioxidative defense mechanism that plays a role in the detoxification of H_2O_2 thereby maintaining equilibrium (Apel & Hirt, 2004; Ghaffari

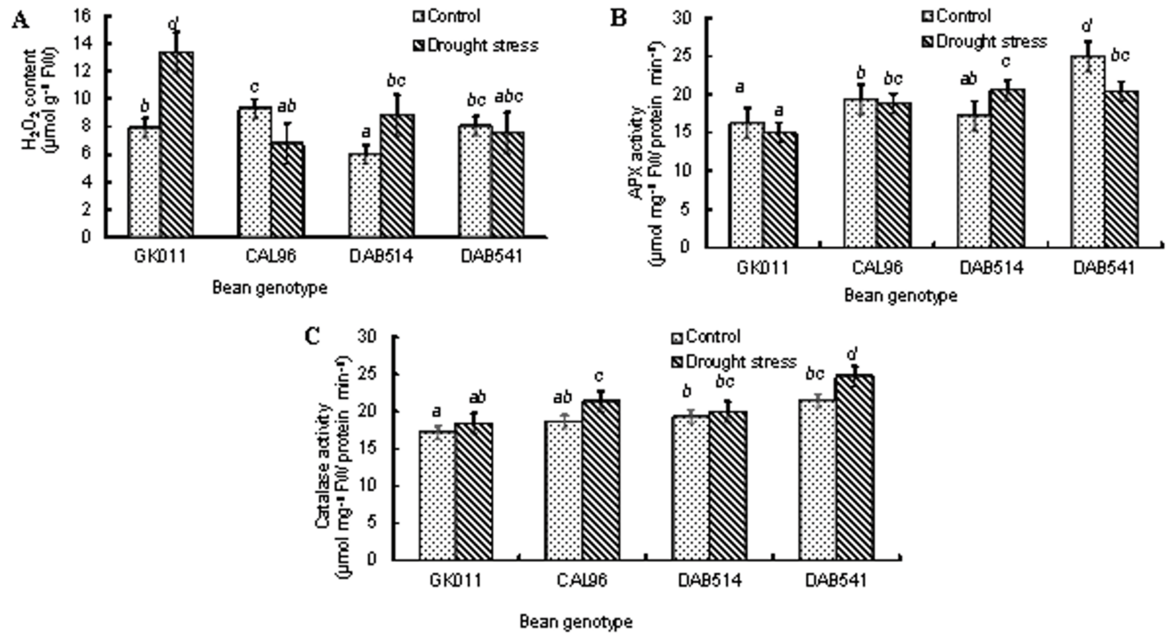

Figure 1: The effect of drought stress on the accumulation of H2O2 (A), APX activity (B) and CAT activity in common bean genotypes. Mean values represent ± SEM (n = 6) of independent biological replications. Different lower-case letters indicate significant ($p \leq 0.05$) differences between mean values according to Tukey's tests made separately for each genotype for the drought stresses against the control.

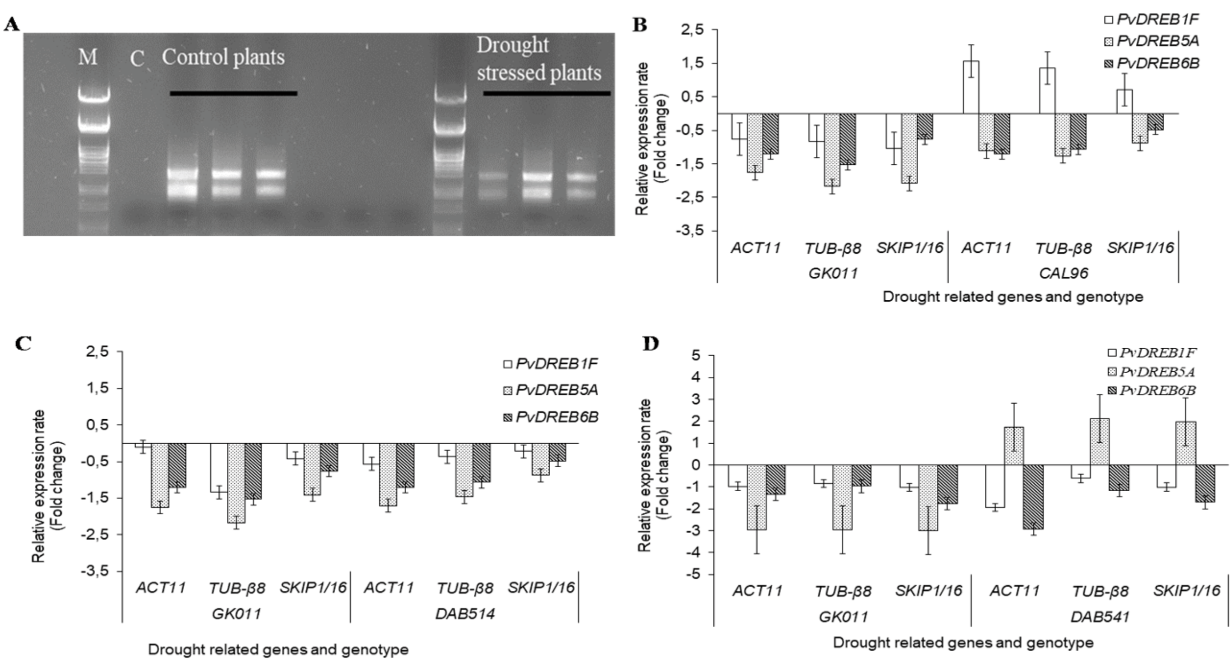

Figure 2: Relative expression of DREB genes run on high intact RNA template (A) for CAL96 (B), DAB514 (C) and DAB541 (D) common bean genotype in response to drought. Real-time PCR was conducted in the respective common bean genotypes against the GK011 tepary bean. Relative expression rates of DREB genes were normalized against three internal reference genes (PvACT11; PvTUB-β8; PvSKIP1/16). Bars represent the mean ± standard error of the mean (SE) of pooled three technical samples from three independent samples. Values sharing a common letter are not significantly different at $p < 0.05$.

et al., 2019; Gomes et al., 2022). The drought tolerance mechanism for CAL96 likely occurs through the expression of *PvDREB1F*, maintenance of ROS homeostasis and prevention of lipid peroxidation-related cell death. These are additional proposed drought stress tolerant mechanisms that also support the previous study that indicated that CAL96 could confer drought tolerance through the promotion of allantoin pathways (Pholo-Tait et al., 2022).

The expression of a DREB 5-A subgroup of transcription factor genes from castor bean in tobacco has been reported to be associated with drought tolerance (do Rego et al., 2021). Similarly, the overexpression of *PvDREB5A* in our study suggested the presence of transcriptionally related drought tolerance mechanisms that resulted in the maintenance of growth in terms of leaf dry biomass. Contrary to inhibited growth as a result of high levels of protein (Arteaga et al., 2020), the growth rate was not affected by an increase in proline in this study. In concert with the previous report (Porch et al., 2009), the increased proline levels might have acted as a component of signal transduction pathways that regulate the overexpression of *PvDREB5A*. In addition to its adaptive role in mediating osmotic adjustment and protecting subcellular structure, an increase in proline in the DAB541 genotype could have played a role in maintaining higher activities of CAT. The latter could have subsequently maintained a steady activity of CAT-enzyme scavenging of H_2O_2 reactive oxygen species (Chen et al., 2022; Molinari et al., 2007; Noctor et al., 2018). Furthermore, the overexpression of *PvDREB5A* correlated with a reduction in MDA in DAB541 and this is in line with the findings of a study on maize (Moussa & Abdel-Aziz, 2008). High levels of proline could have contributed to the reduction in MDA levels (Soares et al., 2019). This promoted an inverse correlation along with an increase in CAT activity, suggested a reduced lipid peroxidation and improved redox buffering as a result of effective scavenging of H_2O_2 (Dong et al., 2018).

On the contrary to CAL96 and DAB541 genotypes, drought stress-induced suppression of the differential expression of *PvDREB1F*, *PvDREB5A*, and *PvDREB6B* in GK011 and DAB514 genotypes. The downregulated differential expression of genes positively correlated with greater levels of proline, MDA, and H_2O_2 in the GK011 and DAB514 genotypes. Contrary to a previous study on common bean (Arteaga et al., 2020), decreased levels of proline resulted in an inhibited growth GK011 bean genotype. Rather, the inhibited growth rate might have been attributed to the promoted lipid peroxidation and eventual cell death due to the high accumulation of MDA and levels and the promoted H_2O_2 content (Ghaffari et al., 2019; Sivakumar et al., 2000; Soares et al., 2019). Previous reports indicated that an increase in MDA content resulted in cell membrane rupture, hence increasing membrane leakage in *P. vulgaris* L. (Zlatev et al., 2006), *Avena* species (Pandey et al., 2010) and wheat (Tatar & Gevrek, 2008). In that respect, the downregulated expression of DREB genes and increased levels of MDA and H_2O_2 suggested a promoted disequilibrium between H_2O_2-related ROS production and H_2O_2 scavenging activity (Yang et al., 2020) due to the stable cooperative activities of APX and CAT (Apel & Hirt, 2004; Gomes et al., 2022). Such disequilibrium could have resulted in an oxidative burst due to lipid peroxidation and protein denaturation (P. Sharma et al., 2012; Yang et al., 2020). This could have resulted in greater levels of oxidative damage possibly through enhanced membrane lipid peroxidation and loss of membrane integrity to withstand the cellular-level effects of water loss and ultimately caused cellular damage and death, hence inhibiting plant growth (Kong et al., 2016; Ripullone et al., 2021; V. Sharma et al., 2019).

An increase in MDA content, APX activity and greater levels of H_2O_2 was accompanied by a decrease in dry biomass in DAB514. As previously discussed above, increased levels of MDA suggested an increase in lipid peroxidation and subsequently promoted plant cell damage. Despite an increase in APX activity, the promoted production of H_2O_2 substantiated the need for APX for cooperative ROS enzymatic scavenging activity. This is in concert with previous studies which reported that increased APX activity in *Salvinia molesta* D. Mitch. and *Vallisneria natans* (Lour.) H. Hara resulted in H_2O_2 accumulation, lipid peroxidation, and subsequently decreased growth rates (Gomes et al., 2022).

5 CONCLUSIONS

The current study demonstrated that CAL96 and DAB541 plants are possibly tolerant to drought stress. The CAL96 common bean genotype could confer drought tolerance through the overexpression of *PvDREB1F* and enhance the scavenging mechanism that involves the active role of proline in maintaining lipid peroxidation and the cooperative scavenging of H_2O_2 by CAT and APX. In the DAB541 common bean genotype, drought stress tolerance is associated with the overexpression of *PvDREB5A* and increased levels of proline, which cooperatively play a major role in the suppression of lipid peroxidation through reduced levels of MDA, hence stabilizing the membrane. In addition, such drought tolerance could have been attributed to H_2O_2 enzymatic scavenging activity, in which increased CAT activity enhanced the maintenance of steady H_2O_2 levels. This finding therefore supported a previous study (Pholo-Tait et al., 2022) that the CAL96 and DAB541 genotypes serve as promising drought-tolerant common bean genotypes. However, future reverse genetic approach studies that involve silencing *PvDREB1F* and *PvDREB5A* will unambiguously conclude their drought-induced tolerance role. This will further substantiate the importance of the inherent genotypic traits to serve as potential parent material in marker-assisted breeding to improve common bean varieties for drought tolerance and stress-induced ROS

6 CONFLICTS OF INTEREST

There is no conflict of interest regarding the manuscript.

7 DATA AVAILABILITY

Original data could be obtained upon reasonable requests from corresponding author.

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Codling moth management by low doses of sugars on ‘Royal Gala’ apple trees

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Codling moth management by low doses of sugars on 'Royal Gala' apple trees

Abstract: Codling moth (CM) is a key pest in apple orchards in Algeria. Its control is difficult because of its ability to develop resistance to pesticides. Besides, concern about the safety of these pesticides for human health and the environment has led to regulatory actions that have reduced the availability of these products to growers. Thus, the use of eco-friendly alternative methods is encouraged. In this context, foliar spraying using fructose (100 ppm) and glucose (100 ppm) against CM larval stages on 'Royal Gala' apple, compared to the control and insecticide (Deltamethrin), was assessed in an orchard located in Beni Fedhala province (Batna-Algeria). The obtained results confirmed that the spraying of glucose and fructose increased the percentage of healthy fruits at harvest, the percentages of healthy fallen fruits and the total healthy fruits. Further, the number of diapausing larvae in corrugated cardboard banding was reduced. In addition, our study shows that the number of chrysalis was significantly lower and different from the number of male and female larvae, which are identical.

Key words: Codling moth, glucose, fructose, Deltamethrin, 'Royal Gala'.

Uravnavanje jabolčnega zavijača z majnimi odmerki sladkorjev na jablanah 'Royal Gala'

Izvleček: Jabolčni zavijač (CM) je ključni škodljivec v sadovnjakih jablan v Alžiriji. Njegovo uravnavanje je težavno zaradi njegove sposobnosti razvoja rezistence na pesticide. Poleg tega je skrb zaradi škodljivosti pesticidov ljudem in okolju privedla k načinom uravnavanja, ki zmanjšujejo dostopnost pesticidov sadjarjem. Vzpodbujane so okolju prijazne metode uravnavanja škodljivca. V tem kontekstu je bilo opravljeno foliarno škroplenje s fruktozo (100 ppm) in glukozo (100 ppm) za zatiranje jabolčnega zavijača v larvalnem štadiju v nasadu jablan 'Royal Gala', v primerjavi s kontrolo in uporabo insekticida (Deltamethrin), v sadovnajaku province Beni Fedhala (Batna-Algeria). Dobljeni rezultati so potrdili, da je škropljenje z glukozo in fruktozo povečalo odstotek zdravih plodov ob obiranju, kot tudi odstotek odpadlih zdravih plodov in celokupni delež zdravih plodov. Zmanjšalo se je tudi število mirujočih gosenic v pasteh iz valovite lepenke. Dodatno je raziskava pokazala, da se je število bub zmanjšalo in da je bilo različno od sicer identičnih moških in ženskih gosenic.

Ključne besede: jabolčni zavijač, glukoza, fruktoza, Deltamethrin, 'Royal Gala'.

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1 INTRODUCTION

Cydia pomonella (Linnaeus, 1758) causes large economic losses for the fruit yield in Algeria. Codling moth resistance to many insecticides has been observed in several areas (Bouvier et al., 2001; Sauphanor et al., 2006; Charmillot and al., 2007; Whalon et al., 2008; Rodríguez et al., 2011 et 2012; Pajac et al., 2019). Currently, it is essential to design eco-friendly control systems. The concept of exogenous application of sugars on apple trees to reduce the damage of *C. pomonella* in commercial orchards has shown increased interest in several countries (France, Italy, Greece, and Algeria) with an efficacy percentage of 40–59 % (Derridj et al., 2011). The studies of Lombarkia (2002) and Lombarkia et al. (2008) and (2013) testing the link between six metabolites (glucose, fructose, sucrose, sorbitol, quebrachitol, and *myo*-inositol) and *C. pomonella* egg-laying behavior to reduce the damage, in addition, Tiffrent and Lombarkia (2022a) showed that the glucose and fructose treatments significantly reduced the number of eggs led to similar results as for the reference chemical treatment during the first and third generation flights on 'Golden Delicious' and during the fourth flight on 'Royal Gala'. Furthermore, according to Tarkowski et al. (2019), the concept of "sweet immunity" postulates that sugar metabolism and signaling influence plant immune networks.

The objective of this study was to determine the effect of the foliar application of sugars specifically, fructose alone and glucose alone, and insecticide compared with a control in apple fruit orchard and to design alternative eco-friendly control systems to substitute conventional chemical programs.

2 MATERIALS AND METHODS

2.1 STUDY SITE AND TREATMENTS

'Royal Gala', is a highly esteemed variety famed for its sweet, crisp apples. According to the Food and Agriculture Organization of the United Nations (FAO), the major apple cultivars can be roughly divided into either red-colored bearing fruit, such as 'Red Delicious', 'Fuji', and 'Royal Gala', among others, and yellow/green-colored bearing fruits, such as 'Golden Delicious', 'Granny Smith', and 'Orin' (Korbon, 2021). 'Golden Delicious' and 'Royal Gala' are the preferred apple varieties for juice production due to their good balance between sweetness, bitterness, and aroma compounds (Ramadan and Farag, 2022).

The experiment was carried out in 2021 in a 'Royal Gala' apple orchard (35°21'21,6" N, 006°01' 16,5" E) located in Batna province (eastern Algeria). The treatments were adjusted in a randomized Latin square with four repetitions against *C. pomonella*. All four modalities are then distributed within each of the four plots, and each plot has three trees. The studied orchard was managed under common practices of the region. It had a surface area of 2.5 ha (three apple varieties, apricot, peach, plum, and nectarine trees) with 450 'Royal Gala' apple trees. The trees were 11 years old, and the plant spacing was 4 m × 4 m.

The treatments were applied using an electrical pressure sprayer (12 V-12 Ah) with a capacity of 16 l. The tested modalities were fructose, glucose, and insecticide Decis (its active ingredient is Deltamethrin), in addition to the unsprayed control. The tested treatments, their doses, and periods of application are reported in Table 1.

2.2 DAMAGE ASSESSMENTS

The following variables were measured; the percentage of healthy fruit at harvest, the percentages of healthy fallen fruits and the percentage of total healthy fruits. All variables where based on the total number of fruits produced per tree.

2.3 COUNTING DIAPAUSING LARVAE

To collect diapausing larvae of the CM, all trees of the four plots were selected, a strip of corrugated cardboard (20 cm wide) was placed around the trunk of each tree and at a height of 20 cm from the ground, installed between the mid-April and the end of September, and the captured diapausing larvae were counted, making it possible to distinguish between male larvae, female larvae, and chrysalis. According to Kuyulu and Genc (2019), the fifth instar larvae were used to determine the sex of the

Table 1: Tested treatments, their doses, and periods of application

Treatments	Doses	Periods of application
Control (Untreated)	Control (Untreated)	The morning treatments (sugars and insecticide) were carried out every 20 days throughout the season from the flowering end until harvest (Derridj et al., 2012).
Fructose (Fluka Biochemika)	10 g 100 l^{-1} (100 ppm)	
Glucose(Fluka Biochemika)	10 g 100 l^{-1} (100 ppm)	
Decis 25 EC 25 g l^{-1} Deltamethrin (Bayer)	(0,5 l) 1000 l^{-1}	

larvae. Male larvae had two unique dark spots near the end of the dorsal side. So the distinction is based on the presence or absence of these dark spots (the male genital system), clearly visible on the dorsal side.

2.4 STATISTICAL ANALYSIS

The statistical procedure for the obtained data was performed with SPSS software. The means between each variable, percentage of healthy fruit at harvest, percentage of healthy fallen fruits and the percentage of total healthy fruits, number and type of diapausing larvae were compared by ANOVA on a rank test, followed by post hoc analysis using Fisher's and Tukey's tests or the Kruskal-Wallis test. A P-value of 0.05 was used to establish statistical differences in all tests.

3 RESULTS AND DISCUSSION

3.1 PERCENTAGE OF HEALTHY FRUITS AT HARVEST

Foliar sprays of glucose have induced a significant increase in the percentages of healthy fruit at harvest compared to the untreated control. On the other hand, fructose generated a percentage of healthy fruits at harvest similar to that of the insecticide. The analysis of variance (Kruskal-Wallis test) ($p < 0.05$) identifies three groups: control (51.18 ± 2.57 %), glucose (78.18 ± 0,80 %), followed by the spraying of fructose and insecticide (82.91 ± 0.52 % and 83.76 ± 0.99 %, respectively) (Figure 1).

3.2 PERCENTAGE OF HEALTHY FALLEN FRUITS

The spraying of glucose and fructose induced a significant increase in the percentages of healthy fallen fruits compared to the untreated control, and their percentages are similar to those of the insecticide. The analysis of variance (ANOVA) followed by the Tukey test ($p < 0.05$) identified two groups: control (41.41 ± 0.79 %), followed by the spraying of glucose, fructose, and insecticide (64.89 ± 2.24 %), 64.87 ± 2.04 % and 62.21 ± 2,55 % respectively (Figure 2).

3.3 PERCENTAGE OF TOTAL HEALTHY FRUITS

Foliar sprays of glucose have increase significantly the percentage of total healthy fruits and fructose generated a percentage of total healthy fruits similar to that of the insecticide. The analysis of variance (ANOVA) followed by the Tukey test ($p < 0.05$) classified the tested treatments in three group: control (47.08 ± 1.48), glucose (75.27 ± 0.85 %), fructose and insecticide (78.73 ± 0.64 %, 80.93 ± 1.01 % respectively (Figure 3).

3.4 COUNTING THE NUMBER OF DIAPAUSING LARVAE

The spraying of glucose and fructose led to a result similar to that of the insecticide. It caused a significant decrease in the number of diapausing larvae compared to the untreated control. The analysis of variance (Kruskal-Wallis test)($p < 0.05$) identified two groups: control (56.92 ± 2.73 %), followed by the spraying of glucose,

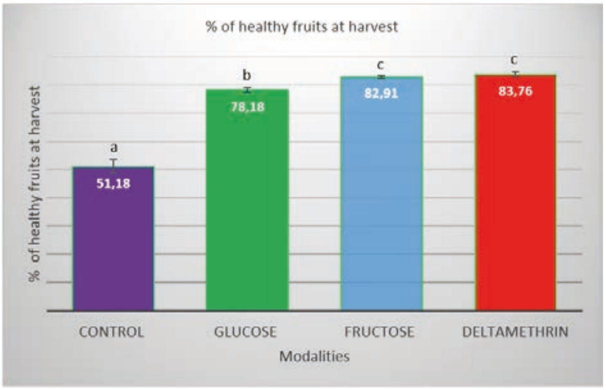

Figure1: Percentage of healthy fruits at harvest in the apple orchard (n = 12) under different modalities (control, fructose, glucose, insecticide). Different letters indicate a significantly different percentage of healthy fruits at harvest ($p < 0.05$).

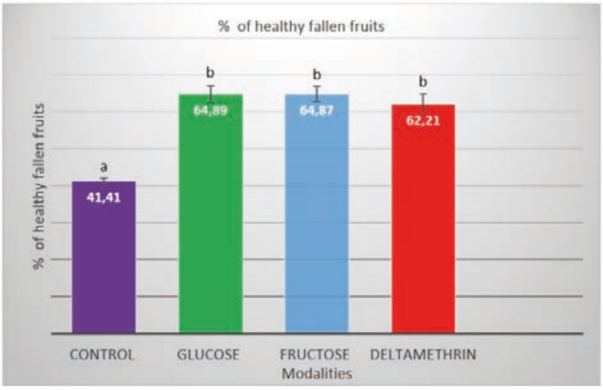

Figure 2: Percentage healthy fallen fruits in the apple orchard (n = 12) under different modalities (control, fructose, glucose, insecticide). Different letters indicate a significantly different percentage of healthy fallen fruits ($p < 0.05$).

fructose, and insecticide (6.17 ± 1.58 %), 10.08 ± 2.58 %, and 4.75 ± 0.82 %, respectively (Figure 4).

3.5 COUNTING THE NUMBER OF MALE AND FEMALE LARVAE AND CHRYSALIS

The number of male and female larvae was not significantly different. Furthermore, the number of chrysalis was significantly lower and different in comparison to the number of male and female larvae. The analysis of variance (Kruskal-Wallis test) (p <0.05) revealed two groups: the number of male and female larvae (7.06 ± 1.15 % and 11.5 ± 2.02 %, respectively), followed by the number of chrysalis, 0.92 ± 0.27 % (Figure 5).

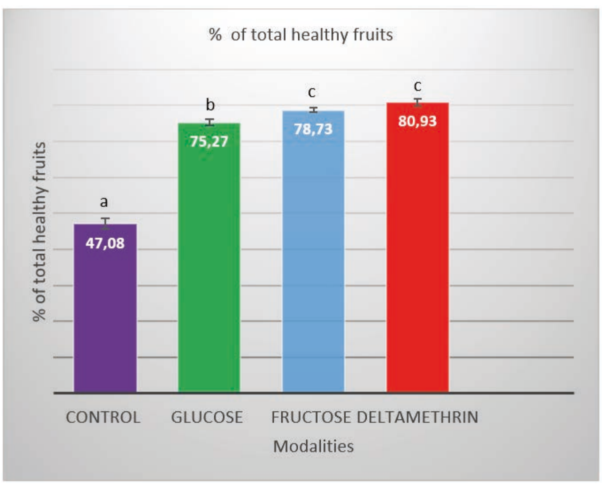

Figure 3: Percentage of total healthy fruits in the apple orchard (n = 12) under different modalities (fructose, glucose, insecticide). .

It is apparent from our findings, that the spray of exogenous foliar application at doses of 100 ppm for glucose and fructose has increased the percentage of healthy fruits and reduced damage compared to the control. These results are partly similar to the results of some previous experiments conducted on 'Starkrimson', 'Golden Delicious', and 'Royal Gala' varieties (Abdesslem, 2016; Arnault et al., 2015 and 2016; Meradi, 2015; Nasri, 2015; Tiffrent and Lombarkia, 2021). As discussed in details in the article of Tiffrent and Lombarkia, (2022b) and their finding that the 'Royal Gala' variety was better suited than the other varieties to the concept of exogenous foliar application at doses of 100 ppm for glucose and fructose, because they have increased the percentage of healthy fruits and reduced the percentage of damaged fruits at harvest (percentage of damaged fruits: 15.27 % and 16.45 %, respectively, compared to the control 76.99 %). The same authors mentioned that the percentage of fallen and damaged fruits at harvest was 31.93 % and 19.16 %, respectively, compared to the control (32.54 %); while, the percentage of healthy fallen fruits was 68,07 % and 80,84 % respectively, compared to the control 67,46 %, also, the spraying of glucose and fructose induced a significant decrease in the number of diapausing larvae compared to the untreated control (glucose 10.08 %, fructose 06.67 %, and control 34.50 %, respectively). Thus, the present study has confirmed the promising results recorded for this variety (Royal Gala).

Meradi (2015) has demonstrated in her study on the 'Starkrimson' variety that that the number of males was more important than that of the female larvae, and that of the chrysalis was lower. Walters et al. (2013) explained that induced resistance is a host response; its expression under field conditions is likely to be influenced by a number of factors, including the environment, genotype, crop

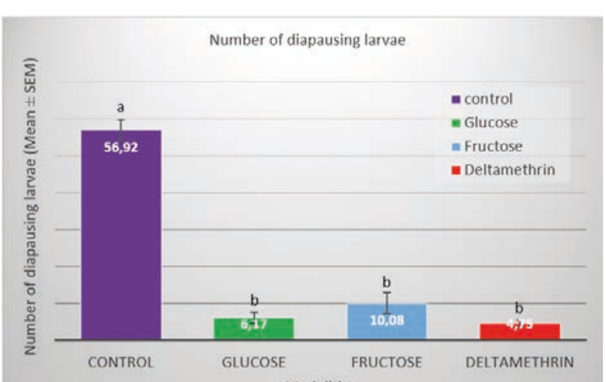

Figure 4: Number of diapausing larvae (n = 12) under different modalities (control, fructose, glucose, insecticide). Different letters indicate a significantly different number of diapausing larvae (p < 0.05).

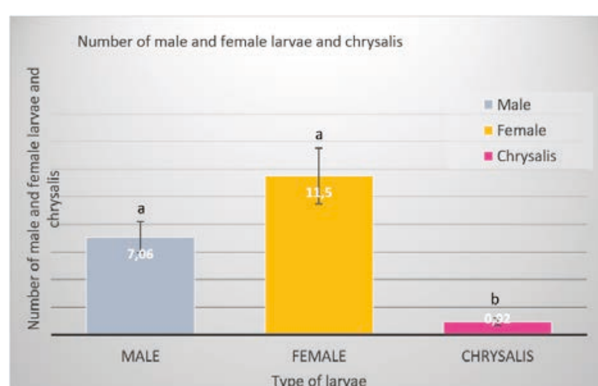

Figure 5: Number of male and female larvae and chrysalis (n = 12) under different modalities (control, fructose, glucose, insecticide). Different letters indicate a significantly different number of diapausing larvae (p < 0.05).

nutrition, and the extent to which plants are already induced.

4 CONCLUSION

In conclusion, our study has shown that codling moth can be effectively managed by using exogenous applications of sugars to reduce damages. However, sugars can induce resistance to *C. pomonella*. These results open up new methods of integrated pest management. In the future, it may be desirable to investigate the effects of foliar application of single sugars on the 'Royal Gala' variety.

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Combined effects of deficit irrigation and biochar application on seed yield and its components in three different sesame varieties grown in sandy soil conditions

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Combined effects of deficit irrigation and biochar application on seed yield and its components in three different sesame varieties grown in sandy soil conditions

Abstract: To answer the question if biochar application was regarded as an effective tool for mitigating the adverse effects of drought stress on sesame production. Thus, two experiments were conducted in the summer season of 2023 and 2024 at Ismailia Agricultural Research Station, Egypt. A randomized complete block design in a split-plot arrangement with three replications was used for each irrigation regime. Biochar application rates were applied to the main plots, while sesame varieties were planted in the sub-plots. In the present study, deficit irrigation with biochar application improved the seed yield and its components significantly in all three sesame varieties. Combination of deficit irrigation with application of biochar proved to be a viable strategy to enhance productivity of sesame under sandy soils. In addition, application of biochar has been able to compensate for the negative deficit irrigation effects, resulting in higher seed yields.

Key words: biochar, seed yield and its components, sesame, varieties, drought stress

Kombinirani učinki uporabe biooglja in deficitnega namakanja na pridelek semena treh sort sezama in njegove komponente, rastočega v peščenih tleh

Izvleček: Raziskava je bila izvedena z namenom, da se ugotovi, če je uporaba biooglja učinkovito sredstvo za preprečevanje negativnih učinkov sušnega stresa na pridelek semena sezama. V ta namen sta bila izvedena dva poskusa v poletnih sezonah 2023 in 2024 na Ismailia Agricultural Research Station, Egypt. Popolni naključni bločni poskus z deljenkami s tremi ponovitvami je bil izveden za vsak način namakanja. Biooglje je bilo dodano na glavnih ploskvah, sorte sezama so bile posejane na podploskvah. Deficitno namakanje in uporaba biooglja sta značilno izboljšala pridelek semena in njegove komponenete pri vseh treh sortah sezama. Kombinacija deficitnega namakanja z uporabo biooglja se je izkazala kot dobra strategija za povečanje pridelka sezama na peščenih tleh. Dodatno je uporaba biooglja kompenzirala negativne učinke deficitnega namakanja, kar je omogočilo večje pridelke semena

Ključne besede: biooglje, pridelek semena in njegove komponenete, sezam, sorte, sušni stres

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1 INTRODUCTION

Sesame seeds are a rich source of essential nutrients, important vitamins, key minerals, and potent antioxidants that all play a very significant role in achieving a balanced diet. In the year 2022, Egyptian sesame seeds reached 48,000 tons harvested from 76,190 feddans, according to a 2022 report by FAO. The consumption of the sesame seeds, therefore, serves to reduce cholesterol levels and further limits inflammation of body organs by improving heart health. Besides, the rich content of calcium in sesame seeds greatly contributes to a strong skeleton and, therefore, is one of the possible healthy additions to a diverse diet. Zubair et al. (2020), Morya et al. (2022), and Sumara et al. (2023) all proved that regular sesame consumption favours cardiovascular and general health.

Drought stress manifested its effect in the form of reduction in biological yield, number of capsule per plant, and harvest index, which resulted in a net fall of 10.26 % in the biological yield of sesame plants. Surprisingly, the seed yield of sesame manifested no significant alteration due to drought stress. Although sesame is considered a drought-resistant crop, cases of drought stress may hamper its growth and reduce seed yield, affecting the components of yield, as found in works by Heidari et al. (2011) and Ebrahimian et al. (2019). Drought stress becomes a crucial factor, which reduces growth, seed yield and yield components in sesame plants. Even though sesame is considered to be relativelly drought-tolerant, under such conditions of the water-scarce regions, it still shows inhibitions in growth and yields, according to Ebrahimian et al. (2019). Also, according to studies by Hailu et al. (2018), large variations in the yield of sesame have been exposed concerning various deficit irrigation levels applied differently. Precisely, the treatments of 50 % ETc with alternate and conventional furrow application methods in both years of 2013 and 2015 had the highest averages. However, the highest yield was obtained from the 100 % ETc treatment under the conventional furrow application method. In another investigation, Qureshi et al. (2023) appraised the performance of 21 accessions collected from 12 different countries and exposed them to two different water-regime treatments, namely non-stressed (NS) and drought stress (DS). The results from the investigation showed a high variation in agronomic characteristics by the accessions. Surprisingly, the yield was the highest under the drought stressed treatment. Also, oil content and oleic acid were recorded at higher levels under the non-stressed treatment, hence giving very interesting findings from this study. Thus, promising genotypes are considered an important component of sustainable agricultural productivity and soil fertility management, especially in arid and semi-arid regions, which are facing the twin challenges of water scarcity and soil degradation. Such as Jabborova et al. (2023) and Ibrahim et al. (2013), biochar is a carbonaceous product produced by pyrolysis from organic materials that can be applied to improve physical and hydraulic conditions in soils for better plant growth with higher yields. Its application could result in savings on water by reducing evaporation losses and improving retention in coarse-textured sandy soils, improving soil quality and productivity with the reduction of irrigation water use without inhibiting crop yield, as suggested by Castellini et al. (2015). However, there was great variation in the impact that biochar had on the hydraulic properties of soils due to the wide variation in source materials, production conditions, soil composition, and experimental conditions in use within individual studies, as elaborated by Mukherjee and Lal (2013). This therefore found for the thorough assessment of the impacts of biochar on clayey soils for full realization of its benefits. Besides, biochar application has been found to increase crop yields, especially in sesame, through retention of water and nutrients in the soil. For this reason, the application of biochar in the case of sandy soil will reduce the problems of limited water by reducing evapotranspiration rates with increased retention of water. Thus, it becomes relevant to examine the potentiality of application of biochar to mitigate the effect of drought stress on seed yield and its attributes in three sesame cultivars that grow under sandy soil conditions. Therefore, the key objective of the present work was to examine if there is any probable impact of applying biochar on the mitigation of drought stress effects on seed yield and its various components using three different varieties of sesame grown under sandy soil conditions.

2 MATERIALS AND METHODS

2.1 SITE DESCRIPTION

Two experiments were carried out at the Ismailia Agricultural Research Station in Egypt during the summer seasons of 2023 and 2024. The geographical coordinates of the station are 30°35’ 41.9” N, 32°16’ 45.8” E, with an elevation of 3 meters above sea level. Prior to the planting phase, soil samples were carefully prepared by air-drying, finely grinding, sieving through a 2 mm sieve, and then stored for further analysis. The soil experiment included a detailed analysis of both physical and chemical parameters, which were documented and presented

Table 1: Physicals and chemicals analyses of experimental sites at 0-30 cm depth of soil.
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Seasons	Available			pH	EC mmh/v	Clay %	Silt %	Fine sand %	Texture
	N	P	K						
2023	10.2	2.11	62	7.33	0.89	3.45	2.58	93.97	Sandy
2024	9.52	2.36	54	7.65	0.95	4.23	2.35	93.42	Sandy

in Table 1 according to Jackson (1973). In both seasons, the crop that was planted earlier was wheat.

2.1.1 Experimental design

For each irrigation regimes *i.e.,* mild (irrigation when 25 % of available soil moisture was depleted (ASMD), T1), moderately (irrigation when 50 % of ASMD, T2) and severe (irrigation when 75 % of ASMD, T3), the experiments were performed in a randomized complete block design (RCBD) using split-plot arrangement with three replications. The main plots received biochar application rates of B0, which represents zero addition and serves as the control; B10, which corresponds to an application rate of 10 (t ha^{-1}); and B20, which represents an application rate of 20 (t ha^{-1}), while sub-plots planted with three different sesame varieties: Shandaweel 3, Giza 32, and Sohag 1, as presented in Figure (1). The experimental unit area was 9 square meters, divided into five ridges with 60 centimeter widths and 3 meters lengths. Sesame varieties that underwent assessment were obtained from the Oil Crops Research Department, Field Crop Research Institute, Agricultural Research Center in Egypt.

2.2 CULTIVATING AND FIELD MANAGEMENT

The sesame varieties evaluated were sown manually on ridge at a spacing of 60 cm between ridges. The distance between hills was according to the variety recommendation. In both seasons, sowing date was on 15th April. Following the recommended guidelines, the sesame seedlings were thinned to maintain a plant density of one or two plants per hill. Besides that, all other agricultural practices and recommendations concerning the crop were carefully followed (Table 2).

2.3 DATA RECORDED

2.3.1 Yield and its component traits

The flowering date was taken for each experimental plot as a number of days taken to have 50 % of the plants within it start flowering. During the harvesting process, a selection of five competitive plants were chosen at random from both the 2nd and 4th ridges for the purpose of assessing seed yield and various attributes including plant height (in cm), fruiting zone length (in cm), number of branches per plant, 1000-seed mass (in grams), and seed mass per plant (in grams). The seed yield per square meter was determined by harvesting plants from central ridge units and converting them to kilograms per feddan.

2.4 STATISTICAL ANALYSIS

The study analyzed the mean values of three sesame varieties affected by different rates of biochar application for all studied traits in the three replications across three irrigation regimes and two seasons using a randomized

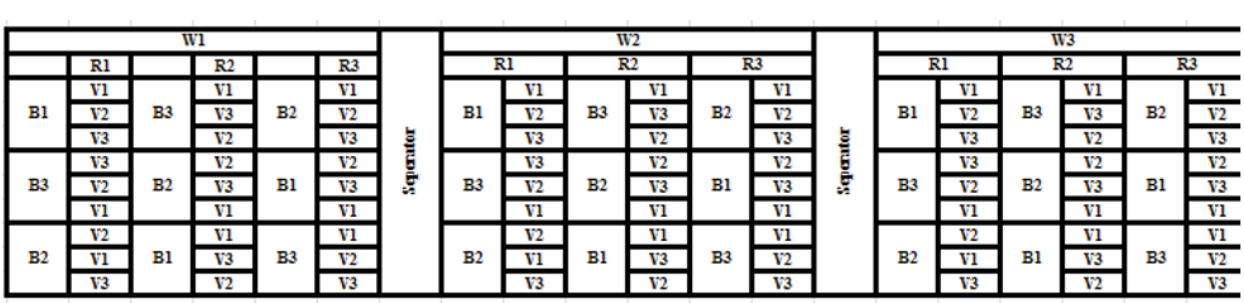

Figure 1: Scheme of Experimental design. W refers to water regimes; B refers to biochar application; V refers to varieties and R refers to replications

Table 2: Dates of planting, tillage, fertilizer, pesticides, and harvesting in the experiments conducted during two sesame seasons

Operation	Date	Date
Planting	15-April	15-April
Tillage application	05-Apr	04-Apr
Fertilizer Application	15 and 30 April	15 and 30 April
Pesticides application	05-May	06-May
Harvesting	01-Sep	01-Sep

complete block design using split-plot arrangement. At each water regime and season, bilateral and trilateral interactive effects were achieved through the combination of field experiments. These effects were further confirmed by the homogeneity of error variance, as stated by Gomez and Gomez (1984). The treatments were assessed by comparing their mean differences using the least significant difference (L.S.D. 5 %), as outlined by Gomez and Gomez (1984).

3 RESULTS AND DISCUSSION

3.1 ANALYSIS OF VARIANCES FOR ALL STUDIED TRAITS

The findings for the various studied traits of the three sesame varieties, which were evaluated in relation to the impact of biochar across different water regimes and seasons, can be found in Table 3. The combined analysis revealed that the main effect of year, as well as its dual and triple interactions with other factors, were found to be statistically insignificant for most studied traits. Consequently, these interactions were excluded from further analysis. In their place, the effects of the main effects of water regimes, biocahar rates, sesame genotypes, and their respective dual and triple interactions were examined. The combined analysis of variance showed that the main effects of water regimes, biochar, and sesame varieties on all the studied traits were highly significant, indicating that the main effect means much in the variation of studied traits. Additionally, for most of the studied traits, the dual interaction of water regimes and biochar rates was highly significant, indicating that the magnitude of the water regime effects varied significantly with varying biochar rate. Similarly, the combined analysis showed a highly significant interaction between water regimes and sesame varieties for all the traits, suggesting that the effect of water regimes on the traits varied significantly across different sesame varieties. In addition, as stated in the combined analysis, the dual influence of biochar and sesame varieties had a significant impact on all the traits that were studied. Similarly, the three-way interaction involving water regimes, biochar rates, and sesame varieties was highly significant for most of the traits, just like in the combined analysis. This meant that the studied water regimes influenced the studied characteristics being greatly dependent on the different biochar application rates, on one hand, and the investigated sesame varieties, on the other.

3.2 MAIN EFFECTS

3.2.1 Water regimes effects

Drought stress has been reported to inversely affect flowering stage of plants concerning 50 % flowering timing (Table 3). However, this treatment regime resulted in a reduction of 3.63 % and 12.89 % in the number of days required for 50 % flowering compared to treatments T2 and T1, respectively. Severe drought stress was found to delay flowering in sesame plants, which bears testimony to proper irrigation management in encouraging all growth parameters of sesame plants. This is further emphasized by the fact that the availability of water dictates the growth and development of these crops. Under water-scarce conditions, sesame plants manipulate the timing of flowering due to a regulation mechanism involved in the gene expression of the flowering pathway. Under drought stress conditions, some of the genes activated during the event of flowering are either overexpressed or suppressed, thereby advancing the onset of flowering. Other plant hormones such as abscisic acid and gibberellins have also been involved in the signaling and induction of early flowering under water-stress conditions. Plant height was considerably influenced by the water regimes. The tallest plants were observed in the well-watered treatment, while the shortest were those under the drought stress treatments (Table 4). However, surprisingly, it has been determined in this study that moderate levels of drought stress may be beneficial to obtain taller plants than by using the control treatment. The drought stress treatment was also responsible for the reduction of plant height by 1.68 % and 8.49 % compared to treatments T2 and T1, respectively. Silva et al. (2016), in their investigation, observed that the plants under the influence of drought stress developed highly reduced height compared to the plants, which had sufficient water supply. The retarded growth of sesame plants results from growth inhibition caused by limited

Table 3: Combined analysis of variance for three sesame varieties as affected by three rates of biochar across three water regimes and two years regarding all studied traits.

S.O.V	df		1st season	2nd season	Combined	1st season	2nd season	Combined
	Individual	Combined	Days to 50 % flowering			Plant height		
Year (Y)		1			83.06**			207.29*
Water regimes (W)	2	4	361.53**	300.48**	331.01**	1358.12**	1170.68**	1264.40**
Reps within W	6	12	0.50	0.42	1.45	1.42	1.95	3.88
Biochar (B)	2	2	159.27**	108.59**	265.19**	3453.58**	2747.33**	6179.11**
Y × B		2			2.67			21.80
W × B	4	8	11.35**	13.85**	12.60**	197.34**	219.39**	208.37**
Pooled error A	12	24	1.47	1.97	1.72	27.09	29.70	28.40
Varieties (V)	2	2	132.09**	71.26**	198.64**	1191.15**	1342.21**	2529.72**
Y × V		2			4.71			3.63
W × V	4	8	11.49**	9.63**	10.56**	113.25**	113.99**	113.62**
B × V	4	4	22.90**	16.91**	38.43**	308.80**	231.74**	533.36**
W × B × V	8	4	23.89**	13.97**	18.93**	101.02**	92.43**	96.72**
Y× B × V		16			1.3765			7.1804
Poled Error B	36	72	0.77	0.88	0.82	14.45	10.30	12.38
S.O.V	Individual	Combined	Fruiting zone length			Number of branches per plant		
Year (Y)		1			64.64**			19.36**
Water regimes (W)	2	4	149.83**	591.73**	370.78**	41.59**	44.90**	43.25**
Reps within W	6	12	0.69	1.27	1.66	0.17	0.70	0.55
Biochar (B)	2	2	2121.76**	1922.92**	4042.13**	12.70**	11.49**	24.02**
Y × B		2			2.54			0.17
W × B	4	8	32.31**	60.55**	46.43**	2.02**	3.31**	2.66**
Pooled error A	12	24	5.33	6.02	5.67	0.27	0.50	0.38
Varieties (V)	2	2	1223.03**	762.67**	1958.59**	107.70**	173.23**	277.06**
Y × V		2			27.12			3.88
W × V	4	8	45.14**	96.33**	70.74**	10.96**	14.66**	12.81**
B × V	4	4	158.49**	252.53**	370.92**	6.63**	6.53**	12.55**
W × B × V	8	4	57.34**	74.93**	66.14**	2.64**	2.96**	2.80**
Y× B × V		16			40.0890			0.6080
Poled Error B	36	72	5.18	5.31	5.25	0.38	0.64	0.51

*, ** Significant at 0.05 and 0.01 probability level, respectively

water availability, which impeded nutrient absorption and metabolic activity. This in turn will result in the ultimate reduction in photosynthesis and nutrient absorption, causing a plant to be short. The increased inhibition of root growth, prioritization towards root growth rather than shoot growth, and reduced cell elongation in shoots also favored the short height of the plants. Also, the plant can make hormones that save water by reducing its loss through transpiration. Water shortage clearly and negatively impacted the length of the fruiting zone of the sesame plants with a reduction of 4.82 % and 8.91 % as compared to T2 and T1, respectively (Table 4). This is in agreement with Abd El-Lattief (2015) findings that showed various irrigation regimes significantly affected plant height, fruiting zone length, branches, capsules, seed mass, and seed and oil yield. This indicates that drought stress adversely affected the fruiting zone length because of reduction in cell division and the rate of elongation contributing to short fruiting zones. This might be explained through resource allocation by the plant, whereby root development and seed production are favored over vegetative growth. Besides this, drought stress could cause hormonal changes which would ultimately result in a retardation of development of a well-thriving fruiting zone. Drought stress had an adverse impact on the number of branches per sesame plant, and it reduced these characteristics substantially compared to T2 and T1 by 43.65 and 108.00 %, respectively (Table 4). This is due to the serious water shortage that reduced water flow and caused an interruption of essential activities such as cell division, elongation, and differentiation. According to Pandey et al. (2021), drought stress interfered with the general growth and development of the plants; this interfered with dry mass and reduced plant yield, as reported by Askari et al. (2018). According to Table 4, drought stress significantly and adversely affected the 1000-seed mass. In comparison to T2 and T1, under drought stress, there was a reduction of 0.79 % and 8.93 %, respectively, in 1000-seed mass. The decrease in 1000-seed mass was caused by the reduced rate of photosynthesis and carbon assimilation due to water stress. Because water was limited, photosynthesis, being an efficient one, was restricted,

Table 3: Continued

S.O.V	df		1st season	2nd season	Combined	1st season	2nd season	Combined
	Individual	Combined		1000-seed mass			Seed mass per plant	
Year (Y)		1			0.99**			29.11**
Water regimes (W)	2	4	0.44**	1.17**	0.81**	2417.69**	2402.02**	2409.86**
Reps within W	6	12	0.02	0.003	0.01	0.98	1.38	1.64
Biochar (B)	2	2	3.67**	2.66**	6.30**	42.84**	53.26**	95.81**
Y × B	0	2			0.04			0.29
W × B	4	8	0.05*	0.08*	0.07**	4.49*	9.21**	6.85**
Pooled error A	12	24	0.01	0.02	0.01	0.84	0.87	0.85
Varieties (V)	2	2	1.73**	0.84**	2.48**	91.86**	79.02**	170.38**
Y × V	0	2			0.08			0.50
W × V	4	8	0.05**	0.14**	0.10**	6.96**	7.82**	7.39**
B × V	4	4	0.11**	0.06*	0.14**	8.40**	6.94**	13.05**
W × B × V	8	4	0.05**	0.15**	0.10**	1.83NS	8.62**	5.22**
Y× B × V	0	16			0.0258			2.2913
Poled Error B	36	72	0.01	0.02	0.01	1.41	0.95	1.18
S.O.V	Individual	Combined		Seed yield per feddan			Seed oil content	
Year (Y)		1			8320.64**			1.71NS
Water regimes (W)	2	4	867786.02**	943556.53**	905671.28**	31.76**	45.29**	38.53**
Reps within W	6	12	56.35	88.91	81.27	0.42	0.59	0.70
Biochar (B)	2	2	12214.47**	17757.93**	29711.77**	107.36**	72.19**	177.46**
Y × B	0	2			260.62			2.08
W × B	4	8	1580.74**	3939.52**	2760.13**	1.82NS	7.30**	4.56**
Pooled error a	12	24	130.64	304.35	217.49	0.67	0.94	0.81
Varieties (V)	2	2	35326.95**	26510.58**	61519.90**	75.69**	49.55**	122.38**
Y × V	0	2			317.63			2.86
W × V	4	8	2668.60**	3344.10**	3006.35**	2.62**	3.26*	2.94**
B × V	4	4	3276.85**	2032.26**	4067.51**	3.51**	7.07**	9.94**
W × B × V	8	4	383.49*	3231.74**	1807.62**	1.29NS	3.72**	2.51**
Y× B × V	0	16			1241.6043			0.6377
Poled Error B	36	72	152.83	121.52	137.17	0.61	0.93	0.77

*, ** Significant at 0.05 and 0.01 probability level, respectively

and hence the seeds were smaller in size and mass. The closing of the stomata restricts the intake of carbon dioxide, hence again affecting the size and mass of the seeds. In addition to that, drought stress disrupted nutrient uptake and transport, adding to the detrimental effects on mass. All these combined to reduce 1000-seed mass in the sesame plants that were exposed to drought stress.

Drought stress significantly and adversely affected seed yield per plant, which was reduced by 45.65 and 164.23 % in comparison with T2 and T1, respectively (Table 4). Such a reduction in seed yield may be due to the harmful influence of drought stress on the metabolism of sesame plants, which affected photosynthesis negatively because of the lack of appropriate water supply. As a result, the seed production and filling reduced, which resulted in reduced flowering and seed setting. Moreover, the drought stress thwarted the photosynthetic rate to lower the capacity of carbon dioxide absorption by the plant for carrying out photosynthesis. Besides, the scanty water further reduced nutrient uptake and its availability, which altogether made the plant inefficient in producing seeds. Drought stress showed the significant negative effect on reducing seed yield per feddan by 48.62 % and 173.12 % compared to T2 and T1, respectively, given in Table 4. This is due to the decline of photosynthesis and nutrient absorption reflected in smaller-sized and weighted seeds. Moreover, drought stress decreases the plant's capability for setting and retaining capsules; this contributed to the reduction of seed yield. Furthermore, the sizes and mass of individual seeds are reduced by drought stress, adding to the overall depression of seed yield per feddan. Water deficiency significantly increased the content of seed oil by 2.16 % and 5.25 % over T2 and T1, respectively (Table 4). This could be due to the action of drought stress on the sesame plants via positive regulation of key genes associated with biosynthesis of oil, lipid biosynthesis, and stress-responsive pathways. Consequently, these genes trigger, and oil production increases in the seed by enhanced accumulation of storage lipid. Further, the stress-responsive pathway increased antioxidant production, improved the expression of the oil biosynthesis genes that enable plants to retain more oil in their seeds to improve yield and quality. Similar results was reported by Abdo and Anton (2009) their study was conducted at Ismailia Agricultural Research Station to study the physiological response of sesame 'Shandaweel-3' to three levels of soil moisture depletion (ASMD): wet (20-25 %), medium (45-50 %), and dry (65-70 %). Results showed that increasing soil moisture stress significantly decreased plant height, and fruiting zone length. Dry treatment reduced 1000-seed mass, number of capsules, seed mass/plant, seed yields/fed and oil contents in seeds. Moreover, Askari, et al (2019) showed that drought stress reduced seed yield and its components.

3.2.2 Biochar application effect

Application of biochar at different levels ranging from zero to 10 up to 20 t ha^{-1} resulted in a gradual increase in all the studied traits, as shown in Table 4. Specifically, when comparing the non-application of biochar, the application of 10 and 20 t ha^{-1} led to significant increases in the following studied traits: days to 50 % flowering by 1.31 % and 7.53 %, plant height by 10.99 % and 13.58 %, fruiting zone length by 13.01 % and 24.26 %, number of branches per plant by 4.88 % and 40.24 %, 1000-seed mass by 8.04 % and 20.73 %, seed mass per plant by 8.02 % and 13.57 %, seed yield per feddan by 7.26 % and 12.70 %, and seed oil content by 3.23 % and 8.54 %, respectively. The above improvement may be attributed to the fact that biochar had a positive effect on soil fertility and nutrient availability in the plants, hence improving growth and development. It improved water and nutrient retention, reduced acidity in the soil by improving microbial activities. This sustainable method, therefore, improved sesame crop yield and quality but also sustained healthier plants with minimal environmental stress. Besides, biochar increased nutrient availability, advanced the plants toward early flower, enhanced plant height, and thus resulted in a higher yield of seeds per

Table 4: The main effect of water regimes on seed yield and its components as combined analysis across the studied sesame varieties, biochar application rates and the two seasons

Traits/ Water regimes	Days to 50 % flowering (N0)	Plant height (cm)	Fruiting zone length (cm)	Number of branches per plant (N0)	1000-seed mass(g)	Seed mass per plant (g)	Seed yield per feddan (kg)	Seed oil content (%)
25 % of ASMD	59.69	165.71	83.69	4.81	3.69	30.40	577.65	42.60
50 % of ASMD	57.59	162.98	79.85	3.35	3.66	20.87	388.68	43.54
75 % of ASMD	52.87	152.74	76.85	2.31	3.39	11.50	211.50	44.96
LSD 0.05	0.52	2.99	1.34	0.35	0.07	0.52	8.28	0.50

ASMD refers to available soil moisture depleted feddan. The favorable impact of biochar on productivity also appeared in the case of sesame plants concerning the 1000-seed mass and the oil content of seeds. Wacal et al. (2019) also announced similar findings while investigating the effect of adding biochar on sesame performance regarding growth and yield, leaf nutrient concentration, seed mineral nutrients, and some soil physicochemical properties. Their results showed that biochar addition increased plant height, yield, and seed number.

3.2.3 Sesame varieties effects

The performance of different sesame varieties varied significantly in terms of all studied traits, as shown

Table 5: The main effect of biochar application rates on seed yield and its components, as combined analysis across the studied sesame varieties, water regimes and the two seasons

Traits/ Biochar application rates	Days to 50 % flowering (N0)	Plant height (cm)	Fruiting zone length (cm)	Number of branches per plant (N0)	1000-seed mass (g)	Seed mass per plant (g)	Seed yield per feddan (kg)	Seed oil content (%)
B 0 (t ha^{-1})	55.09	148.33	71.27	3.04	3.27	19.52	368.12	42.05
B20 (t ha^{-1})	55.81	164.63	80.55	3.19	3.53	21.08	394.84	43.41
B20 (t ha^{-1})	59.24	168.48	88.56	4.26	3.94	22.17	414.88	45.64
LSD 0.05	0.52	2.99	1.34	0.35	0.07	0.52	8.28	0.50

Table 6: Agronomic performance of tested sesame varieties, as combined analysis across biochar application rates, water regimes and the two seasons

Traits/ Sesame varieties	Days to 50% flowering (N0)	Plant height (cm)	Fruiting zone length (cm)	Number of branches per plant (N0)	1000-seed mass (g)	Seed mass per plant (g)	Seed yield per feddan (kg)	Seed oil content (%)
Shandaweel 3	58.15	165.98	86.11	0.89	3.82	22.74	425.93	45.30
Giza 32	57.46	162.64	80.21	5.00	3.52	20.85	393.47	43.48
Sohag 1	54.54	152.81	74.06	4.59	3.40	19.19	358.44	42.32
LSD 0.05	0.35	1.91	1.24	0.39	0.06	0.59	6.35	0.48

Table 7: The dual interactive of water regimes with biochar application rates on seed yield and its components, as combined analysis across the studied sesame varieties, and the two seasons

Interaction	Traits	Days to 50% flowering (N0)	Plant height (cm)	Fruiting zone length (cm)	Number of branches per plant (N0)	1000-seed mass (g)	Seed mass per plant (g)	Seed yield per feddan (kg)	Seed oil content (%)
	B 0 (ton ha^{-1})	57.39	153.84	74.14	4.06	3.30	28.09	534.31	40.64
25 % of ASMD	B20 (ton ha^{-1})	58.89	166.99	82.48	4.22	3.71	30.71	583.69	42.04
	B20 (ton ha^{-1})	62.78	176.31	94.45	6.17	4.06	32.39	614.97	45.12
	B 0 (ton ha^{-1})	55.67	148.45	72.14	2.89	3.34	19.76	370.84	42.02
50 % of ASMD	B20 (ton ha^{-1})	57.83	166.09	81.66	3.00	3.62	20.64	384.09	43.03
	B20 (ton ha^{-1})	59.28	174.39	85.74	4.17	4.02	22.20	411.11	45.58
	B 0 (ton ha^{-1})	52.22	142.69	67.54	2.17	3.16	10.71	199.22	43.50
75 % of ASMD	B20 (ton ha^{-1})	50.72	160.80	77.50	2.33	3.26	11.90	216.73	45.16
	B20 (ton ha^{-1})	55.67	154.73	85.50	2.44	3.75	11.91	218.56	46.23
LSD0.05		0.90	5.18	2.32	0.60	0.11	0.90	14.35	0.87

ASMD refers to available soil moisture depleted, and B refers to biochar rates in Table 6. Among these varieties, 'Sohag1' had the shortest period to 50 % flowering, with a duration of 54.54 days, compared to the other varieties. 'Giza 32' and 'Shandaweel 3' demonstrated superiority over 'Sohag 1' in plant height by 8.62 % and 6.43 %, respectively. In addition, 'Shandaweel 3' and 'Giza 32' surpassed 'Sohag 1' in all the other studied traits, viz., length of fruiting zone, mass of 1000 seeds, mass of seed per plant, yield per feddan, and seed oil content. The percentage difference between the best and 'Sohag 1' varied between 3.58 % and 18.83 %. However, 'Shandaweel 3' had the lowest number of branches per plant in the comparison to others. Probably, differences in means for seed yield and its components that exist in sesame varieties Shandaweel 3, Giza 32, and Sohag1 may be described by diversity in the genetic structure of these cultivars. In other words, the genetic features associated with the variety would be major factors

Table 8: The dual interactive of water regimes with studied sesame varieties on seed yield and its components, as combined analysis across biochar application rates, and two seasons

Interaction	Traits	Days to 50% flowering (N0)	Plant height (cm)	Fruiting zone length (cm)	Number of branches per plant (N0)	1000-seed mass (g)	Seed mass per plant (g)	Seed yield per feddan (kg)	Seed oil content (%)
25 % of ASMD	Shandaweel 3	61.94	169.25	87.60	0.94	3.95	32.27	611.52	44.44
	Giza 32	59.22	170.14	86.34	6.78	3.62	30.93	592.43	42.39
	Sohag 1	57.89	157.75	77.13	6.72	3.50	27.99	529.01	40.97
50 % of ASMD	Shandaweel 3	58.06	166.72	88.56	0.89	3.89	22.99	429.01	45.20
	Giza 32	59.44	167.23	77.82	4.33	3.69	20.81	387.54	43.61
	Sohag 1	55.28	154.98	73.17	4.83	3.40	18.81	349.50	41.82
75 % of ASMD	Shandaweel 3	54.44	161.96	82.17	0.83	3.61	12.95	237.26	46.27
	Giza 32	53.72	150.55	76.48	3.89	3.26	10.80	200.44	44.46
	Sohag 1	50.44	145.71	71.89	2.22	3.30	10.77	196.81	44.16
LSD0.05		0.60	3.31	2.15	0.67	0.11	1.02	11.01	0.83

ASMD refers to available soil moisture depleted

Table 9: The dual interactive of biochar application rates with studied sesame varieties on seed yield and its components, as combined analysis across water regimes, and two seasons

Interaction	Traits	Days to 50 % flowering (N0)	Plant height (cm)	Fruiting zone length (cm)	Number of branches per plant (N0)	1000-seed mass (g)	Seed mass per plant (g)	Seed yield per feddan (kg)	Seed oil content (%)
B 0 (t ha^{-1})	Shandaweel 3	55.06	153.05	80.09	0.89	3.47	20.82	395.65	43.59
	Giza 32	56.06	148.72	68.77	4.61	3.25	18.89	355.88	41.66
	Sohag 1	54.17	143.21	64.96	3.61	3.08	18.85	352.83	40.90
B20 (t ha^{-1})	Shandaweel 3	57.11	169.89	85.59	0.83	3.69	22.89	427.26	45.78
	Giza 32	56.50	162.94	85.55	5.00	3.51	21.22	401.99	43.24
	Sohag 1	53.83	161.06	70.51	3.72	3.39	19.14	355.26	41.21
B20 (t ha^{-1})	Shandaweel 3	62.28	175.00	92.65	0.94	4.29	24.50	454.87	46.54
	Giza 32	59.83	176.26	86.31	5.39	3.81	22.43	422.54	45.55
	Sohag 1	55.61	154.17	86.72	6.44	3.73	19.58	367.23	44.84
LSD0.05		0.60	3.31	2.15	0.67	0.11	1.02	11.01	0.83

B refers to biochar rates

that will determine the varieties' overall productivity regarding flowering time, the number of capsules per plant, and size of seeds. Nevertheless, it is important to emphasize that environmental conditions and farming practices have a contribution to making the final seed yield. Among them, 'Shandaweel 3' showed higher productivity due to an efficient system of nutrient absorption and transfer inside the plant. It might be because of the breeding selection and genetic modification done through the years to focus on these traits. This could encourage farmers to buy seeds like 'Shandaweel 3', as they end up with a far more rewarding harvest than with other varieties, such as Giza 32 and Sohag 1. Its success with 'Shandaweel 3' in terms of yield and quality of the seeds proved that research and development was truly significant in enhancing crop varieties toward sustainable agriculture. The same trends were reported by Abdelsatar et al. (2021), who recorded significant differences in the performance of three sesame varieties, Giza 32, Sohag 1, and Shandaweel 3, based on seed yield and its components.

Table 10: Effect of triple interaction among water regimes, biochar application rates and sesame varieties on all studied traits as combined analysis across two seasons

Interaction	Traits		Days to 50% flowering (day)	Plant height (cm)	Fruiting zone length (cm)	Number of branches per plant	1000-seed mass (g)	Seed mass per plant (g)	Seed yield per feddan (kg)	Seed oil content (%)
25 % of ASMD	B 0 (ton ha^{-1})	Shandaweel 3	57.33	158.10	80.04	0.83	3.45	28.74	548.44	42.04
		Giza 32	58.00	152.44	74.37	5.50	3.24	29.58	561.69	40.39
		Sohag 1	56.83	150.97	68.02	5.83	3.22	25.94	492.79	39.50
	B20 (ton ha^{-1})	Shandaweel 3	61.00	171.46	82.35	0.83	4.06	32.65	622.05	44.28
		Giza 32	58.50	168.58	91.16	7.33	3.62	30.90	594.79	41.76
		Sohag 1	57.17	160.94	73.94	4.50	3.47	28.59	534.23	40.10
	B20 (ton ha^{-1})	Shandaweel 3	67.50	178.19	100.40	1.17	4.36	35.43	664.07	47.02
		Giza 32	61.17	189.40	93.50	7.50	4.00	32.32	620.81	45.02
		Sohag 1	59.67	161.34	89.45	9.83	3.82	29.44	560.02	43.32
50 % of ASMD	B 0 (ton ha^{-1})	Shandaweel 3	55.83	152.82	82.17	0.67	3.52	21.79	413.76	43.50
		Giza 32	56.33	148.71	70.09	4.83	3.45	18.32	338.82	42.16
		Sohag 1	54.83	143.82	64.16	3.17	3.06	19.19	359.95	40.39
	B20 (ton ha^{-1})	Shandaweel 3	57.67	170.19	91.17	0.83	3.79	22.98	423.70	45.27
		Giza 32	62.33	164.11	82.32	3.50	3.58	20.68	389.16	43.06
		Sohag 1	53.50	163.98	71.50	4.67	3.49	18.26	339.41	40.76
	B20 (t ha^{-1})	Shandaweel 3	60.67	177.17	92.33	1.17	4.37	24.21	449.55	46.84
		Giza 32	59.67	188.87	81.04	4.67	4.05	23.43	434.64	45.61
		Sohag 1	57.50	157.14	83.84	6.67	3.65	18.97	349.13	44.30
75 % of ASMD	B 0 (t ha^{-1})	Shandaweel 3	52.00	148.24	78.05	1.17	3.44	11.94	224.76	45.24
		Giza 32	53.83	145.00	61.86	3.50	3.05	8.77	167.13	42.43
		Sohag 1	50.83	134.83	62.71	1.83	2.97	11.42	205.77	42.82
	B20 (t ha^{-1})	Shandaweel 3	52.67	168.00	83.25	0.83	3.23	13.05	236.03	47.80
		Giza 32	48.67	156.14	83.16	4.17	3.35	12.09	222.02	44.91
		Sohag 1	50.83	158.26	66.09	2.00	3.20	10.56	192.13	42.76
	B20 (t ha^{-1})	Shandaweel 3	58.67	169.65	85.22	0.50	4.15	13.86	250.99	45.76
		Giza 32	58.67	150.51	84.41	4.00	3.37	11.53	212.17	46.03
		Sohag 1	49.67	144.04	86.88	2.83	3.73	10.33	192.53	46.89
LSD 0.05			1.04	5.73	3.73	1.17	0.19	1.77	19.06	1.43

ASMD refers to available soil moisture depleted, and B refers to biochar rates

3.3 DUAL AND TRIPLE INTERACTIONS EFFECTS ON STUDIED TRAITS

The combined effect of water regimes and biochar application significantly impacted the performance of the traits under study (Table 3 and 6). The highest values for these traits were recorded when biochar was applied at a rate of 20 t ha^{-1} under normal irrigation conditions. Also, increased application rates of biochar under the different water regimes continued to improve the performance of the studied traits in the betterment in plants' ability to tolerate the water stress conditions studied. As evident from Table 3 and 8, the interactions of dual water regimes significantly influenced various investigated trait performances in the tested varieties. Maximum values for most of the investigated traits were recorded when 'Shandaweel 3' was planted under a normal irrigation conditions. Unexpectedly, the best performance was shown by 'Shandaweel 3' variety when sown under both moderate and severe levels of water stress over other varieties. This may partly be due to the great capability of its adaptation to unfavorable conditions such as drought stress. Numerous studies had demonstrated that the growth of the shoot system could be influenced either positively or negatively by changes in water scarcity, soil type, and plant species. Decrease in plant growth under high drought stress levels may be due to inhibition in hydrolysis of reserved food and its translocation to the growing shoots.

It showed from the research that there was a significant and positive interaction between biochar application and sesame varieties in all the studied traits reflected from Table 3 and 9. Maximum values of most of these traits were exhibited while using Shandaweel 3 variety followed by Giza 32 and Sohag 1 varieties. Additionally, these positive effects were observed with increasing levels of biochar application, up to 20 tons per hectare.

In addition, the combination of water regimes and biochar application with different sesame genotypes had a significant impact on all studied traits (Table 3 and 10). This presents an opportunity to identify the most favorable triple interactions for these traits. Based on this perspective, the most favorable triple interaction was observed when 'Shandaweel 3' was sown under normal irrigation and biochar was applied at a rate of 20 tons per hectare. Furthermore, 'Shandaweel 3' outperformed other varieties, even when it was sown under different water regimes with the highest application rate of biochar at 20 tons per hectare.

4 CONCLUSION

It can be concluded that investigated deficit irrigation and application of biochar on seed yield and its components in three diverse varieties of sesame grown under a sandy soil environment, thus providing realistic overviews of the methods to be used for sustainable agricultural practices that would enable farmers to have maximum yields in water-scarce regions. 'Shandaweel 3' was more tolerant and adaptable to a wide range of environmental conditions compared to the other varieties. The results of this study clearly indicate the potentiality for a positive role of biochar treatment in agricultural practice, both to improve the performance in crops and to enhance tolerance against drought stress. 'Shandaweel 3' demonstrated the most favorable triple interaction when sown with a biochar rate of 20 tons per hectare in water-limiting environments. More studies are recommended in order to explain, in full, the mechanisms responsible for such improvements and, secondly, to optimize biochar application rates in view of crop varieties. In a nutshell, the results obtained from this study provided meaningful insight into how farmers and practitioners may work to improve sesame productivity in a water-limiting environment.

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Functional analysis of drought tolerance QTLs in two barley populations using BLAST on associated SNP sequences

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Functional analysis of drought tolerance QTLs in two barley populations using BLAST on associated SNP sequences

Abstract: This study explored the relationship between QTLs associated with drought tolerance and functional SNPs using two populations: Vada × Susptrit (V × S) and Cebada Cappa × Susptrit (C. Cappa × S). Bioinformatics tools were employed to analyze significant SNPs within QTL regions, revealing markers on chromosomes 1H, 2H, 3H, 5H, and 7H for the V × S population, and on 4H and 6H for C. Cappa × S. In total, 24 proteins/enzymes related to drought tolerance were characterized in the V × S population, while 10 were identified in C. Cappa × S. Notable proteins, including phytochrome B and SAPK7, were located on chromosome 4H. The identified proteins are integral to the plant's adaptive response to drought stress, mediating essential regulatory mechanisms that enhance resilience. Gene ontology analysis delineated four primary cellular components—membrane, nucleus, chloroplast, and proteasome complex—linked to drought resistance pathways. Additionally, five critical biological processes, including oxidation-reduction and protein phosphorylation, were identified as pivotal in these adaptive responses. This comprehensive understanding underscores the potential application of these proteins in breeding strategies aimed at developing drought-tolerant barley cultivars. Overall, the study highlights potential functional SNP markers for validating QTLs related to drought tolerance in barley.

Key words: barley, bioinformatics, gene ontology, QTL validation, single nucleotide polymorphism

Funkcionalna analiza odpornosti na sušo (QTL) v dveh populacijah ječmena z uporabo BLAST analize na povezanih SNP zaporedjih

Izvleček: Raziskava preučuje razmerje med s toleranco na sušo povezanimi zaporedji (QTL) in funkcionalnimi SNP mesti v dveh populacijah ječmena: Vada × Susptrit (V × S) in Cebada Cappa × Susptrit (C. Cappa × S). Bioinformacijska orodja so bila uporabljena za analizo pomembnih SNP znotraj QTL območij za odkritje markerjev na kromosomih 1H, 2H, 3H, 5H in 7H za V × S populacijo in na kromosomih 4H in 6H za C. Cappa × S populacijo. Celokupno je bilo opisanih 24 proteinov/encimov, povezanih s tolerance na sušo v V × S populaciji med tem, ko jih je bilo v populaciji C. Cappa × S deset. Pomembni proteini, ki vključujejo fitokrom B in serein treonin kinazo (SAPK7) so bili locirani na kromosomu 4H. Ti proteini so sestavni del rastlinskega prilagoditvenega odziva na sušni stres, ki je bistven za sprožitev mehanizmov uravnavanja procesov, ki povečuje odpornost. Analiza delovanja genov je odkrila štiri glavne celične oddelke, ki so povezani s procesi odpornosti na sušo in sicer celično membrano, celično jedro, kloroplast in proteasom. Dodatno je bilo v tem prilagoditvenem odzivu prepoznanih pet ključnih bioloških procesov, ki so med drugimi obsegali oksidacijo, redukcijo in fosforilacijo proteinov. Takšno celostno razumevanje poudarja pomen potencilane uporabe teh proteinov v strategijah žlahtnenja z namenom vzgojiti na sušo toleratne sorte ječmena. Raziskava osvetljuje uporabo potencialnih funkcionalnih SNP markerjev za ovrednotenje QTL povezanih s toleranco na sušo pri ječmenu.

Ključne besede: ječmen, bioinformatika, genska ontologija, ovrednotenje QTLs, SNP (polimorfizem posameznih nukleotidov)

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1 INTRODUCTION

Drought stress is an unavoidable factor present in various environments, affecting plant biomass production, quality, and energy without regard for borders or providing clear warnings. Its pervasive nature hampers agricultural productivity by challenging plants' ability to thrive under water-limiting conditions.(Seleiman, 2021). Understanding the complex regulatory pathways guarantees in-depth consideration of a biological system. These challenges are closely related to informatics in biology, in other words "bioinformatics". The field of multi-omics has witnessed unprecedented growth, converging multiple scientific disciplines and technological advances. This surge is evidenced by a more than doubling in multi-omics scientific publications within just two years (2022–2023) since its first referenced mention in 2002, as indexed by the National Library of Medicine. (Moher et al., 2024). Bioinformatics actually manages the data collected through various techniques -omics, including genomics, transcriptomics, proteomics and metabolomics. Systems Bioinformatics is the framework in which systems approaches are applied to such data (oulas et al.,, 2017). Access to plant genome sequencing technology, the development of mapping populations, genetic diversity, and molecular markers with wide genomic coverage has led researchers to accelerate the identification of important QTLs (quantitative trait locus) and their responsible

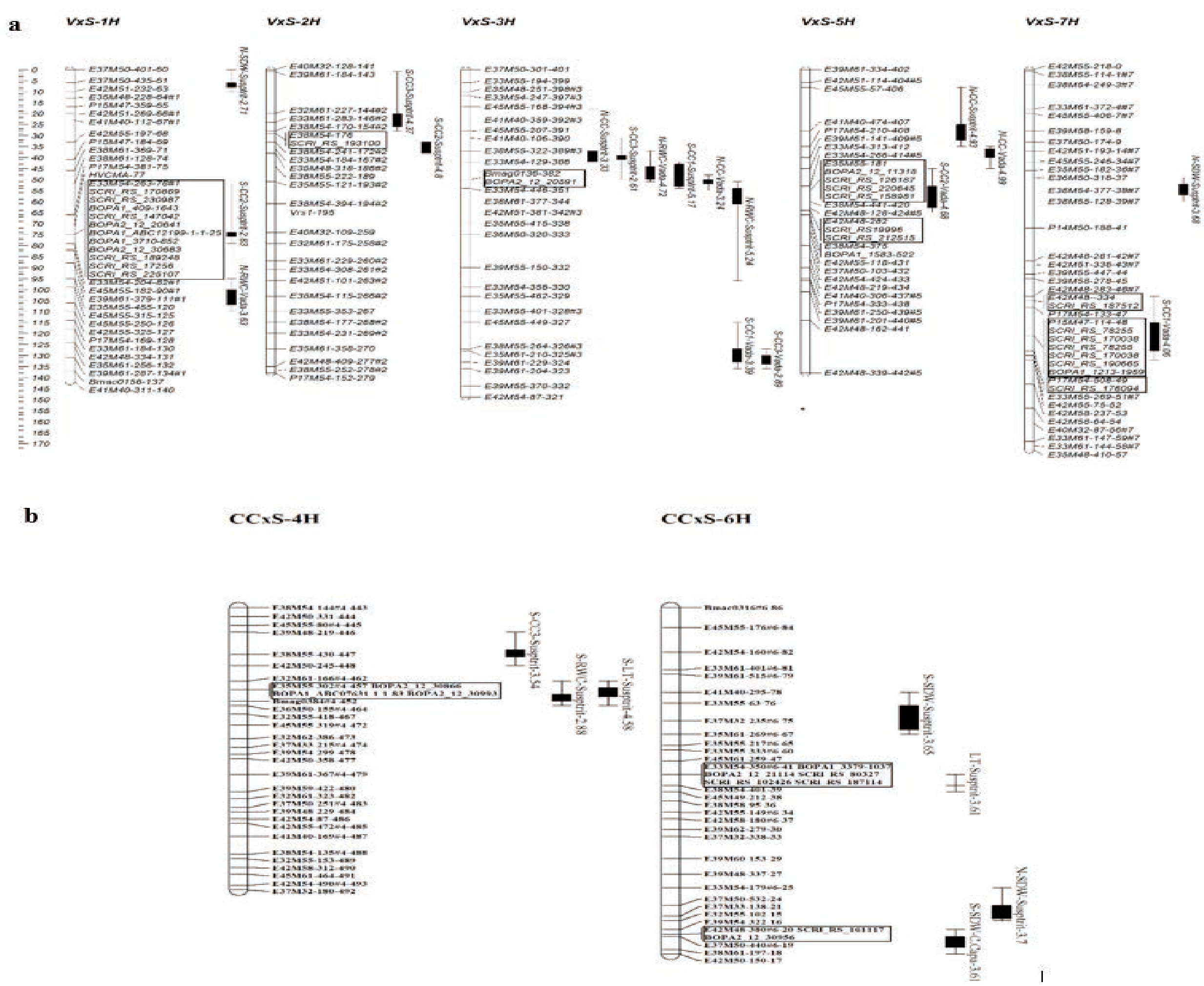


Figure 1: QTLs related to physiological traits under drought stress and normal irrigation in barley populations V × S (a) and C. Capa × S (b) and SNP markers associated with AFLP/SSR markers related to the QTLs. AFLP/SSR markers and the associated SNP markers are shown inside the rectangle. The letters S and N indicate drought and normal irrigation conditions, respectively. The numbers in the QTL area indicate the maximum LOD. Bold areas have significant LOD. CC1, CC2 and CC3 indicate chlorophyll content at 50 %, 30 %, and 20 % of field capacity, respectively. FC: field capacity; RWC: Relative leaf water content and SDM: Shoot dry mass and LT: Leaf temperature.

genes (Kurakawa et al., 2007). Among recent advances in -omics technology, the emergence of high-efficiency methods for genomic sequencing and high-saturation genotyping using DNA markers such as single nucleotide polymorphism (SNP), has proven to be very effective (Kuromori et al., 2009). Including the bioinformatics implements, are Sequence Analysis and Similarity Searching Tools. In bioinformatics, sequence alignment is a method of arranging DNA, RNA, or protein sequences to identify "similar regions" that can reveal functional, structural, or evolutionary relationships between sequences (Stormo, 2000). In the aligning method, there are strong evidences that two similar sequences have the same nucleotids (or identical amino acids). Due to the large number of gaps, multiple alignments can occur between the two sequences (Vassilev et al., 2005). Dynamic algorithms identify the optimal alignment. In the BLAST method, statistical techniques are used to assess the probability of a specific alignment between two sequences (Neumann et al., 2014). Alignments are widely used in bioinformatics to identify sequence similarity, prepare phylogenetic trees and homology models of protein structures (Dubey et al., 2010). The NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/), is the most popular tool to search for align sequences (Altschul et al., 1990). Bioinformatics method has been used in many studies to identify or predict the proteins or enzymes involved in the response of different plants to drought stress (Faghani et al., 2015; Landi et al., 2017; Neumann et al., 2014; Shaar-Moshe et al., 2015; Wehner et al., 2015). Barley, scientifically known as Hordeum vulgare L., ranks fourth in cereals in terms of production. The ability to grow barley in harsh and low-yield environments is higher than other cereals, and this crop is best adapted to environmental stresses such as drought, salinity and cold (Jogaiah et al., 2013). The present study was performed to BLAST analysis on SNP sequences have significant correlation with QTL regions related to drought tolerance identified in our previous study (Mohammadi et al., 2018) as well as prediction of related genes and proteins/enzymes and their ontology study.

2 MATERIALS AND METHODS

In our previous study (Mohammadi et al., 2018), drought tolerance chromosomal regions in seedling stage were identified and reported in two barley populations: 'Vada' ×' Susptrit' (V × S) and 'Cebada Cappa' × 'Susptrit' (C. Cappa × S). BLAST analysis was performed on SNP sequences have significant correlations with some AFLP/SSR markers associated with QTLs related to physiological traits under drought conditions. SNP markers were developed in the University of Wagningen, Netherlands. The QTLs and their associated SNP markers are shown in Figure 1. In order to study of functional genomics, BLAST analysis was conducted against the NCBI non redundant (nr) nucleotide collection (www.ncbi.nlm.nih.gov). The UNIPROT database (www.uniprot.org) was used to predict proteins and their function. Gene ontology information was also extracted through the European Bioinformatics Institute website (EBI = www.ebi.ac.uk). In order to plot three types of ontologies, including cellular components, molecular functions, and biological processes, EXCEL software was used. Bioinformatics pipeline, is shown in Figure 2.

3 RESULTS AND DISCUSSION

3.1 BLAST ANALYSIS SNPS ASSOCIATED TO QTL REGIONS IDENTIFIED IN V × S POPULATION

Summary of BLAST results SNP sequences with significant correlations with QTL regions identified in the V × S population are shown in Table 1. In this population, a total of 24 types of proteins / enzymes related to QTL regions were identified whose sequences of genes encoding them had very low FDR (near zero), which indicates

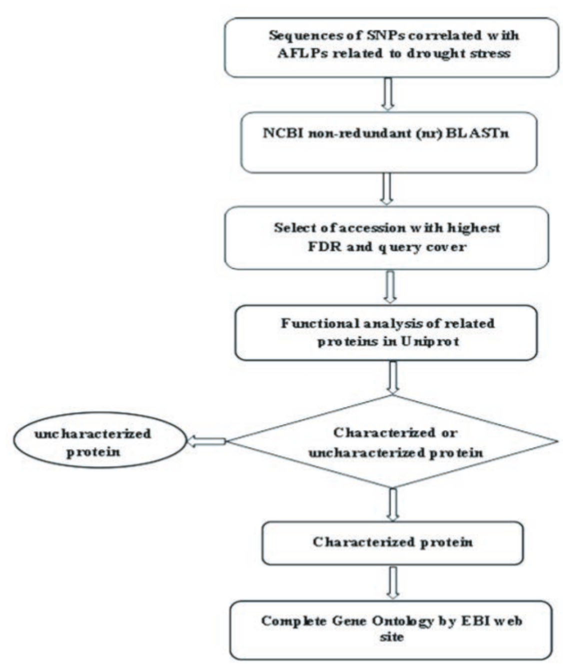


Figure 2: Bioinformatics pipeline used in this work

a very high similarity between SNP sequences and their alignments. These genes were identified on chromosomes 1H, 2H, 3H, 5H and 7H. On chromosome 1H, proteins/enzymes glycosyltransferases, protein dehydration-induced 19 homolog 5, dihydrolipoyl dehydrogenase, hexosyltransferase, salt tolerant protein-GSK-like kinase, peroxidase, glycine-rich RNA-binding protein RZ1B, mitogen-activated protein kinase, pectinesterase, cinnamoyl-CoA reductase-like SNL6, ribosomal protein, were identified. Two proteins/enzymes include S-adenosylmethionine decarboxylase proenzyme and ATP-dependent Clp protease proteolytic subunit, were predicted on chromosomes 2H and 3H, respectively. On chromosome 5H: pectinesterase, protoporphyrinogen oxidase, beta-carotene hydroxylase, zinc finger A20 and AN1 domain-containing stress-associated protein 1, chlorophyll a-b binding protein, probable anion transporter 5; and on chromosome 7H: proteasome subunit beta type, lactoylglutathione lyase, xyloglucan endotransglucosylase/hydrolase, phosphoglycerate kinase, D-3-phosphoglycerate dehydrogenase were identified. Pectin esterase and zinc finger proteins play crucial roles in plant responses to drought stress. Identifying these proteins can aid in developing new strategies for enhancing drought tolerance in barley and other crops. Given their involvement in key processes like water regulation and plant metabolism, a deeper understanding of their functions could assist farmers in improving crop yields under challenging environmental conditions, ultimately contributing to food security. Pectin esterase was detected on chromosomes 1H and 5H and also zinc finger A20 and AN1 domain-containing stress-associated protein 1 was identified in two regions on chromosome 5H. Based on BLAST results, it was observed that most genes were found in barley (Hordeum vulgare subsp. vulgare Spenn.) (Table 1). A summary of the results of former studies on the effect of identified proteins/enzymes on drought tolerance in different plants is given in Table 2. A review of the relationship between the identified proteins/enzymes and drought stress in different studies showed well, that all the proteins/enzymes identified in the present study, were directly involved in the drought stress response in previous studies conducted on different plants. The results confirm the QTLs reported in our previous study (Nadarajah and Sidek, 2010). In most studies, increase or induction of identified proteins, enhanced drought stress tolerance (Table 2). Rollins (2012) studied effect of heat stress in barley, that two dihydrolipoyl dehydrogenase proteins named F2E5U7 and F2E2T3 have been reported, which one of them, F2E5U7, was also identified in present study. As a result of BLAST on SNP marker named “BOPA2_12_20641” located on chromosome 1H correlated with AFLP marker E33M54-263, a glycogen synthase kinase enzyme called Q8LK43, which is the same protein reported by Talami et al. (2007) (Table 2). Qin et al. applied drought stress in rice and Arabidopsis and studied BLAST on sequence of genes related to chlorophyll a/b binding proteins in barley genomic database, that 17 genes expressing chlorophyll a/b binding proteins were reported. In this study, we identified MLOC_44755, which corresponds to the protein F2CRC1, through BLAST analysis of SNP “BOPA1_1583-522” located on chromosome 5. The identification of this protein suggests its potential role in biological processes related to the SNP. Understanding the functional implications of MLOC_44755 can provide insights into its contribution to phenotypic traits or disease susceptibility. Further exploration of this relationship may reveal important connections that enhance our understanding of the underlying genetic mechanisms involved (Table 1).

3.2 BLAST ANALYSIS OF CORRELATED SNP SEQUENCES OR QTL REGIONS IDENTIFIED IN C.CAPA × S POPULATION

Summary of BLAST analysis on SNP sequences have significant correlations with QTL regions identified in C.Cappa × S population are shown in Table 2. A total of 10 proteins/enzymes associated with QTL regions were identified, and their coding gene sequences exhibited a very low false discovery rate (near zero). This indicates a high level of similarity between the SNP sequences and the identified alignments. In other words, these findings suggest a strong and meaningful connection between the SNPs and the identified proteins. These genes were identified on chromosomes 4 and 6. On chromosome 4, proteins/enzymes phytochrome B, serine/threonine-protein kinase SAPK7, o-methyltransferase were identified. Proteasome subunit alpha type, nuclear cap-binding protein subunit 2, 3-ketoacyl-CoA synthase, heat shock protein 16.9C, calcium-dependent protein kinase 4, beta-carotene hydroxylase and HGWP repeats were detected on chromosome 6H.

3.3 REVIEW OF PREVIOUS STUDIES ON IDENTIFIED PROTEINS / ENZYMES AND THEIR RELATIONSHIP WITH DROUGHT STRESS IN DIFFERENT PLANTS

The results of this section in V × S and C. Capa × S populations are given in Tables 3 and 4, respectively. As can be seen, in both populations direct relationship of all

Table 1: Briefing of BLASTn analysis on SNP sequences having significant correlation with AFLP/SSR markers linked to QTLs related to drought stress in V × S population. Note. SNP: Single Nucleotide Sequence; AFLP: Amplified Fragment Length Polymorphism; SSR: Single Sequence Repeat; FDR: False Discovery Rate

Chromosome number, AFLP marker	SNPs correlated to associated AFLP marker	Correlation rate between SNPs and AFLPs	Accession	Species	FDR	Query cover	Gene	Uniprot name	Protein
1H,E33M54-263	SCRI_RS_170869	0.88435955	AK250861.1	*Hordeum vulgare* subsp. *vulgare*	2.00E-51	100 %	pglcat4	Q7XHJ7	Glycosyltransferases
	SCRI_RS_230987	0.90629731	AP014957.1	*Oryza sativa japonica*	4.00E-15	82 %	DI19-2	Q5JME8	Protein DEHYDRATION-INDUCED 19 homolog 5
	BOPA1_409-1643	0.92889292	AK371521.1	*Hordeum vulgare* subsp. *vulgare*	3.00E-116	100 %	N/A	F2E5U7	Dihydrolipoyl dehydrogenase
	SCRI_RS_147042	0.928893	AK362608.1	*Hordeum vulgare* subsp.. *vulgare*	7.00E-50	100 %	N/A	F2DFE1	Hexosyltransferase
	BOPA2_12_20641	0.9043956	AF525086.1	*Triticum aestivum (bread wheat)*	1.00E-40	89 %	N/A	Q8LK43 AAM77397	=salt tolerant protein-GSK-like kinase
	BOPA1_ABC12199-1-1-25	0.9043956	XM_006654903.2	*Oryza brachyantha*	4.00E-28	100 %	N/A	J3M3R4	Peroxidase
	BOPA1_3710-852	0.88129947	AK250786.1	*Arabidopsis thaliana (Mouse-ear cress)*			RZ1B	O22703	Glycine-rich RNA-binding protein RZ1B
	BOPA2_12_30683	0.88129947	AK356908.1	*Hordeum vulgare* subsp.. *vulgare*	2.00E-51	100 %	N/A	F2CZ52	Mitogen-activated protein kinase
	SCRI_RS_189248	0.83214722	AK371220.1	*Hordeum vulgare* subsp.. *vulgare*	2E-51	100%	N/A	F2E4Z6	Pectinesterase
	SCRI_RS_17256	0.80792947	XM_020338974.1	*Oryza sativa subsp. japonica (Rice)*			SNL6	Q0JKZ0	Cinnamoyl-CoA reductase-like SNL6
	SCRI_RS_225107	0.71110956	AK359627.1	*Hordeum vulgare* subsp. *vulgare*	2.00E-51	100 %	N/A	F2D6W5	Ribosomal protein
2H,E38M54-176	SCRI_RS_193100	0.47013575	AP014960.1	*Oryza sativa japonica*	7.00E-12	98 %	SAMDC	Q0JC10	S-adenosylmethionine decarboxylase
3H,Bmag0136	BOPA2_12_20591	0.95060956	AK249478.1	*Hordeum vulgare* subsp. *vulgare*	2.00E-51	100 %	clpP	P48883 (CLPP_HORVU)	ATP-dependent Clp protease proteolytic subunit
5H,E35M55-181	BOPA2_12_11318	0.711	AK365601.1	*Hordeum vulgare* subsp. *vulgare*	2.00E-49	100%	N/A	F2DNY3	Pectinesterase
	SCRI_RS_126187	0.711	AK356154.1	*Hordeum vulgare* subsp.. *vulgare*	7.00E-50	100%	N/A	F2CX01	Protoporphyrinogen oxidase
	SCRI_RS_220645	0.8	AP014959.1	*Oryza sativa japonica*	8E-22	96%	Os03g0125100	Q10SE7	Beta-carotene hydroxylase, putative, expressed
	SCRI_RS_158981	0.879	AP014965.1	*Oryza sativa japonica*	4.00E-34	98%	SAP1	A3C039	Zinc finger A20 and AN1 domain-containing stress-associated protein 1
5H,E38M54-375	BOPA1_1583-522	0.815	AK354173.1	*Hordeum vulgare* subsp.. *vulgare*	9.00E-116	100%	N/A	F2CRC1 MLOC_44755, MLOC_44755.2	:Chlorophyll a-b binding protein, chloroplastic
5H,E42M48-282	SCRI_RS_199964	0.7082	AK059357.1	*Oryza sativa japonica*	1E-14	89%	PHT4;5	Q0IZQ3	Probable anion transporter 5, chloroplastic
	SCRI_RS_212515	0.7082	AP014965.1	*Oryza sativa* subsp.. *japonica*	1.00E-28	90%	SAP1	A3C039	Zinc finger A20 and AN1 domain-containing stress-associated protein 1
7H,P15M47-184	SCRI_RS_78255	0.767	AK365413.1	*Hordeum vulgare* subsp.. *vulgare*	2.00E-36	79%	N/A	F2DNE5	Proteasome subunit beta type
	SCRI_RS_170038	0.921	AP014964.1	*Oryza sativa japonica*	4.00E-21	86%	GLYI-11	Q948T6	Lactoylglutathione lyase
	SCRI_RS_78255	0.767	AK365413.1	*Hordeum vulgare subsp. vulgare*	2.00E-36	79%	N/A	F2DNE5	Proteasome subunit beta type
	SCRI_RS_170038	0.921	AP014964.1	*Oryza sativa japonica*	4.00E-21	86%	GLYI-11	Q948T6	Lactoylglutathione lyase
	SCRI_RS_190665	0.948	AK371075.1	*Hordeum vulgare* subsp.. *vulgare*	2.00E-51	100%	N/A	F2DXV8	Xyloglucan endotransglucosylase/hydrolase
	BOPA1_1213-1959	0.817	FN179374.1	*Hordeum vulgare* subsp. *vulgare*	3.00E-116	100%	SSI	C3W8L3	Starch synthase, chloroplastic/amyloplastic
7H,E17M54-169	SCRI_RS_176094	0.757	AK354003.1	Hordeum vulgare subsp.vulgare	8.00E-49	100%	N/A	F2CQV1	Phosphoglycerate kinase
7H,E42M48-334	SCRI_RS_187512	0.751	AK371688.1	Hordeum vulgare subsp. vulgare	3.00E-48	99%	N/A	F2DG93	D-3-phosphoglycerate

N/A: Indicates that the gene name is not available in the database.

Table 2: Briefing of BLASTn analysis on SNP sequences having significant correlation with AFLP/SSR markers linked to QTLs related to drought stress in barley C. Cappa×S population.

Chromosome number, AFLP/SSR markers	SNPs correlated to associated AFLP/SSR markers	Correlation rate between SNPs and AFLPs/SSRs	Accession	Species	FDR	Query cover	Gene	Uniprot name	Protein
4H, E35M55-302	BOPA2_12_30866	0.623126467	DQ201144.1	*Hordeum vulgare* subsp. *vulgare*	2E-51	100 %	PhyB	Q2I7M0	Phytochrome B
	BOPA1_ABC07631-1-1-83	0.667539	AP014960.1	*Oryza sativa japonica*	6E-22	67 %	SAPK7	Q7XQP4	Serine/threonine-protein kinase SAPK7
	BOPA2_12_30993	0.712802	AY177404.1	*Secale cereale*	2e-19	77 %	N/A	Q84XW5	O-methyltransferase
6H, E33M54-350	BOPA1_3379-1037	0.655509896	AK360106.1	*Hordeum vulgare* subsp. *vulgare*	3.00E-116	100 %	N/A	F2D894	Proteasome subunit alpha type
	BOPA2_12_21114	0.724140479	AK355532.1	*Hordeum vulgare* subsp. *vulgare*	2.00E-51	100 %	N/A	F2CV80	Nuclear cap-binding protein subunit 2
	SCRI_RS_80327	0.704318358	AK362351.1	*Hordeum vulgare* subsp. *vulgare*	5.00E-51	100 %	N/A	F2DEN4	3-ketoacyl-CoA synthase
	SCRI_RS_102426	0.60858085	L14444.1	*Triticum aestivum*	2.00E-43	100 %	hsp16.9C	Q41561	Heat shock protein 16.9C
	SCRI_RS_187114	0.701762419	AP014958.1	*Oryza sativa japonica*	7.00E-31	99%	CPK4	Q6Z2M9	Calcium-dependent protein kinase
6H, E42M48-380	SCRI_RS_161117	0.575180587	AP014959.1	*Oryza sativa japonica*	7e-12	89 %	Os03g0125100	Q10SE7	Beta-carotene hydroxylase, putative, expressed
	BOPA2_12_30956	0.649579	AP008220.2	*Oryza sativa japonica*		96 %	OSJNOa246I10.1	Q6EP25	HGWP repeat containing protein-like

Table 3: Results of studies on proteins/enzymes identified in the population V × S under drought stress and other stresses.

Protein/enzyme	Species studied	Treated stress	Gene/peotein expressed	Expression type, effect	Reference
Glycosyltransferase	Arabidopsis	Drought	UDP-Glycosyltransferase	Down-regulation	[Li et al., 2015]
	Rice	Drought	Gene LOC_Os01g68324.3 encoding a glicosyltransferase named Dolichyl-diphosphooligosaccharide	Suppression	[Landi et al., 2017]
Protein DEHYDRATION-INDUCED 19 homolog 5	Arabidopsis	Drought	Gene DI19	Up-regulation, increasing drought tolerance	[Liu et al., 2013]
	Wheat	Drought	Gene TiDI19-2	Induction, enhancing drought tolerance	[Li & Chen, 2000]
Dihydrolipoyl dehydrogenase (LPD)	Sugarcane	Drought	Gene LPD	Up-regulation in tolerant cultivars	[da Silva et al., 2017]
	Barley	High temperature	Two LPD proteins: F2E5U7 and F2E2T3, that F2E5U7 identified in our study under drought stress	Induction, enhancing tolerance to high temperature	[Rollins, 2012]
Hexosyltransferase	Wheat	Drought	One hexosyltransferase gene	Induction, enhancing drought tolerance	[Ajigboye et al., 2016]
	Chickpea (Cicer arietinum)	Drought	11 hexosyltransferase genes related to drought tolerance QTLs included 7 sucrose synthase genes (SuSy) and 4 sucrose phosphate synthase (SPS)	Induction, enhancing drought tolerance	[Nagesh Nayak, 2010]
Salt-tolerant protein-GSK-like kinase	Rice	Drought and salinity	Mutation in gene OsGSK1 that was caused to enhanced expression of special stress respond genes and increased to drought and salinity	Up-regulation, increasing drought and salinity tolerance	[Koh et al., 2007]
	Barley	Dehydration and drought then rehydration	One glicosyle synthase kinase named AAM77397 that is same to Q8LK43 identified in our study.	Induction, enhancing drought tolerance	[Talamé et al., 2007]
Peroxidase	Barley	Drought	Peroxidase	Up-regulation in tolerante cultivares	[Hellal et al., 2018]
	Wheat	Osmotic stress	Peroxidase (TaPrx)	Up-regulation in tolerante cultivares	[Csiszár et al., 2012]
Glycine-rich RNA-binding protein RZ1B (GRP)	Transgenic tobacco	Salinity	GRP gene belong to Limonium bicolor	Up-regulation in tolerante cultivares	[Wang et al., 2014]
	Transgenic rice	Drought	Two GRP gene belong to Arabidopsis included AtGRP2 and AtGRP7	Enhancing drought tolerance	[Yang et al., 2012]
Mitogen-activated protein kinase	*Arabidopsis*	Drought	Two genes AtMPK4 and AtMPK6	Up-regulation, increasing drought tolerance	[Nadarajah and Sidek, 2010.]
	Rice	Drought	Two genes OsMAPK2 and OsMAPK5	Up-regulation, increasing drought tolerance	[Rohila & Yang, 2007]
Pectinesterase	Barley	Drought	Gene PME49	Up-regulation, increasing drought tolerance	[Wendelboe-Nelson et al., 2012]
	Rice	Drought and salinity	Three root genes that encoded pectinesterase	Up-regulation, increasing drought and salinity tolerance	[Koh et al., 2007]
Cinnamoyl-CoA reductase-like SNL6 (CCR)	Transgenic tobacco species *Nicotiana benthamiana*	Drought	Three CCR genes belong to sorghum including to SbCCR1, SbCCR2-1 and SbCCR2-2	Up-regulation, increasing drought tolerance	[Li et al., 2016]
	Tea	Drought	One CCR gene	Up-regulation, increasing drought tolerance	[Gupta et al., 2012]
Ribosomal protein	Maize	Drought	Ribosomal protein S18	Up-regulation, increasing drought tolerance	[Benešová et al., 2012]
	Bermuda grass	Drought	Two ribosomal protein S1 and L12	Down-regulation in sensitive cultivares	[Zhao et al., 2011]
S-adenosylmethionine decarboxylase proenzyme (SAMDC)	Transgenic *Arabidopsis* lines	Drought	One SAMDC gene from *Capsicum annuum*	Enhanced tolerance in transgenic plants than wiled types	[Wilkins et al., 2010]
	Wheat	Drought, salinity and external ABA treatment	TaSAMDC gene	Induction and increasing tolerance	[Li & Chen, 2000]

ATP-dependent Clp protease proteolytic subunit	Wheat	Drought	Two ATP-dependent chlroplastic protease proteolytic subunits	Up-regulation, increasing drought tolerance	[Cheng et al., 2016]
	Parthenium hysterophorus	Drought and salinity	ATP-dependent chlroplastic protease proteolytic subunit	Induction and increasing tolerance	[Ahmad et al., 2017]
Beta-carotene hydroxylase, putative, expressed	Mutant rice	Drought	Mutation in beta-carotene hydroxylase gene (BCH)	Decreased tolerance in mutant than wiled type	[Du et al., 2010]
	Transgenic tobacco	Drought	Overexpression of beta-carotene hydroxylase gene from arabidopsis (chyB)	Increasing beta-carotene and drought tolerance	[Zhao et al., 2014]
Probable anion transporter 5, chloroplastic	*Populus trichocarpa*	Drought	Two genes PtPHT1.2 and PtPHO9	Up-regulation	[Zhang et al., 2016]
	Apple	Drought	Genes MdPHT3;6, MdPHT3;7, MdPHT4;5, MdPHT1;12 and MdPHT4;7	Up-regulation in stressed plants than controls and enhanced drought tolerance	[Sun et al., 2017]
Zinc finger A20 and AN1 domain-containing stress-associated protein 1	Rice	Drought	Overexpression of OsiSAP1 gene	Enhanced drought tolerance	[Dansanaet al., 2014]
	Alfalfa	Drought	MtSAP1 gene	Enhanced drought tolerance	[Gimeno-Gilles et al., 2011]
Protoporphyrinogen oxidase	Transgenic rice	Drought	Overexpression of two Protoporphyrinogen oxidase genes (PPO) from *Arabidopsis thaliana* and *Myxococcus xanthus*	Enhanced drought tolerance	[Thu-Ha et al., 2011]
Chlorophyll a-b binding protein, chloroplastic	Rice and Arabidopsis	Drought, dark, high temperature and salinity	Based on BLAST analysis of sequences of light-harvesting complex (LHC) genes in barley genomic database, 17 LHC genes encoding Chlorophyll a-b binding proteins (HvLHC) were identified that one of them (MLOC_44755) is same to F2CRC1 characterized in our study.	Induction	[Qin et al., 2017]
	Morus indica L.	Drought	Two chloroplastic chlorophyll a-b binding protein	Up-regulation and increasing drought tolerance	[Guha et al., 2013]
Starch synthase, chloroplastic/amyloplastic	Rice	Drought	LOC_Os07g22930 (AT1G32900) as a starch synthase gene	Up-regulation and increasing drought tolerance	[Basu & Roychoudhury, 2014]
	Sorghum	Drought	Starch synthase enzyme	Down-regulation and decreasing drought tolerance	[Yi et al., 2014]
Proteasome subunit beta type	Common bean	Drought	Proteasome subunit beta type-3-A	Down-regulation	[Zadražnika et al., 2013]
	Barley	Drought	Proteasome subunit beta type	Up-regulation	[Wehner et al., 2015]
Lactoylglutathione lyase	Eucalyptus	drought	Lactoylglutathione lyase gene	Down-regulation	[Ghosh & Dharanishanthi, 2017]
	Humulus lupulus L.	Drought	Lactoylglutathione lyase protein related to ROS pathwey		[Kolenc et al., 2016]
Xyloglucan endotransglucosylase/hydrolase	Transgenic tomato	Drought	CaXEH3 gene from *Capsicum annum* L.	Overexpression	[Choi et al., 2011]
	Medicago truncatula	Drought	21 XEH genes	Induction	[Yi et al., 2014]
Phosphoglycerate kinase	Soybean	Drought and high temperature	Phosphoglycerate kinase protein	Up-regulation	[Das et al., 2016]
	Barley	Drought	Phosphoglycerate kinase protein	Down-regulation	[Pieczynski et al., 2012]
D-3-phosphoglycerate dehydrogenase	Arabidopsis	H2O2 and ABA treatment	D-3-phosphoglycerate dehydrogenase protein	Induction	[Cramer et al., 2013]
	Grapevine (*Vitis vinifera* L.)	Drought	D-3-phosphoglycerate dehydrogenase protein	Down-regulation	[Alqurashi et al., 2017]

identified proteins/enzymes with drought stress has been proven in previous studies.

As can be seen, all the proteins/enzymes identified in both populations were directly involved in the response to drought stress in several studies on different plants, which indicates the confirmation of the validity of the QTLs found in our previous study (Mohammadi et al., 2018). In order to identify genes related to drought stress in rice, Gorantla et al. (2007), prepared ESTs from the leaf tissue cDNA library of a rice cultivar under drought treatment, and one of the genes expressed in response to drought stress, was heat shock protein C16.9, which is consistent with the present research, and also Szűcs et al. (2006) reported 6 QTLs related to photoperiod in barley, and one of genes, HvPhyB, located on chromosome 4, i.e.

Table 4: Results of studies on protein/enzymes identified in the population C. Cappa × S under drought stress and other stresses.

Protein/enzyme	Species studied	Treated stress	Gene/protein expressed	Expression type, effect	Reference
Phytochrome B	Rice PhyB mutant	Drought	PhyB mutant protein	Increased drought tolerance	[Liu et al., 2012]
	Barley	Drought	The HvPhyB gene located on chromosome 4H, i.e. accession "DQ201144", which was also identified in the present study.	Increased drought tolerance	[Talamé et al., 2007]
Serine/threonine-protein kinase SAPK7	Rice	Dehydration	SAPK5 gene	Increased expression	[Basu & Roychoudhury, 2014]
	Groundnut	Drought	Serine/threonine-protein kinase HT1	Induced expression	[Ding et al., 2014]
O-methyltransferase	Sugarcane	Drought	O-methyltransferase 2	Increased expression and drought tolerance	[da Silva et al., 2017]
	Ocimum basilicum	Drought	Chavicol O-methyltransferase and eugenol O-methyltransferase genes	Increased expression	[Abdollahi Mandoulakanieet al., 2017]
	Tea	Drought	Caffeic acid 3-O-methyltransferase	Decreased expression	[Wang et al., 2017]
Proteasome subunit alpha type	Alfalfa	Drought and salt	Two proteins related to the proteasomal subunit, including the alpha-7 and beta-2-B subunits	Decreased expression	[Ma et al., 2016]
	Soybean	Drought	Proteasome subunit alpha type	Increased expression	[Pour Mohammadi et al., 2012]
	Common bean	Drought	Proteasome subunit alpha type	Decreased expression	[Zadražnika et al., 2013]
	Wheat	Drought	Proteasome subunit alpha type	Decreased expression	[Jiang et al., 2012]
Nuclear cap-binding protein subunit 2	Mutant barley in HvCBP20 gene	Drought	HvCBP20 gene	Increased drought tolerance	[Daszkowska-Golec et al., 2017]
	Potato CBP20 and CBP80 mutants	Drought	CBP20 and CBP80	Increased drought tolerance	[Pieczynski et al., 2012]
3-ketoacyl-CoA synthase	Cotton	Drought	3-ketoacyl-CoA synthase gene	Induction	[Wang et al., 2010]
	Two varieties of silver fir (Abies alba Mill.)	Drought	Two 3-ketosyl-CoA synthase genes	The expression of these two genes increased under drought conditions in one cultivar and decreased in the other cultivar	[Behringer et al., 2015]
Heat shock protein 16.9C	Rice	Drought	Heat shock protein 16.9C	Induction	[Gou et al., 2017]
	Wheat	Drought and high temperature	HSP proteins	Induction	[Guha et al., 2013]
Calcium-dependent protein kinase	Barley	Drought	HvCPK2a	Increased expression	[Ciésla et al., 2016]
	Rice	Drought	OsCPK4	Overexpressed	[Campo et al., 2014]
HGWP repeat containing protein-like	Maize, wheat and barley	Drought	Six genes encoding HGWP repeat containing protein-like	Increased tolerance	[Swamy et al., 2011]
	Two sensitive and drought-resistant varieties of barley	Drought	HGWP repeat containing protein-like	Increased expression in the sensitive variety	[Wendelboe-Nelson, 2012]

accession "DQ201144", which has been identified in the present study in the C. Cappa × S population on chromosome 4H (Tables 3 and 4).

3.4 GENE ONTOLOGY

3.4.1 Ontology results in V × S population

The results of gene ontology in population V × S are shown in Figures 3 and 4. The frequencies of three GO description, including cellular components, biological processes, and molecular functions, were 25 %, 32 %, and 43 %, respectively (Figure 3). Based on the results of complete gene ontology, four types of cell components including membrane, integral component of membrane, nucleus and chloroplast had the highest frequencies: 4.9 %, 12.4 %, 12.4 % and 9.1 % respectively (Fig. 4a). Two types of biological processes, including oxidation-reduction process and protein phosphorylation, had the highest frequencies: 11.5 % and 5.1 %, respectively (Figure 4b). The study of the frequency distribution of molecular functions showed that ATP binding, oxidoreductase activity, transferase activity, DNA binding, and metal ion binding, were most frequent: 9.0 %, 7.1 %, 5.69 %, 5.2 % and 4.74 % respectively (Figure 4c).

Wilkins et al. (2010) studied drought stress-induced transcripts in *Arabidopsis*, and based on ontology analysis, reported that transferase activity as a molecular function; chloroplast and membrane as cellular components had the highest frequency. Kokas et al. (2016) investigated the transcripts of wild barley in response to drought stress and as a result of the ontology study, they reported molecular functions such as binding, catalytic activity and binding to nucleic acid (Kokas et al., 2016). Similar results have been reported in other studies on different plants in drought conditions (Bedada et al., 2014; Zeng et al., 2016; Gou et al., 2017). All these studies confirm the results of the present research.

3.4.2 Ontology results in C. Capa × S population

The results of gene ontology in C. Capa × S population are shown in Figures 5 and 6. The frequency of GO descriptions including molecular functions, biological processes, and cellular components were 40.2 %, 30.4 %, and 29.4 %, respectively (Figure 5). Based on the analy-

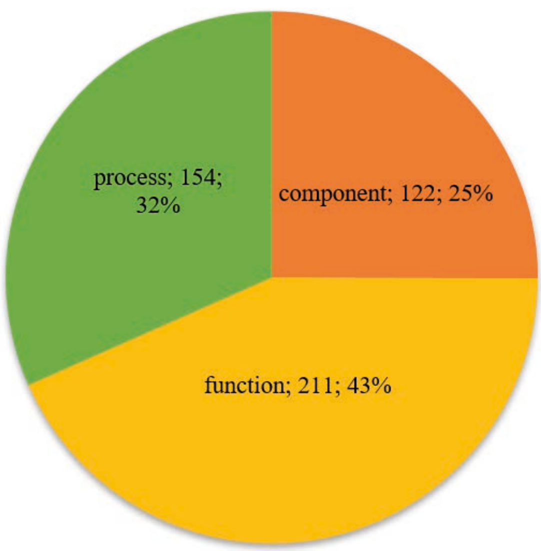
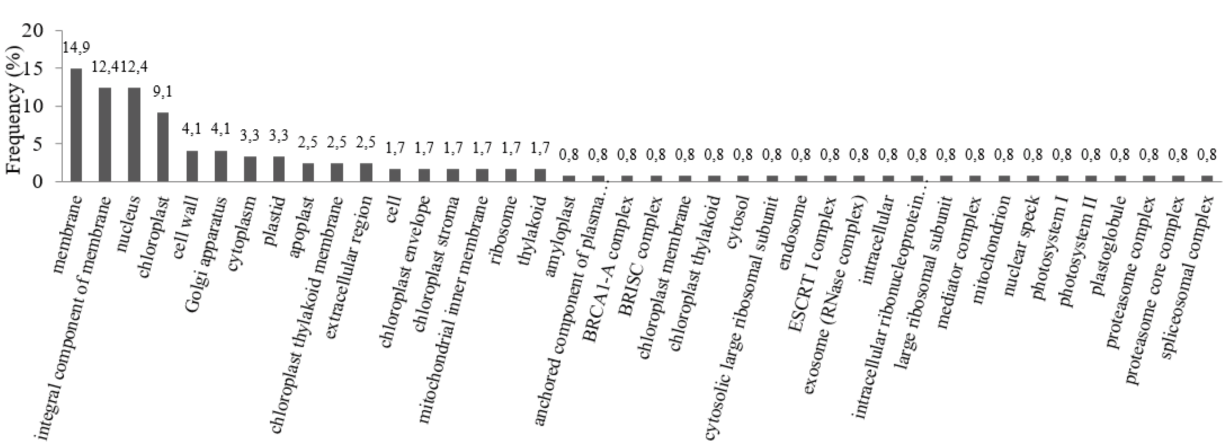


Figure . Frequency distribution of GO description types (number, percentage) in population V×S



GO terms of Cellular Components (a)

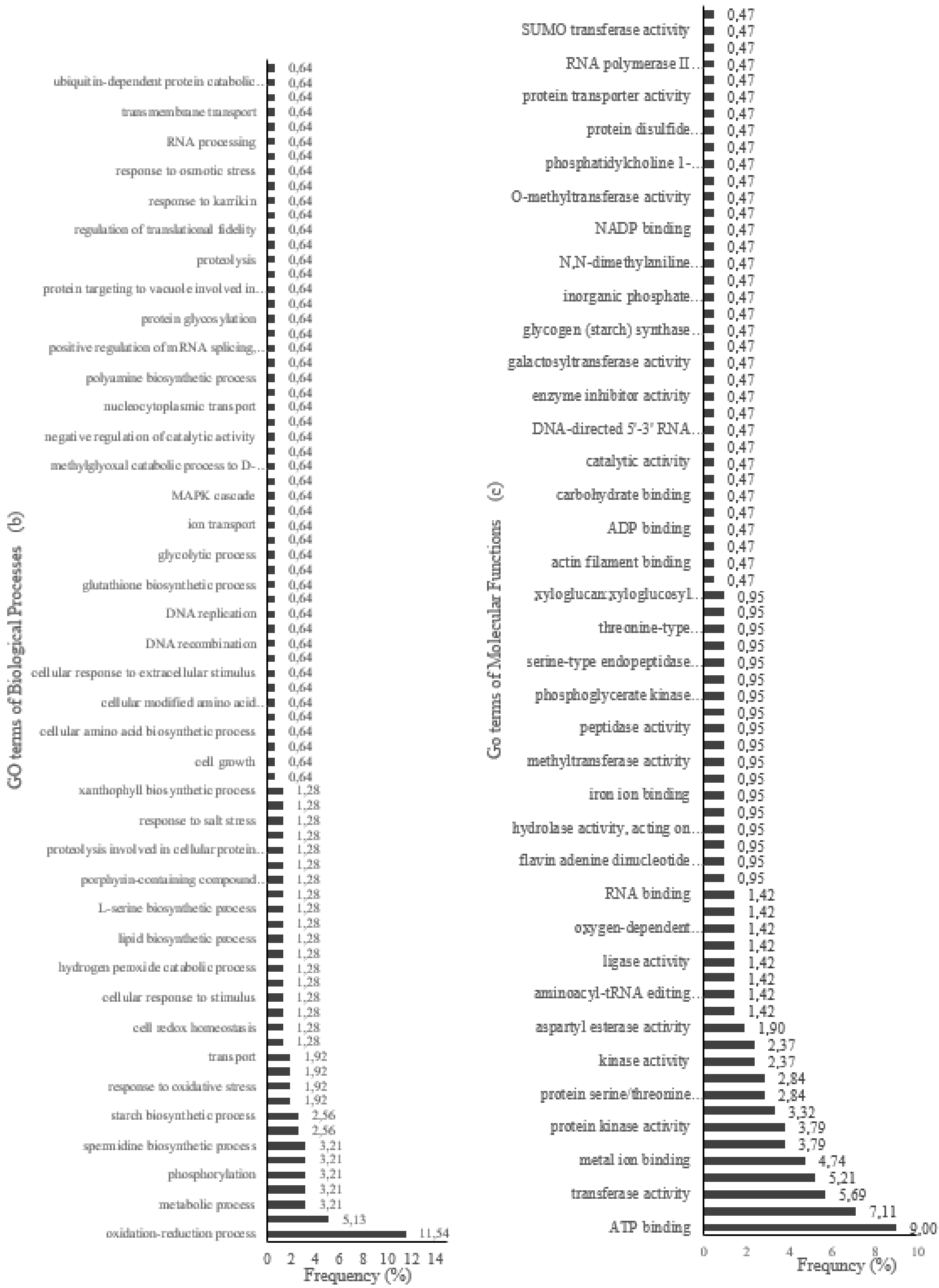


Figure 4: Frequencies of three types of GO descriptions characterized in V × S population; a: Cell Components, b: Biological Processes and c: Molecular Functions

sis results, the highest frequency of cellular components was associated with the nucleus, comprising 26.7 % of the total. Additionally, the membrane and cytoplasm accounted for 20 %, while the proteasome core complex and integral membrane components each represented 10 %. These findings highlight the predominant roles of these cellular structures in the context of the study, emphasizing their significance in cellular functions and processes (Figure 6a). Also 9 types of molecular functions including ATP binding and transferase activity (with a frequency of 9.8 %); thereonine-type endopeptidase activity and protein serine/thereonine kinase activity (with a frequency of 7.3 %); RNA binding, transferase activity, transferring acyl groups, kinase activity, protein kinase activity and methyltransferase activity (with a frequency of 4.9 %) had the highest frequency (Figure 6b). Among the biological processes, the highest frequencies were related to five types of biological processes, including protein phosphorylation (12.9 %), fatty acid biosynthetic process (6.5 %), proteolysis (6.5 %), intracellular signal transduction (6.5 %) and oxidation-reduction process (6.5 %) (Figure 6c). In Saavedra experience the highest frequencies were related to oxidation-reduction process (tree time of occurrence) (Saavedra et al., 2017).

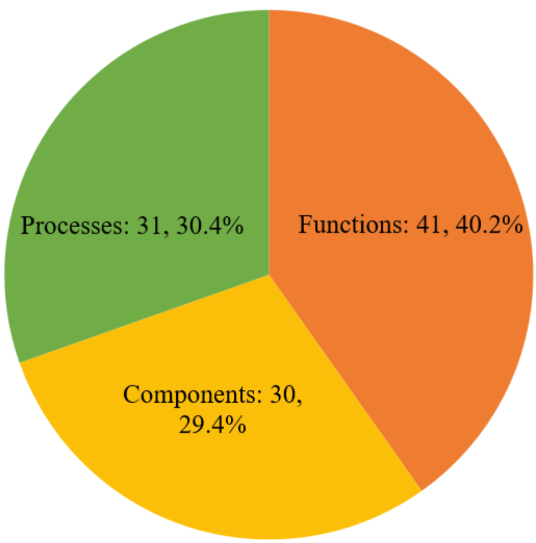


Figure 5: Frequency distribution of GO description types (number, percentage) in population C. Cappa × S

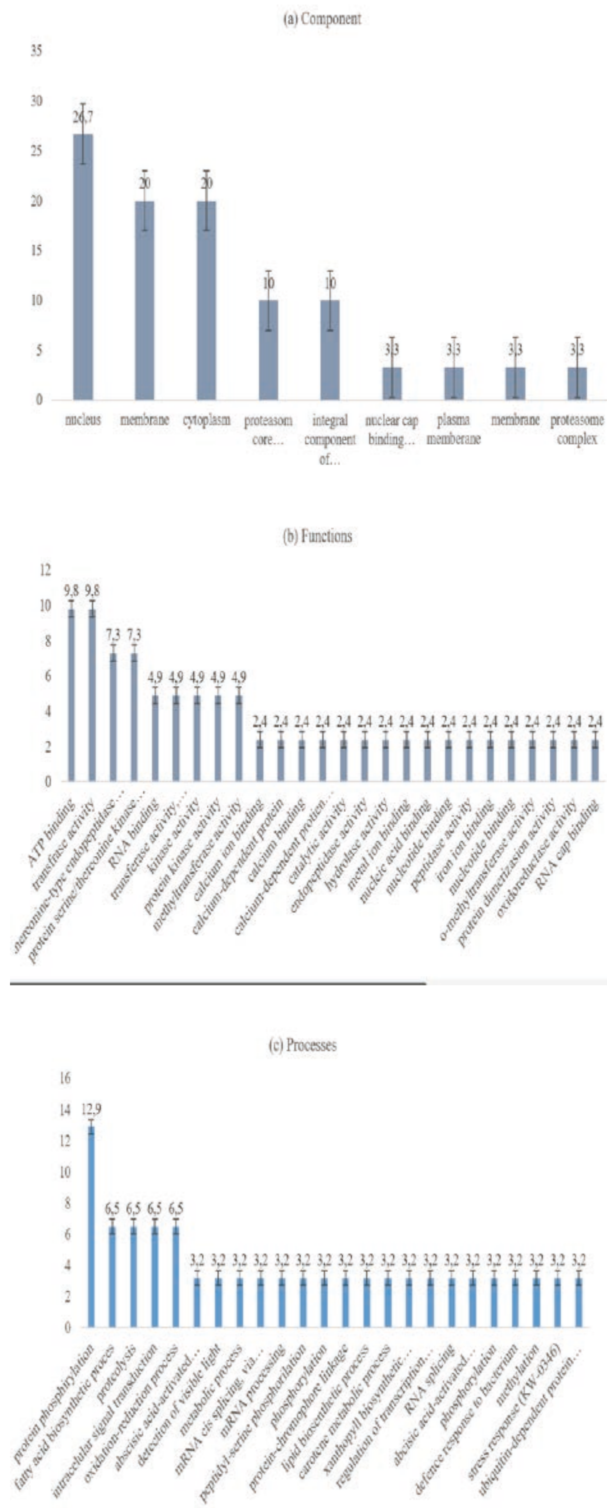


Figure 6: Frequencies of three types of GO descriptions characterized in C. Cappa × S population; a: Cell Components, B: Biological Processes and c: Molecular Functions

4 CONCLUSIONS

The results of this study, as well as previous similar studies, showed that BLAST analysis on SNP sequences is a very effective tool for validating the identified QTLs. Based on the results of the present study, all proteins/enzymes identified in two populations are directly involved in the response to drought stress in different plants. Both up-regulation (increased expression) and down-regulation (decreased expression) have been reported for the identified proteins/enzymes in response to drought stress in different plants. The gene ontology results showed that the identified genes are significantly involved in drought stress. Practical result is that these QTLs, particularly those identified on chromosomes 1H, 4H, and 5H, along with related genes like glycosyltransferases, phytochrome B, and pectinesterase, can be very useful for developing drought tolerance in barley and other plants.

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Utilization of nitrogen-fixing endophytic bacteria to improve tuber yield and nitrogenous nutrient uptake of Cassava plant (*Manihot esculenta* Crantz)

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Utilization of nitrogen-fixing endophytic bacteria to improve tuber yield and nitrogenous nutrient uptake of Cassava plant (*Manihot esculenta* Crantz)

Abstract: Cassava (*Manihot esculenta* Cratz) yield remains low due to various environmental stressors, yet sustainable strategies for enhancing productivity, especially through microbial interventions, are underexplored in current literature. This study investigated the potential of endophytic bacteria isolated from native cassava (*M. esculenta* KM98-7) to enhance plant growth and yield under greenhouse and field conditions. Eleven bacterial strains (TL1 to TL11) were isolated and assessed for nitrogen fixation, phosphate solubilization, and indole acetic acid (IAA) synthesis. TL4 and TL8 exhibited superior capabilities, with TL8 producing the highest levels of $NH4^+$ (14.95 mg l^{-1}) and IAA (46.57 mg l^{-1}). Molecular identification revealed that TL4 and TL8 were closely related to *Burkholderia cenocepacia* Vandamme et al. 2003 and *Prestia aryabhattai* (Shivaji et al. 2009) Gupta et al. 2020. Greenhouse trials showed that inoculation with TL8 significantly increased plant height, leaf number, and tuber yield, comparable to 90 kg urea ha^{-1} application. Field experiments confirmed these findings, with the 60 kg urea ha^{-1} + TL8 treatment achieving similar yields to 90 kg urea ha^{-1} without bacterial inoculation. This study demonstrates that integrating nitrogen-fixing bacterial inoculants, particularly strain TL8, with reduced nitrogen fertilization can maintain high cassava productivity while promoting sustainable agricultural practices.

Key words: biofertilizers, crop productivity, endophytic bacteria, plant-growth promotion, sustainable agriculture

Uporaba endofitskih bakterij, ki vežejo dušik za izboljšanje pridelka in privzema hranil manioke (*Manihot esculenta* Crantz)

Izvleček: Pridelek manioke (*Manihot esculenta* Cratz) ostaja majhen zaradi različnih okoljskih stresnih dejavnikov a kljub obstoječim trajnostnim strategijam za povečanje pridelka, še posebej z mikrobi, ostajajo te neuporabljene glede na obstoječe vire. V raziskavi je bil preučevan potencial endofitskih bakterij izoliranih iz lokalne manioke (*M. esculenta* KM98-7) na vzpodbujanje rasti in povečanje pridelka v rastlinjaku in poljskih razmerah. Enajst sevov bakterij (TL1 do TL11) je bilo izoliranih in ocenjenih na vezavo dušika, raztaplanje fosfata, in sintezo indol acetne kisline (IAA). Seva TL4 in TL8 sta pokazala najboljšo sposobnost, pri čemer je sev TL8 proizvedel največ $NH4^+$ (14,95 mg l^{-1}) in IAA (46,57 mg l^{-1}). Molekularno preverjanje je odkrilo, da sta bila seva TL4 in TL8 zelo sorodna vrstama *Burkholderia cenocepacia* Vandamme et al. 2003 in *Prestia aryabhattai* (Shivaji et al. 2009) Gupta et al. 2020. Poskus v rastlinajku je pokazal, da je inokulacija s sevom TL8 značilno povečala višino rastlin, število listov in pridelek gomoljev, ki je bil primerljiv uporabi 90 kg urea ha^{-1}. Poljski poskus je potrdil ta odkritja, kjer je obravnavanje s 60 kg urea ha^{-1} + TL8 doseglo podoben pridelek kot uporaba 90 kg urea ha^{-1} brez bakterijske inokulacije. Raziskava kaže, da vključevanje inokulov bakterij, ki vežejo dušik, še posebej seva TL8, z zmanjšanjem gnojenja z dušikovimi gnojili, lahko ohranja velike pridelke manioke in hkrati pospešuje trajnostno kmetijsko pridelavo.

Ključne besede: biognojila, pridelek gojenih rastlin, endofitke bakterije, pospeševanje rasti rastlin, trajnostno kmetijstvo

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1 NTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a crucial staple food crop in tropical regions, particularly in sub-Saharan Africa, Latin America, and Southeast Asia, providing a primary source of carbohydrates for millions of people. However, achieving high yields, such as 30 tons of cassava tubers per hectare, requires substantial nutrient removal from the soil - approximately 180-200 kg N ha^{-1}, 15-22 kg P_2O_5 ha^{-1}, and 140-160 kg K_2O ha^{-1} (Susan et al., 2010). Consequently, cassava productivity is often constrained by poor soil conditions, such as alum soil, characterized by high acidity and low nutrient availability (Omondi et al., 2018). Traditional agricultural practices aimed at improving soil fertility frequently fall short, necessitating alternative, sustainable solutions to enhance cassava growth and yield.

One promising approach is the use of plant endophytic bacteria, which inhabit plant tissues without causing harm and can confer various benefits to their host plants. These bacteria promote plant growth through mechanisms such as nitrogen fixation, phosphate solubilization, production of growth hormones, and induction of systemic resistance to pathogens (Ferreira et al., 2021; Do et al., 2023; Ferreira et al., 2024). The potential of these bacteria to improve plant health and productivity under stress conditions has been increasingly recognized (Do et al., 2023).

Recent studies have highlighted the positive impacts of endophytic bacterial inoculation on various crops under different stress conditions. For instance, inoculation with *Bacillus* sp. has been shown to enhance the growth and yield of rice in the presence of the bacterium *Xanthomonas oryzae* pv. *Oryzae* (Xoo) that caused bacterial leaf blight disease (Do et al., 2023). Similarly, *Pseudomonas fluorescens* (Flügge 1886) Migula, 1895 strains have been reported to improve the growth of wheat under drought stress by enhancing root development and water uptake (Vurukonda et al., 2016). These findings suggest that endophytic bacteria could be a valuable tool in mitigating abiotic stresses and improving crop productivity on challenging soils.

In the context of cassava, research on the role of endophytic bacteria is still emerging. Recent studies (Ferreira et al., 2021; Feng et al., 2023; Ferreira et al., 2024) demonstrated that endophytic bacteria isolated from cassava plants could promote plant growth and enhance resistance against phytopathogens such as *Phytopythium* sp., a causal agent of soft root rot in cassava, and *Xanthomonas phaseoli* pv. *Manihotis* (Xpm) which causes cassava bacterial blight. Furthermore, the study by Zhang et al., (2022) indicated that cassava-associated endophytes could improve nutrient uptake, leading to better growth and higher yields.

Given these promising findings, the present study aims to investigate the effect of plant endophytic bacterial inoculation on cassava growth and yield. By understanding how these bacteria interact with cassava plants and influence their growth under adverse soil conditions, we can develop sustainable agricultural practices that leverage natural microbial relationships to enhance crop productivity and resilience.

2 MATERIALS AND METHODS

2.1 ISOLATION OF ENDOPHYTIC BACTERIA FROM THE CASSAVA PLANT

A total of 50 cassava (*Manihot esculenta* KM98-7) plant samples, including roots, stems, and leaves, were collected from Tuy Lai commune in My Duc district, Ha Noi, Vietnam. These samples were cleaned and cut into 1 cm pieces. Surface sterilization involved immersion in 70 % ethanol with gentle shaking for 3 minutes, followed by rinsing with sterile distilled water. The samples were then treated with 0.1 % $HgCl_2$ for 1 minute with shaking, rinsed again, treated with 3 % hydrogen peroxide for 3 minutes, and thoroughly rinsed with sterile distilled water four times. To ensure no residual microorganisms, 100 μl of the final rinse water was plated onto Luria-Bertani (LB) medium. Successful sterilization was confirmed by the absence of colonies after 24-48 hours of incubation.

The sterilized samples were ground using a sterile mortar and pestle with 1 ml of sterile distilled water. A 100 μl aliquot of the homogenized sample was inoculated into test tubes containing 3 ml of semi-solid LB medium and incubated at 30 °C for 24-48 hours. The presence of endophytic microorganisms was indicated by a thin film on the medium surface, which was transferred to a solid LB medium and incubated at 30 °C for 24-48 hours. Pure cultures were obtained through subculturing and preserved on slant LB agar tubes at 4 °C.

2.2 CHARACTERIZATION OF ENDOPHYTIC BACTERIA FROM THE CASSAVA PLANT

2.2.1 Nitrogen fixation activity assay

All bacterial strains were screened for nitrogen fixation abilities by culturing in 50 ml nitrogen-free Burk's liquid medium at room temperature for 3 days with shaking at 160 rpm, followed by centrifugation at 10,000 rpm for 10 minutes. The NH_4^+ content in the supernatant was quantified using Nessler's reagent colorimetric method at 420 nm with NH_4Cl as the standard (Franche et al., 2009). Specifically, 0.1 ml of sodium potassium tartrate solution and 0.1 ml of Nessler's reagent were added to 5 ml of the supernatant. After mixing and allowing it to sit for 20 minutes, the absorbance was recorded at 420 nm. A calibration curve for concentration versus absorbance was created using a series of standard NH_4Cl solutions with varying concentrations. The resulting curve demonstrated a strong linear correlation between absorbance and NH_3 concentration.

2.2.2 IAA production

Indole-3-acetic acid (IAA) synthesis by bacteria was determined using the Salkowski method (Do et al., 2023). Bacteria were cultured in 25 ml of LB medium supplemented with 0.1 g l^{-1} tryptophan, incubated at 30 °C for 48 hours with shaking at 120 rpm. The IAA content in the supernatant was quantified using the Salkowski reagent with colorimetric measurement at 530 nm and IAA as the standard.

2.2.3 Phosphate solubilization

Phosphorus solubilization capability was assessed by culturing bacteria in 100 ml National Botanical Research Institute's phosphate growth (NBRIP) liquid medium at room temperature for 7 days with agitation at 120 rpm (Yanlei and Xiaoping, 2018). The supernatant was collected by centrifugation at 10,000 rpm for 15 minutes at 4 °C. Solubilized phosphorus was quantified using the molybdenum blue method with ammonium molybdate at 880 nm and KH_2PO_4 as the standard.

2.2.4 Molecular identification of bacterial isolate

The molecular method was used to identify the species of strain TL8. Bacteria were grown overnight in LB broth at 30 °C with shaking (120 rpm). Genomic DNA was extracted using the NucleoSpin® Tissue extraction kit (Macherey-Nagel, Germany). The 16S rRNA gene was amplified via PCR with the primer pair 27F and 1492R. PCR conditions were: initial denaturation at 94 °C for 2 minutes, followed by 35 cycles of 94 °C for 30 seconds, 55 °C for 20 seconds, 72 °C for 1 minute, final extension at 72 °C for 5 minutes, and hold at 4 °C. PCR products were cleaned and sequenced by 1st BASE Company (Malaysia). The 16S rRNA gene sequence of strain TL8 was compared to sequences in the GenBank database.

2.3 EVALUATION OF ENDOPHYTIC BACTERIA ON CASSAVA PLANT GROWTH UNDER GREENHOUSE CONDITIONS

The greenhouse experiment was conducted in the greenhouse of the Vietnam National University of Forestry (VNUF) during the winter-spring cropping season of 2022-2023 (November 2022 - May 2023) using cassava cuttings (15-20 cm).

Soil from Tuy Lai commune, My Duc district, Ha Noi, Vietnam, was used. The soil analysis criteria include pH and EC, extracted with distilled water at a ratio of 1:2.5 (soil: water). pH is measured with a pH meter, and EC is measured with an EC meter. Available phosphorus (using the Bray II method) is determined by extracting soil with 0.1N HCl + 0.03N NH_4F at a soil-to-water ratio of 1:7, then measured on a spectrophotometer at a wavelength of 880 nm. Exchangeable potassium is extracted using 0.1M $BaCl_2$ and measured with an atomic absorption spectrometer. Soil texture composition is determined using the Robinson pipette method. The properties of soil were pH_{KCl} 4.69, EC = 1.91 mS cm^{-1}, total N 0.11%, organic matter 3.02 %, soluble P_2O_5 = 20.12 mg P kg^{-1}, soluble K_2O = 0.27 meq $100g^{-1}$.

The bacterial strains with strong abilities to fix nitrogen, solubilize phosphate, and produce IAA were chosen. The selected bacteria strains were cultured in LB media at 30 °C for 24 hrs. The culture was centrifuged at 8000 rpm for 10 minutes at 4 °C, and the cells were resuspended in sterilized water to OD600 = 1.0. Cassava cuttings (15-20 cm) were disinfected and immersed in bacterial suspension for 1 hour before planting in pots (0.3 m × 0.4 m) filled with prepared soil (Zhang et al., 2022).

The experiment followed a two-factor completely

Table 1: Summary of treatment combination under greenhouse conditions

Urea fertilizer	Without bacterial inoculation	With bacterial inoculation of strain TL4	With bacterial inoculation of strain TL8
No urea fertilizer	T1	T5	T9
30 kg urea ha^{-1}	T2	T6	T10
60 kg urea ha^{-1}	T3	T7	T11
90 kg urea ha^{-1}	T4	T8	T12

Table 2: Period and dosage of fertilizer for experiments

Fertilization period	Describe
On the day of planting	Apply whole phosphate fertilizer
1st application (25 DAP)	Apply 1/3 urea fertilizer + 1/3 potassium fertilizer
2nd application (50 DAP)	Apply 1/3 urea fertilizer + 1/3 potassium fertilizer
3rd application (80 DAP)	Apply all the remaining urea and potassium fertilizer

DAP: day after planting

randomized block design: factor A (urea fertilizer levels: 0, 30, 60, and 90 kg ha^{-1}) and factor B (bacterial strains: with and without bacteria) with three replications (Table 1).

Fertilizers used were urea (46 % N), Lam Thao superphosphate (16 % P2O5), and Kali Phu My MOP (60 % Growth parameters (plant height, number of leaves, diameter of base, trunk, and stem) were measured at 90 days after planting (DAP), and tuber yield, number of tubers, tuber length, and tuber diameter were measured at harvest.

The Kjeldahl method was applied to determine the total nitrogen content in cassava plants' leaves, roots, and stems from T1 (without nitrogen fertilizer and bacterial TL8 inoculation) and T9 (with bacterial TL8 inoculation only) experiments. Approximately 0.5 g of dried and finely ground plant tissue from each part was placed in a digestion flask. To each sample, 10 ml of concentrated H_2SO_4 was added along with 0.5 g of a catalyst mixture consisting of K_2SO_4 and selenium (0.005 g). The digestion process was done by heating the mixture until the solution became clear, indicating the complete breakdown of organic matter. After digestion, the solution was cooled and diluted with 50 ml of distilled water. To neutralize the acid and convert ammonium ions (NH_4^+) to ammonia (NH_3), 40 ml of 10 mol l^{-1} NaOH was added to the mixture. The ammonia was then distilled into a receiving flask containing 25 ml of 2 % boric acid solution, with the distillation process continuing until approximately 100 ml of distillate had been collected. The collected distillate was titrated with 0.1 mol l^{-1} HCl until the endpoint was reached, as indicated by a color change of the pH indicator. The volume of HCl used in the titration was recorded, and the total nitrogen content was calculated based on the titration results. This procedure allowed for the accurate quantification of total nitrogen in the different plant tissues according to the Kjeldahl method described by Bremner (1996).

2.4 EVALUATION OF PROMISING ENDOPHYTIC BACTERIA ON CASSAVA PLANT GROWTH UNDER FIELD CONDITIONS

The field experiment was conducted during the summer-autumn crop of 2023 at Tuy Lai commune, My Duc district, Ha Noi, Vietnam. The soil was prepared by plowing to a depth of 15-20 cm and arranging into beds (100 cm width, 50 cm height, 10 m length) with 30 cm spacing between beds. Cuttings were planted in single rows per bed, with 80 cm spacing between cuttings.

The experiment followed a completely randomized block design with one factor, comprising 8 different treatments, each replicated three times (summarized in Table 3). Fertilizer application and growth monitoring were conducted as in the greenhouse experiment (see Table 2).

2.5 DATA ANALYSIS

Data were statistically analyzed using Excel and IRRISTAT software. Tukey's Honestly Significant Difference (HSD) tests were used for pairwise comparisons, maintaining a 95 % confidence level.

Table 3: Summary of treatment combination under field conditions

Treatments	Describe
F1	Without Urea fertilizer and bacterial inoculation
F2	With 30 kg urea ha^{-1}
F3	With 60 kg urea ha^{-1}
F4	With 90 kg urea ha^{-1}
F5	Without urea fertilizer + with bacterial inoculation of TL8
F6	With 30 kg urea ha^{-1}+ with bacterial inoculation of TL8
F7	With 60 kg urea ha^{-1}+ with bacterial inoculation of TL8
F8	With 90 kg urea ha^{-1}+ with bacterial inoculation of TL8

3 RESULTS AND DISCUSSION

3.1 ISOLATION AND CHARACTERIZATION OF ENDOPHYTIC BACTERIA FROM CASSAVA

Eleven strains of endophytic bacteria (TL1 to TL11) were isolated from root, stem, and leaf samples of native cassava (Table 4). Two strains were obtained from stem samples, four from leaf samples, and six from root samples. The isolated bacterial strains predominantly displayed round colonies with small sizes ranging from 0.2 to 0.6 cm, appearing glossy, convex, and translucent white.

Research into endophytic bacteria from cassava plants has demonstrated their significant potential in promoting plant growth and health through various mechanisms, such as nitrogen fixation, phosphate solubilization, and IAA production (Ferreira et al., 2021; Feng et al., 2023; Ferreira et al., 2024).

In terms of nitrogen fixation, only four isolated bacterial strains produced NH_4^+, with strain TL8 showing the highest production at 14.95 mg l^{-1}. This NH_4^+ production was higher than that reported by Zhang et al., (2022), where strain A02 produced 13.38 mg l^{-1}. Therefore, strain TL8 might play a critical role in providing nitrogenous compounds for plant development.

Phosphate solubilization is another critical trait observed in cassava-associated endophytic bacteria. Many isolates could solubilize inorganic phosphate, enhancing phosphorus availability in the soil, which is essential for plant growth. This trait is particularly beneficial for cassava, often cultivated in low-fertility soils with limited phosphorus availability (Omondi et al., 2018; Zhang et al., 2022; Feng et al., 2023; Ferreira et al., 2024). Strains TL4 (15.51 mg l^{-1}) and TL8 (14.27 mg l^{-1}) showed the highest phosphorus solubilization. According to Zhang et al. (2022), strain A02 produced 101.23 mg l^{-1} of phosphorus solubilization in 8 days after incubation. The data suggest that strains TL4 and TL8 present significant promise for the biofertilizer industry.

Additionally, the ability of endophytic bacteria to produce IAA, a plant hormone regulating growth and development, has been well-documented (Zhang et al., 2022; Feng et al., 2023; Ferreira et al., 2024). Many isolates, especially from cassava roots, showed positive results for IAA production (Ferreira et al., 2021; Zhang et al., 2022). IAA stimulates root elongation and improves nutrient uptake, supporting overall plant growth and resilience. Six endophytic bacterial strains in our study synthesized IAA, with the highest synthesis by TL8 (46.57 mg l^{-1}), followed by TL7 (30.51 mg l^{-1}) and TL3 (21.15 mg l^{-1}). The lowest IAA synthesis was by TL1 at 11.23 mg l^{-1}. Ferreira et al. (2021) reported strain A02 produced 1.56 mg l^{-1} of IAA in 2 days. These results indicate that strain TL8 could potentially be applied to produce IAA, promoting plant growth.

Based on their superior abilities in nitrogen fixation, phosphorus solubilization, and IAA synthesis, strains TL4 and TL8 were selected for further study.

Table 4: Characteristics of endophytic bacteria isolated from the cassava plant

Isolates	Source	Amount of NH_4^+ (mg l^{-1})	Amount of soluble PO_4^{3-} (mg l^{-1})	Amount of IAA (mg l^{-1})
TL1	Stem	-	-	11.23 ± 0.08^{d}
TL2		-	6.43 ± 0.12^{cd}	-
TL3	Leaf	-	-	21.15 ± 0.04^{c}
TL4		4.31 ± 0.11^{b}	15.51 ± 0.09^{a}	15.12 ± 0.05^{d}
TL5		-	-	-
TL6		3.24 ± 0.03^{c}	8.71 ± 0.12^{c}	-
TL7	Root	-	3.09 ± 0.13^{e}	30.51 ± 0.09^{b}
TL8		14.95 ± 0.21^{a}	14.27 ± 0.21^{b}	46.57 ± 0.11^{a}
TL9		-	-	-
TL10		3.21 ± 0.04^{c}	3.12 ± 0.11^{e}	14.23 ± 0.07^{d}
TL11		-	5.25 ± 0.21^{d}	-

Data present means ± SD (n = 3). Values in the same column with the same letter(s) are not significantly different as determined by the least significant difference (HSD) test ($p < 0.05$).

Molecular identification indicated that TL4 and TL8 were closely related to *Burkholderia cenocepacia* and *Priestia aryabhattai*, with percentage identities of 98.85 % and 98.74 %, respectively. The sequences were deposited in GenBank with accession numbers PQ113673 and PQ119839. These endophytic bacterial strains were further studied under greenhouse and field conditions to highlight their potential as biofertilizers in sustainable agriculture practices.

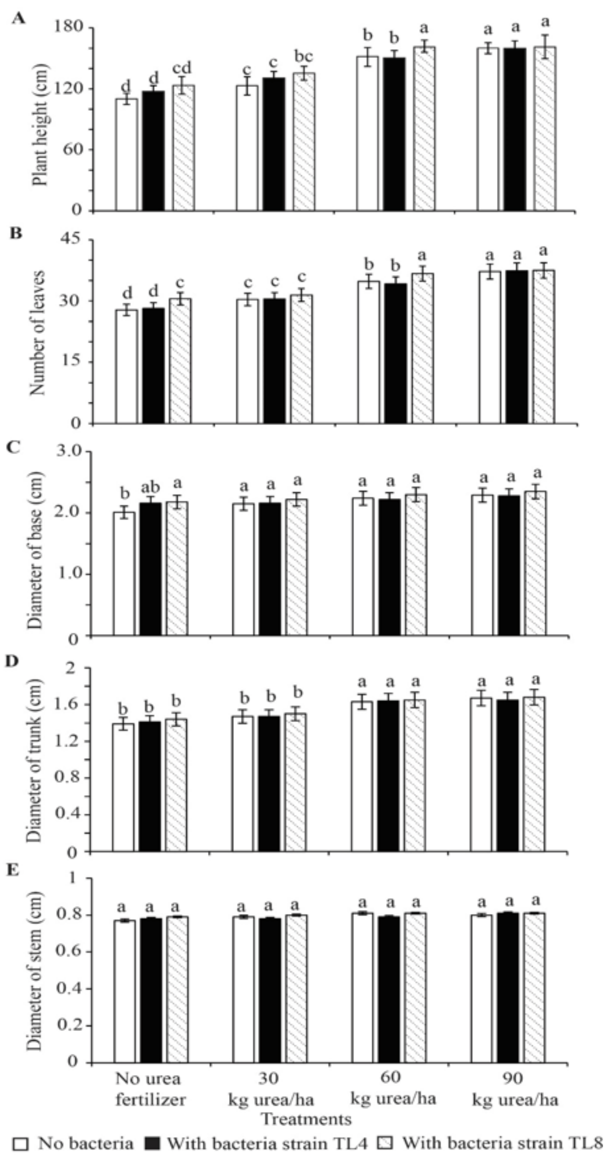

Figure 1: Effects of endophytic bacterial strains TL4 and TL8 combined with the doses of nitrogen fertilizer on (A) cassava plant height; (B) number of leaves; (C-E) Diameter of base, trunk, and stem at 90 DAP under greenhouse conditions. Plotted data present means ± SD (n = 3), the different letter(s) indicate significant differences as determined by the least significant difference (HSD) test ($p < 0.05$).

3.2 EFFECTS OF ENDOPHYTIC BACTERIAL STRAIN TL4 AND TL8 COMBINED WITH NITROGEN FERTILIZER DOSES ON THE GROWTH AND YIELD OF CASSAVA UNDER GREENHOUSE CONDITIONS

The results of this study underscore the significant role that nitrogen fertilization and bacterial inoculation play in the growth and yield of cassava under greenhouse conditions. At 90 days after planting (DAP), statistically significant differences in plant height were observed between the nitrogen fertilization and bacterial inoculation experiments (Figure 1A). Plant height in the nitrogen fertilization experiments ranged from 110.13 to 153.19 cm, with the lowest heights in the non-nitrogen-fertilized plants. Among the bacterial inoculation treatments, plant height ranged from 122.34 to 151.26 cm, with the highest in plants inoculated with strain TL8 (151.26 cm). Additionally, the number of leaves (Figure 1B), base diameter (Figure 1C), trunk diameter (Figure 1D), and stem diameter (Figure 1E) showed statistically significant differences among the nitrogen fertilization treatments. The lowest number of leaves, base diameter, trunk diameter, and stem diameter were observed in the non-nitrogen fertilized plants. This is because nitrogen significantly influences cassava growth and development, promoting robust leaf, stem, and root development compared to nitrogen-deficient plants (Rafikova et al., 2016; Xing et al., 2016).

Moreover, the concentrations of total N in cassava plants' leaves, stems, and roots inoculated with endophytic bacteria TL8 were increased compared to the control. Total N in the stems and roots did not statistically differ between experiments; however, total N in the leaves differed, and the inoculation of TL8 had the highest concentrations (Table 5). These results are in agreement with the results of previous reports (Szilagyi-Zecchin et al., 2014; Li et al., 2017) which demonstrated that cassava stems inoculated with endophytic bacteria led to a higher amount of nitrogen content in the leaves. Nitrogen uptake is crucial for cassava productivity as it enhances photosynthesis, supports protein and enzyme synthesis, and maintains a balanced carbon-to-nitrogen ratio. Adequate nitrogen improves chlorophyll content, promotes root development, and leads to greater tuber biomass and starch accumulation (Feng et al., 2023). Studies confirm that nitrogen-deficient cassava shows reduced growth and yield, while optimal nitrogen fertili-

Table 5: Effect of endophytic bacteria TL8 on the nitrogen content of cassava plant in a greenhouse experiment

Treatment	Nitrogen content (mg g^{-1})		
	Leaves	Stems	Roots
With bacterial TL8 inoculation only	46.87 ± 0.43a	13.58 ± 0.51a	13.08 ± 0.61a
Without nitrogen fertilizer and bacterial TL8 inoculation	35.96 ± 0.74b	13.04 ± 0.46a	12.73 ± 0.58a

Data present means ±SD (n = 3). Values in the same column with the same letter(s) are not significantly different as determined by the least significant difference (HSD) test ($p < 0.05$).

zation improves nutrient use efficiency, boosting productivity sustainably (Feng et al., 2023).

Bacterial inoculation with strain TL8 led to the highest plant height (151.26 cm), suggesting superior nitrogen-fixation capabilities compared to strain TL4. This finding aligns with studies highlighting the potential of specific endophytic bacterial strains to enhance plant growth by improving nitrogen availability (Zhang et al., 2022). The observed statistical differences in the number of leaves, base diameter, and stem diameter further support the importance of adequate nitrogen supply (Feng et al., 2023).

Tuber characteristics, including the number of tubers per pot (Figure 2A), tuber diameter (Figure 2B), and tuber length (Figure 2C), differed statistically among the nitrogen fertilization treatments. The combination of 60 kg urea ha^{-1} with bacterial inoculation showed significant differences compared to the no-nitrogen treatment and the 30 kg urea ha^{-1} treatment with bacterial inoculation, but not from the 90 kg urea ha^{-1} treatment combined with bacterial inoculation. Among the bacterial inocula-

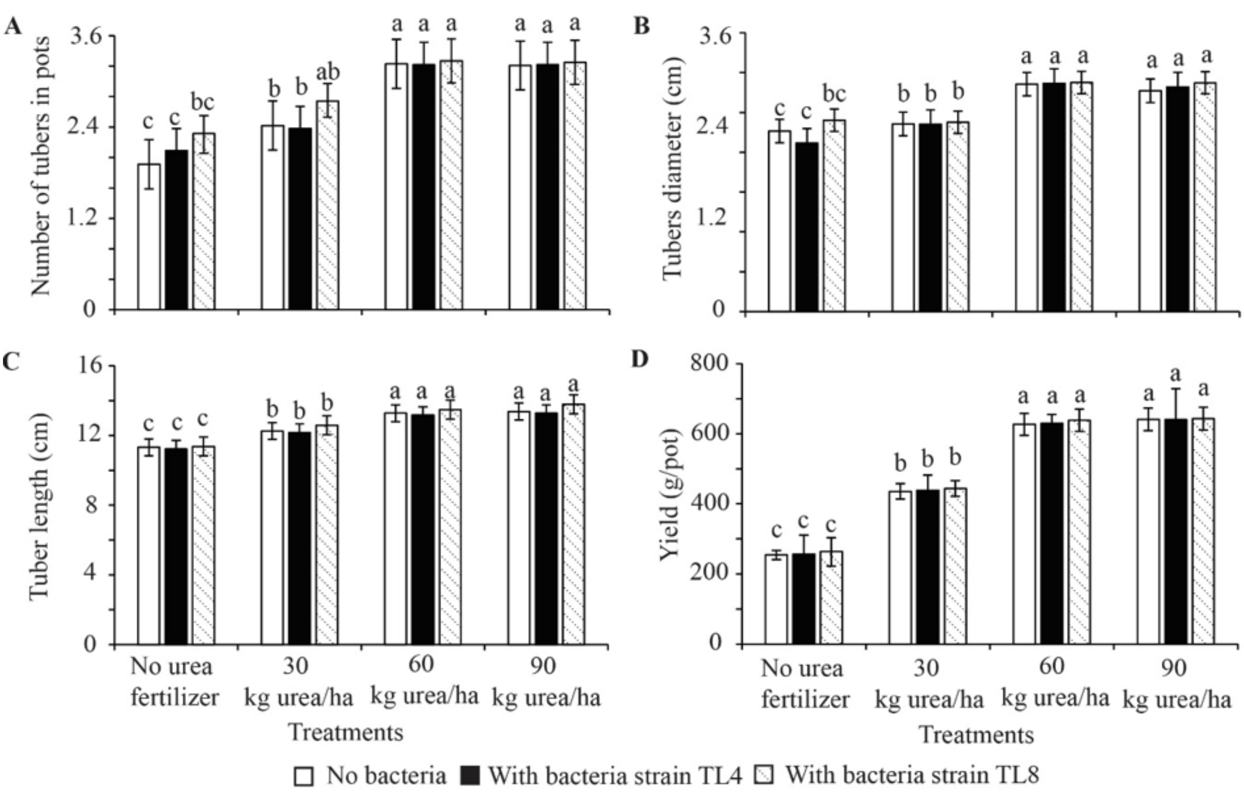

Figure 2: Effects of endophytic bacterial strains TL4 and TL8 combined with the doses of nitrogen fertilizer on (A) the number of tubers per 10 m2; (B) Length of tubers; (C) Diameter of tuber; and (D) Yield at harvest under greenhouse conditions. Plotted data present means ± SD (n = 3), the different letter(s) indicate significant differences as determined by the least significant difference (HSD) test ($p < 0.05$).

tion treatments, significant differences were observed for the number of tubers per pot, tuber diameter, and tuber length, with the highest values for these parameters in the TL8 inoculation.

Tuber yield per pot also differed significantly between the nitrogen fertilization and bacterial inoculation treatments (Figure 2D), with yields ranging from 254.37 to 640.94 g pot^{-1}. The lowest yield was observed without nitrogen fertilization (254.37 g pot^{-1}), and the lowest yield among bacterial inoculation treatments was without bacterial inoculation (441.69 g pot^{-1}). Providing sufficient nitrogen enhances cassava growth and increases tuber yield (Uwah et al., 2013; Zhang et al., 2022). Among the two experimental bacterial strains, TL8 increased the number of tubers, tuber diameter, and tuber length, enhancing cassava yield compared to TL4. This superior performance may be attributed to TL8's higher nitrogen-fixation activity, IAA production, and phosphate solubilization, which enhance nutrient availability and uptake by the plant (Biswas et al., 2022; Argotte-Ibarra et al., 2022; Do et al., 2023).

Overall, this study demonstrates that nitrogen fertilization and bacterial inoculation, particularly with strain TL8, significantly improve cassava growth and yield under greenhouse conditions. Further research exploring the long-term effects of these treatments with strain TL8 under field conditions is necessary to refine cassava cultivation practices.

3.3 EFFICACY OF BACTERIA TL8 ON THE GROWTH AND YIELD OF CASSAVA UNDER FIELD CONDITIONS

At 90 DAP, plant heights across the experiments were statistically different (Figure 3A), ranging from 108 to 153 cm. The application of 60 kg urea ha^{-1} combined with TL8 inoculation (F6) produced plant heights similar to those achieved with 90 kg urea ha^{-1} without bacterial inoculation (F7). The lowest plant height was observed in treatments without nitrogen fertilization and bacterial inoculation (F1). The number of leaves also showed statistically significant differences (Figure 3B), with the lowest number (30.28 leaves) in the non-nitrogen fertilization experiments (F1). These data indicate the potential application of strain TL8 to enhance the nitrogen efficiency of cassava plants under field conditions.

Base and trunk diameters were significantly different (Figures 3C and 3D), with the highest diameters found in the treatments with 60 kg urea ha^{-1} + strain TL8 (F6), 90 kg urea ha^{-1} + no bacterial inoculation (F7), and 90 kg urea ha^{-1} + strain TL8 (F8). This aligns with previous research demonstrating that adequate nitrogen supply, especially when combined with beneficial bacterial inoculants, can significantly enhance structural growth parameters (Biswas et al., 2022; Zhang et al., 2022, Aasfar et al., 2024).

The diameter of the stem across the experiments did not show any statistically significant differences, ranging from 0.79 to 0.88 cm (Figure 3E). This consistency suggests that while nitrogen and bacterial treatments influence overall plant height and biomass, they might not significantly alter certain morphological traits under the given experimental conditions. Similar findings have been reported in studies on other crops, where the impact of nitrogen-fixing bacteria was more pronounced on overall growth and yield metrics rather than specific morphological characteristics (Aasfar et al., 2024). Additionally, applying 60 kg urea ha^{-1} combined with TL8 bacterial inoculation (F6) resulted in cassava growth not statistically different from that achieved with 90 kg urea ha^{-1} without bacterial inoculation (F7). This is particularly relevant for sustainable agriculture, where reducing the dependency on chemical fertilizers can have significant environmental benefits (Do et al., 2023; Aasfar et al., 2024). The ability of TL8 to enhance nitrogen efficiency could lead to more sustainable cassava production practices, aligning with global efforts to minimize agricultural inputs while maintaining high yields.

Further supporting these findings, previous research has shown that the application of nitrogen-fixing bacteria can lead to increased plant height and dry weight in various crops, highlighting their role in improving nitrogen availability and utilization (Xu et al., 2018; Do et al., 2023; Aasfar et al., 2024). These studies suggest that the beneficial effects of bacterial inoculants are not limited to cassava but extend to other rooted crops as well.

The number of tubers differed significantly among the experiments (Figure 4A), ranging from 31.35 to 58.17 tubers. The lowest number of tubers (31.35) was observed in the no-nitrogen fertilization treatment (F1). This finding aligns with the general understanding that nitrogen is a key nutrient for promoting tuber growth and overall plant productivity (Zhang et al., 2022; Aasfar et al., 2024).

Tuber length also showed statistically significant differences, with the highest lengths recorded in the treatments including F6 (60 kg urea ha^{-1} + TL8 inoculation), F7 (90 kg urea ha^{-1} without bacterial inoculation), and F8 (90 kg urea ha^{-1} + TL8 inoculation) (Figure 4B). Similarly, tuber diameter varied significantly at the 5 % level, ranging from 4.55 to 5.48 cm, with the smallest diameter (4.55 cm) in the F1 treatment (Figure 4C). These results highlight the beneficial effects of adequate nitrogen supply, whether through fertilization or bacterial inoculation, on tuber size and quality. Moreover, the results indicated that applying 60 kg urea ha^{-1} combined with strain TL8 (F6) achieved similar numbers, lengths, and diameters of tubers as the 90 kg urea ha^{-1} without bacterial inoculation (F7). This suggests that the nitrogen-fixing ability of strain TL8 contributed additional nitrogen to the cassava plants, allowing the F6 treatment to match the performance of the F7 treatment. Previous research supports these findings, showing that nitrogen-fixing microbial fertilizers can enhance plant growth and yield (Zhang et al., 2022; Aasfar et al., 2024).

Combining nitrogen fertilization with bacterial inoculation significantly impacts cassava tuber yield, demonstrating the potential for optimizing fertilizer use while maintaining high productivity. Figure 4D indicates that tuber yield did not differ significantly between the treatments of F7 (18.49 t ha^{-1}), F6 (18.42 t ha^{-1}), and F8 (19.15 t ha^{-1}). However, these treatments showed a 1.12% higher yield compared to the no-nitrogen treatments (F1 at 9.83 t ha^{-1} and F2 at 10.86 t ha^{-1}), as well as the F4 (15.93 t ha^{-1}), F3 (13.69 t ha^{-1}), and F5 (15.79 t ha^{-1}) treatments. These results suggest that using 60 kg urea ha^{-1} combined with strain TL8 achieved similar yields to the application of 90 kg urea ha^{-1} without bacterial inoculation, indicat-

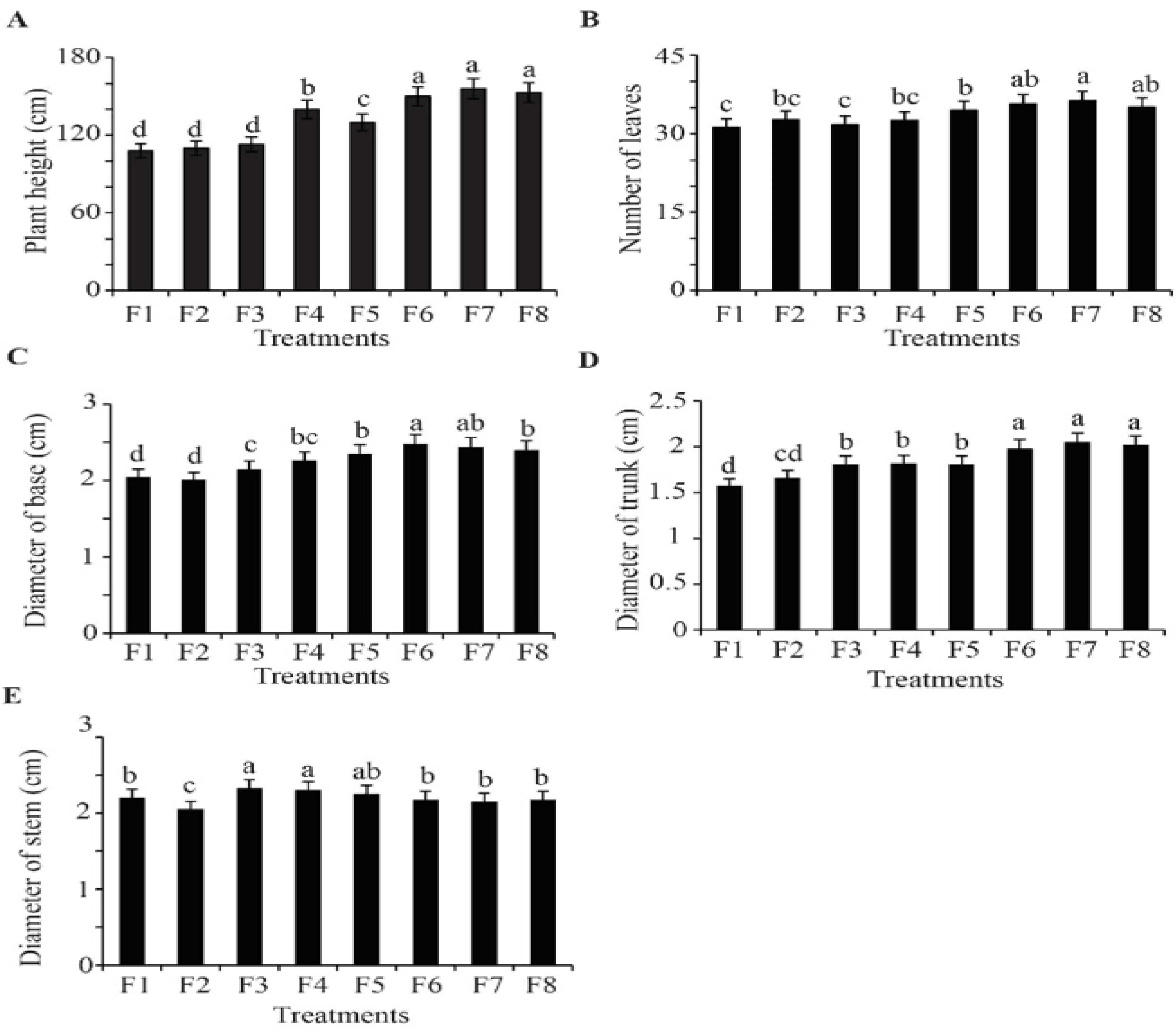

Figure 3: Effects of bacterial strain TL8 combined with the doses of nitrogen fertilizer on (A) cassava plant height; (B) number of leaves; (C-E) Diameter of base, trunk, and stem at 90 DAP under field conditions. F1: without urea fertilizer and bacterial inoculation; F2: without urea fertilizer and with bacterial strain TL8; F3: with 30 kg urea ha^{-1} and without bacterial strain TL8; F4: with 30 kg urea ha^{-1}and with bacterial strain TL8; F5: with 60 kg urea ha^{-1} and without bacterial strain TL8; F6: with 60 kg urea ha^{-1} and with bacterial strain TL8; F7: with 90 kg urea ha^{-1} and without bacterial strain TL8; F8: with 90 kg urea ha^{-1} and with bacterial strain TL8. Plotted data present means ± SD (n = 3), the different letter(s) indicate significant differences as determined by the least significant difference (HSD) test ($p < 0.05$).

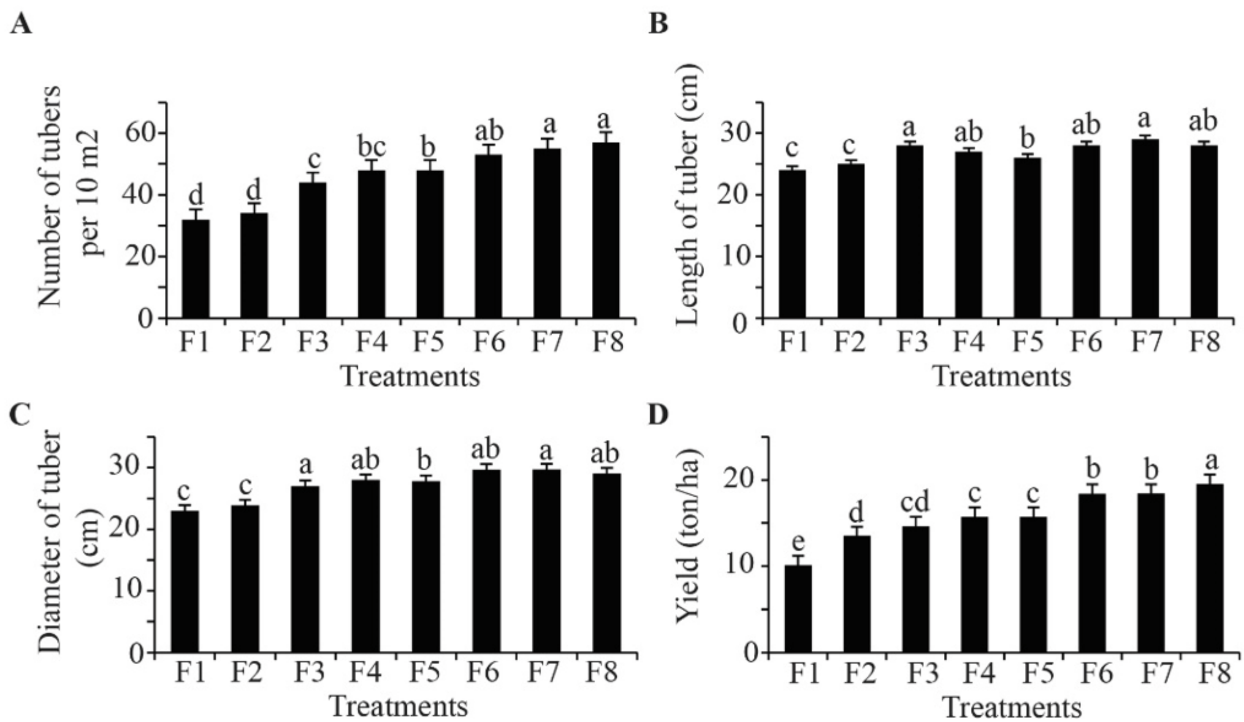

Figure 4: Effects of bacterial strain TL8 combined with nitrogen fertilizer doses on (A) the number of tubers per 10 m2; (B) Length of tubers; (C) Diameter of tuber; and (D) Yield at harvest under field conditions. F1: without urea fertilizer and bacterial inoculation; F2: without urea fertilizer and with bacterial strain TL8; F3: with 30 kg urea ha^{-1} and without bacterial strain TL8; F4: with 30 kg urea $ha^{-1}$and with bacterial strain TL8; F5: with 60 kg urea ha^{-1} and without bacterial strain TL8; F6: with 60 kg urea ha^{-1} and with bacterial strain TL8; F7: with 90 kg urea ha^{-1} and without bacterial strain TL8; F8: with 90 kg urea ha^{-1} and with bacterial strain TL8. Plotted data present means ± SD (n = 3), the different letter(s) indicate significant differences as determined by the least significant difference (HSD) test ($p < 0.05$).

ing that a reduction of 30 kg urea ha^{-1} could be feasible for cassava cultivation. This finding aligns with studies by Zhang et al. (2022) and Aasfar et al. (2024), which suggest that nitrogen-fixing bacteria can reduce the reliance on nitrogen fertilizers, emphasizing their secondary role when effective bacterial inoculation is applied.

The observed results underscore the benefits of integrating nitrogen-fixing bacterial inoculants into fertilization practices. By reducing the reliance on chemical fertilizers, farmers can achieve cost savings and minimize environmental impacts, such as nitrogen leaching and soil degradation. This approach aligns with sustainable agricultural practices and supports the shift toward more eco-friendly farming methods (Zhang et al., 2022).

4 CONCLUSIONS

The integration of nitrogen-fixing endophytic bacteria, specifically strain TL8, with nitrogen fertilization demonstrates significant potential for enhancing cassava growth and yield. The study's findings highlight the superior performance of TL8 in nitrogen fixation, phosphate solubilization, and IAA synthesis, contributing to improved plant height, leaf number, and tuber yield under both greenhouse and field conditions. The ability of TL8 to achieve similar yields with reduced nitrogen fertilization (60 kg urea ha^{-1}) as conventional higher fertilization rates (90 kg urea ha^{-1}) underscores its role in promoting sustainable agricultural practices. This approach not only enhances crop productivity but also reduces the reliance on chemical fertilizers, offering environmental and economic benefits. Further research is warranted to explore the long-term impacts and optimize the application of these bacterial inoculants in diverse agricultural settings.

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Effect of boron spraying, potassium levels, and irrigation interval on some physiological traits and cabbage productivity

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Abstract: Water shortages directly impact human malnutrition and agricultural productivity. However, the ideal irrigation times must accommodate crop seasons in various climates and address the effects of climate change. Thus, this research aimed to quantify the impact of fertilizers, including boron and potassium, and irrigation intervals on cabbage. In 2021, two irrigation intervals, 3 and 6 days were used in a field trial. Potassium fertilizer was applied at 0. 75 and 150 kg K ha^{-1}. Boron was sprayed at 0. 50, and 100 mg B l-1 using boric acid. The data showed that antioxidants were increased at the 6-day irrigation interval compared to the 3-day irrigation interval. Proline, peroxidase, carotene, and catalase enzymes increased at the longer irrigation interval. Since plants use potassium to maintain their water content, potassium was associated with increased antioxidants and the rate of boron fertilizer application. The highest proline content in plant tissue was in the control treatment 22.90 µg g^{-1} FM (fresh mass) and 31 .23 µg g^{-1} FM for 3 and 6-day irrigation intervals, respectively. The control treatment achieved a higher peroxidase content value at the 6-day irrigation interval (47.20 µg g^{-1} FM). The highest total cabbage yield was observed at the 3-day irrigation interval.

Key words: boron spraying, potassium levels, irrigation interval, cabbage, proline content, carotene, peroxidase enzyme

Učinki škropljenja s pripravki bora, dodatki kalija in intervalov namakanja na nekatere fiziološke lastnosti zelja

Izvleček: Pomankanje vode neposredno prizadane prehrano ljudi in pridelavo v kmetijstvu. Pri namakanju morajo biti časovni presledki namakanja prilagojeni fenološkim fazam poljščin v različnih podnebnih razmerah, upoštevajoč spremembe podnebja. Namen raziskave je bil opredeliti učinke gnojenja z borom in kalijem ter intervale namakanja na rast zelja. V rastni sezoni 2021 sta bila v poljskem poskusu uporabljena 3 in 6 dnevni interval namakanja. Kalijeva gnojila so bila uporabljena v odmerkih 0, 75, in 150 kg K ha$^{-1.}$ Škropljenja z borom so bila v odmerkih 0, 50 in 100 mg B l^{-1} z uporabo borove kisline. Podatki iz raziskave so pokazali, da se je vsebnost antioksidantov povečala pri šestdnevnih intervalih namakanja v primerjavi s tridnevnimi intervali. Vsebnosti prolina, peroksidaze, karotena in katalaze so se povečale pri daljših intervalih namakanja. Vsebnosti odmerkov kalija in gnojenja z borom so bile povezane s povečanjem vsebnosti antioksidantov zaradi vloge kalija pri ohranjanju vsebnosti vode v rastlinah. Največja vsebnost prolina v rastlinskih tkivih je bila izmerjena v kontrolnem obravnavanju in sicer 22,90 µg g^{-1} in 31,23 µg g^{-1} na svežo maso pri 3 in 6-dnevnih intervalih namakanja. Kontrolna obravnavanja so imela večjo vsebnost peroksidaze pri 6-dnevnem intervalu namakanja, 4720 µg g^{-1} na svežo maso. Največji pridelek zelja je bil izmerjen pri 3-dnevnem intervalu namakanja.

Ključne besede: škropljenej z borom, vsebnosti kalija, intervali namakanja, zelje, vsebnost prolina, karotena, peroksidaze

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1 INTRODUCTION

Cabbage is a leafy crop belonging to the Brassicaceae family. It can be planted for the leafy head and the bud as a yield in cold weather. According to Ahmed et al. (2023), cabbage leaves can be cooked, simmered, or immersed in vinegar. Cabbage is grown in Iraq in an area estimated at 1170 ha, with total productivity valued at 16.24 Mg ha^{-1} UNESCO (2019). Cabbage is deemed rich in nutrients, including vitamins C and E and the minerals potassium and calcium, Altıntaş et al. (2024). Moreover, cabbage is an outstanding source of B vitamins, including thiamine, folate, and riboflavin. Given that it contains about 4 g of carbohydrates per 100 g of fresh mass, 15 g of protein per 100 g of fresh mass, 0.2 g of fatty acids per 100 g of fresh mass, and 93 mL of water per 100 g of fresh mass, it can be a good source of protein, carbohydrates, and hydration. Ibukunoluwa (2015).

Water reservoirs have experienced a progressive reduction, notably in the last two decades, mostly owing to the increased water use by urban, industrial, and agricultural sectors. This is especially evident in arid and semi-arid regions, where climate change is a significant concern and new irrigation techniques for vegetable production exist. (Al-Lami et al.,2023). Establishing periodic irrigation schedules is crucial for maintaining optimal soil moisture levels in crops and enhancing crop yield Abdel-All and Seham (2013). Several studies have provided evidence about irrigation scheduling (Abdel-All and Seham, 2013; Bhattacharyya et al., 2022). A study performed by Pandey et al. (2023) has shown that the timing of irrigation intervals may significantly impact cabbage production, depending on the growth stage. Maximum yield can be achieved when the soil is well-watered and unaffected by water stress. In this scenario, irrigation timing can be employed to mitigate excessive irrigation or under-irrigation. Mndela et al. (2023) have demonstrated that the sustainability of water resources has been enhanced by irrigation schedules for various crops cultivated throughout the year in small-scale fields.

Potassium is an essential macronutrient found in plants, that exerts a substantial influence on the growth and physiological processes of cells throughout crop development (Abdel-All and Seham, 2013; Mahmood et al., 2019; Shahinul et al., 2020). Hydraulic equilibrium, photosynthesis, and nutrition intake are notable contributors (Ahmad et al., 2020; Battie-Laclau et al., 2014; Juma et al., 2024). The inadequacy of K suplay in the edaphic environment interrupts these processes, diminishing crop yield and lessening the photosynthesis process (Battie et al., 2014; He et al., 2022; Salem et al., 2022). In addition to the fertility preference of forage crops, the scarcity of K impairs the absorption of essential nutrients such as phosphorus and nitrogen, which subsequently creates disarray in element equilibrium (Choudhary et al., 2024; Soumare et al., 2023). In addition to the considerable role of nutrient constituents in the soil environment, low K levels have a precise impact on the development of the crops' roots, which is a critical aspect of nutrient and moisture acquisition (Kumar et al., 2015; Xu et al., 2020). Moreover, the soil contains potassium (K) that is unavailable to plants. It can be categorized into three pathways: available K, which includes soluble K and exchangeable K; non-exchangeable K; and mineral K (Abood and Sherif, 2022; Ahmad et al., 2020). Mostly, soil K is introduced to the soil through fertilizers or verdant and decomposed manure (Das et al., 2022). The K demand can make a crop's absorption of K a core concern (El-Mageed et al., 2022). Soil erosion, leaching in porous soils, extensively irrigated soils, and even inattentive soil management practices are all potential causes of K loss from the soil (Guo et al., 2019; Thakur et al., 2023). Although K can be reintroduced into the soil through soil balancing, it can be adversely impacted by excessive nitrogen and phosphorus applications (Das et al., 2022).

Boron is an indispensable micronutrient that is required by most if not all cereals (Farrag et al., 2021; Shahinul et al., 2022). Boron is essential for the transportation of sugar within plants (Bankar et al., 2024; Farag et al., 2022). It boosts the formation of roots and stimulates the development of the meristematic cells. (Kumar et al., 2023). Further, boron is involved in synthesizing proteins, amino acids, and carbohydrates, along with improving ATPase. Moreover, it is necessary for the formation and development(Al-Kahachi et al., 2024; Mostafa et al., 1999; Trees & Iraqi, 1999). Oxidative damage can occur from an insufficient amount of boron (Shahinul et al., 2020). Unquestionably, the accumulation of materials from lipid peroxidation and cell membrane seepage is frequently observed in the presence of low boron availability (Hopkins, 2015; Kumar et al., 2023; Meena et al., 2023). The cells can extrude sugars, ions, and phenolic forms once membranes are damaged (Ullah et al., 2024; Yadav and Kumar, 2023). A concealed hunger is a condition in which crop boron deficiency can manifest without any obvious signs on the plants. Boron shortages can occur in soils that are particularly sandy, calcareous, leached, or contain significant amounts of zinc (Khatun and Reza, 2024 ; Akhtar et al., 2022). Likewise, the xylem and phloem vessels, which play a crucial role in the transportation of water throughout plants, are susceptible to destruction due to the scarcity of boron (Gs et al., 2023; Ullah et al., 2024). Furthermore, a lack of boron has a detrimental impact on the rates of transpiration and the density of stomata. It additionally contributes to the degradation of guard cells, which subsequently damages the process of photosynthesis (Hopkins, 2015). This may imply that plants lacking in boron become more prone to drought stress than plants that have sufficient amounts of boron.(Riwad and Alag, 2023; Shahinul et al., 2020). This study aimed to ascertain how irrigation interval impacts cabbage production and assess the optimal administration of potassium and boron at the most suitable irrigation interval.

2 MATERIALS AND METHODS

2.1 STUDY SITE DESCRIPTION

In the 2021-2022 season, one-year research was conducted at the University of Baghdad, College of Agricultural Engineering Sciences Station. The precipitation throughout the cabbage growth period ranged from 100 to 150 mm, while the temperature ranged from 15 to 25 °C. The soil physiochemical characteristics have been described in Table 1.

2.2 EXPERIMENTAL DESIGN

The experimental design encompassed a split-split design with three replications. The primary plots were allocated to irrigation intervals of 3 and 6 days. The secondary plots consisted of three distinct potassium rates: 0 kg K ha^{-1}, 75 kg K ha^{-1}, and 150 kg K ha^{-1}. These rates were applied three times during the beginning of the season: on the planting day, 20 days after planting, and 40 days after planting time. The potassium was applied as potassium sulfate, which contained 41.5 % K. In the sub-secondary plots, plants were sprayed with boron at dosages of 0, 50, and 100 mg B l^{-1}. This spraying procedure was performed 20, 40, and 60 days after the plants were planted, using boric acid containing a 17.4 % boron concentration. The cabbage ('Glob Master') was sown in a container and subsequently transplanted to the field after 30 days. The cabbage was planted in rows, with a spacing of 70 cm between rows and 40 cm between plants inside the row. A distance of around 2 meters was preserved between replicates to avoid the movement of nutrients or irrigation water between treatments. Urea was applied at 100 kg N ha^{-1}, while diammonium phosphate (DAP) was utilized to apply 75 kg P ha^{-1}. Weeds were removed manually as needed. The experimental treatments for both irrigation intervals of 3 and 6 days are set as follows:

Treatments	Description
T1	Control (0 kg K ha^{-1} + 0 mg B l^{-1})
T2	(0 kg K ha^{-1} + 50 mg B l^{-1})
T3	(0 kg K ha^{-1} + 100 mg B l^{-1})
T4	(75kg K ha^{-1} + 0 mg B l^{-1})
T5	(75 kg K ha^{-1} + 50 mg B l^{-1})
T6	(75 kg K ha^{-1} + 100 mg B l^{-1})
T7	(150 kg K ha^{-1} + 0 mg B l^{-1})
T8	(150 kg K ha^{-1} + 50 mg B l^{-1})
T9	(150 kg K ha^{-1} + 100 mg B l^{-1})

2.3 SOIL SAMPLE ANALYSIS

Soil samples were gathered at the study site at a depth of 0–25 cm before planting cabbage and continued to be collected for 80 days after the cabbage planting date. The soil samples are placed through the process of air drying out, crushing, and then passing through a sieve with a mesh size of 2 mm. The combined concentration of nitrogen in the form of nitrate ($N\text{-}NO_3^-$) and ammonium ($N\text{-}NH_4^+$) was determined as available nitrogen using the Kjeldahl method. The phosphorus concentration was measured using the Olsen method with a spectrophotometer at an absorption wavelength of 882 nm. Phosphorus (P), a macronutrient, was quantified using a flame photometer.

2.4 PLANT SAMPLING AND ANALYSIS

Five plants were picked for each treatment of nitrogen (N), phosphorus (P), potassium (K), and boron (B) analysis. Four inner leaves of cabbage were selected from each plant before being matured. The leaves were rinsed with distilled water to remove any dust or contaminants that could potentially impact the accuracy during the analysis. The leaves were dried in an oven with airflow at a temperature of 65 °C for 96 hours, and subsequently ground. The crushed material was prepared for analysis.

2.5 CABBAGE YIELD DETERMINATION

The cabbage head was randomly selected for six plants in each plot. Several harvesting dates were used to calculate the cabbage yield. The cabbage yield per hectare was determined by measuring the kilograms obtained from each experimental patch.

Table 1:. Physiochemical and fertility characteristics of soil

Soil Properties	Value	Unit
pH	7.26	-
EC	2.95	dS m^{-1}
$CaCO_3$	327.19	g kg^{-1} soil
O. M	1.19	%
N	17.80	mg kg^{-1} soil
P	11.23	mg kg^{-1} soil
K	135.01	mg kg^{-1} soil
B	1.12	mg kg^{-1} soil
Texture	Silty loam	

2.6 PROLINE ACID CONCENTRATION DETERMINATION

The analysis of proline was conducted by the methodology outlined by Tamayo & Bonjoch (2006). The proline concentration in the leaves was analyzed using toluene dye (methylbenzene). An acid-ninhydrin solution was made by dissolving 1.25 grams in 30 milliliters of glacial acetic acid and 20 milliliters of 6 M phosphoric acid. The solution was then stored in a refrigerator at a temperature of 4 degrees Celsius in a dark location for one day. Ground 0.5 grams of fresh plant tissue with 10 milliliters of a 3 % solution of sulphosalicylic acid to precipitate proteins. Afterward, the mixture was filtered using filter paper. Two milliliters of filtered materials, which were previously prepared, are inserted in a test tube. The test tubes were treated with 2 ml of glacial acetic acid and 2 ml of acid ninhydrin reagents. The test tubes were immersed in a water bath at a temperature of 95 degrees Celsius, while other test tubes were directly placed in an ice bath. Each test tube was filled with four milliliters of toluene dye and then shaken. The mixture was left undisturbed for approximately thirty minutes. The color transitioned to a red hue, and the samples were prepared for measurement using a spectrophotometer at an absorbance wavelength of 520 nm.

2.7 CAROTENE CONCENTRATION DETERMINATION

Beta carotene was determined following the methodology described by Srivastava and Kumar (2004). Pulverized 10 g of recently harvested plant tissue using a ceramic mortar. Introduced a small amount of anhydrous sodium sulfate and 10 ml of acetone. A volume of 10 ml was extracted from the sample, thereafter, subjected to filtration, and then mixed with 15 ml of petroleum ether. The plant samples were combined with reagents and then immersed in a water bath. After approximately 10 minutes, the mixture was transferred into a test tube. The volume was adjusted up to 100 ml by adding 10 ml of petroleum ether, and then it was measured using a spectrophotometer at an absorption wavelength of 452nm.

2.8 VITAMINE C DETERMINATION

The measurement of Vitamin C was accomplished according to the methodology stated by Yurena et al. (2006). One gram of fresh plant tissue was crushed, followed by the application of oxalic acid. The samples were refrigerated at a temperature of 4 degrees Celsius for 12 hours in a location devoid of light. The materials underwent filtration and were then combined with 0.5 ml of a mixture containing phosphoric and acetic acid. Two milliliters of ammonium molybdate were initially added, and subsequently, the volume was adjusted to reach a total of 25 ml. The measurement of this sample was conducted using a spectrophotometer, specifically at a wavelength absorbance of 760 nm.

2.9 DETERMINATION OF PEROXIDASE

The primary reagent utilized is Guaiacol liquid, which is prepared by adding 1.36 ml to a flask and then completing the volume to 250 ml with distilled water. A spectrophotometer was used to measure the absorbance of one milliliter of hydrogen peroxide at a wavelength of 420 nm. Enzyme activity was quantified by adding 2 ml of the reaction mixture(Shabnam et al., 2016).

2.10 TOTAL CHLOROPHYLL DETERMINATION

One gram of freshly crushed plant tissue was combined with 20 ml of cooled acetone (85 %). The sample, which has been filtered, has a volume of 100 ml after being finished with water. Centrifuge the previously applied samples at 5000 rpm for 5 minutes in the centrifuge. The samples were analyzed using a spectrophotometer at certain wavelengths of absorbance, namely 663 nm and 644 nm (Witham et al., 1971), and then calculated using the following formula:

$$\text{Total Chlorophyll} = [20.2 * D\ (644\ nm) + 8.02 * D\ (663\ nm)] * V / 1000 * M$$

D denotes the light intensity.
V is the final volume of extraction 100 m
M is the mass of plant tissue.

2.11 STATISTICAL ANALYSIS OF DATA

The study dataset tested at the least significant differences (LSD) at a probability level of $p < 0.05$. The software used to analyze the data was R Studio version 2024.04.0+735, Boston, MA, USA. All figures were performed using Microsoft Excel (Microsoft Corporation, USA).

3 RESULTS

3.1 IMPACT OF BORON, POTASSIUM, AND IRRIGATION INTERVALS ON PROLINE CONTENT, PEROXIDASE, AND CAROTINE

The proline concentration in cabbage leaves was measured, and it was found that the highest average value

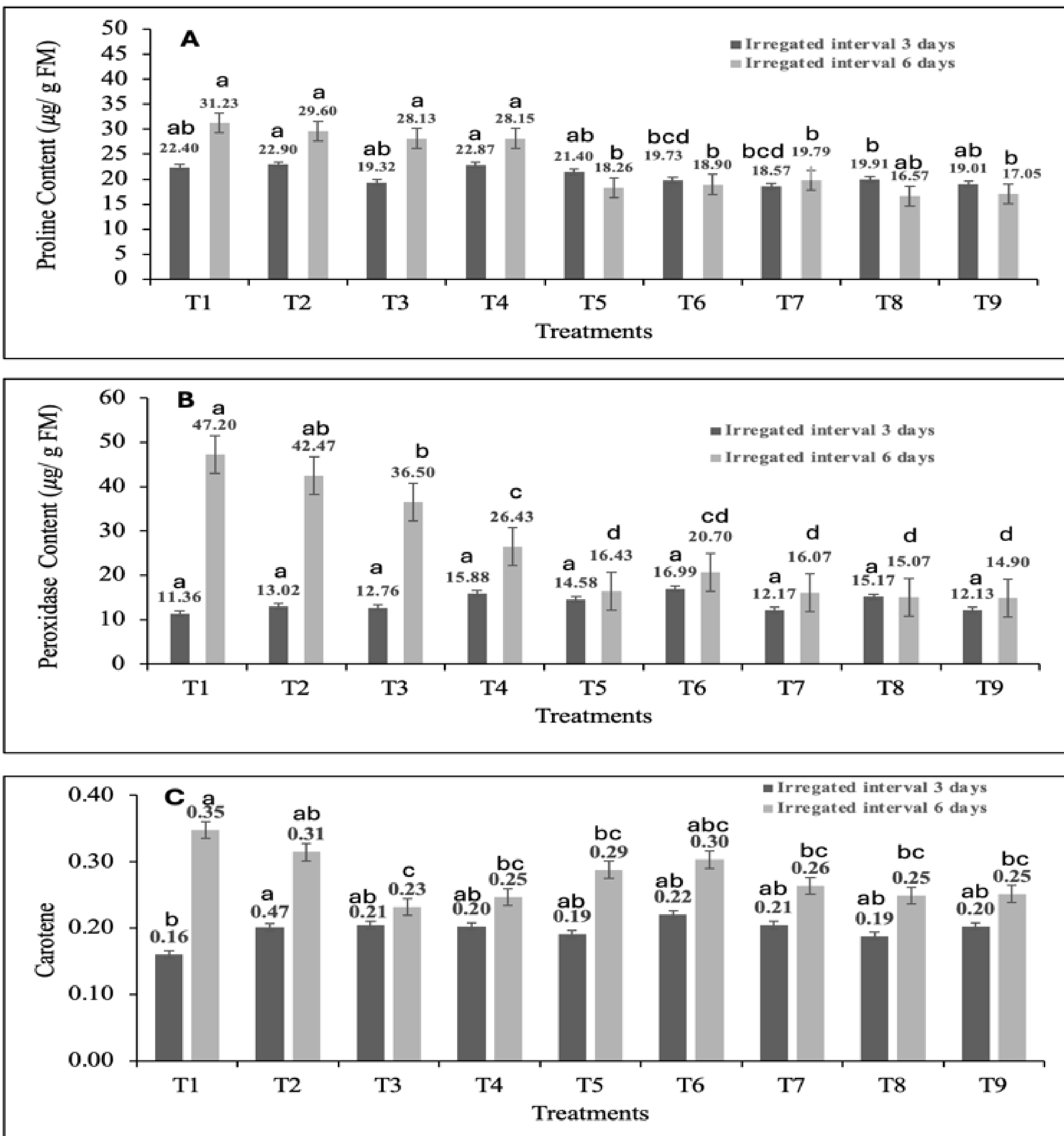

Figure 1: Effect of irrigation intervals, potassium, and boron on (A.) proline content, (B.) peroxidase content, (C.) carotene. The different letters denote significant differences among the mean values of treatments.

was observed at T2 and T1, which were 22.90 μg g^{-1} FM and 31.23 μg g^{-1} FM, respectively. These values represent the control and K0B1 (No potassium + 50 mg B l^{-1}) treatments with irrigation intervals of three and six days. The proline content reached its minimum value at treatment T5 with K1B1 (75kg K ha^{-1} and 50 mg B l^{-1} application), measuring 18.26 μg g FM. There are no significant variations among the T1, T2, T3, T4, and T8 treatments when irrigated every six days, as shown in Figure 1a. The only notable distinction observed was in the T6 and T7 treatments when there was a six-day delay between irrigations compared to the other treatments. However, no substantial distinction was observed between them. Nevertheless, independent of the treatments, the values obtained from irrigating at six-day intervals were more valuable

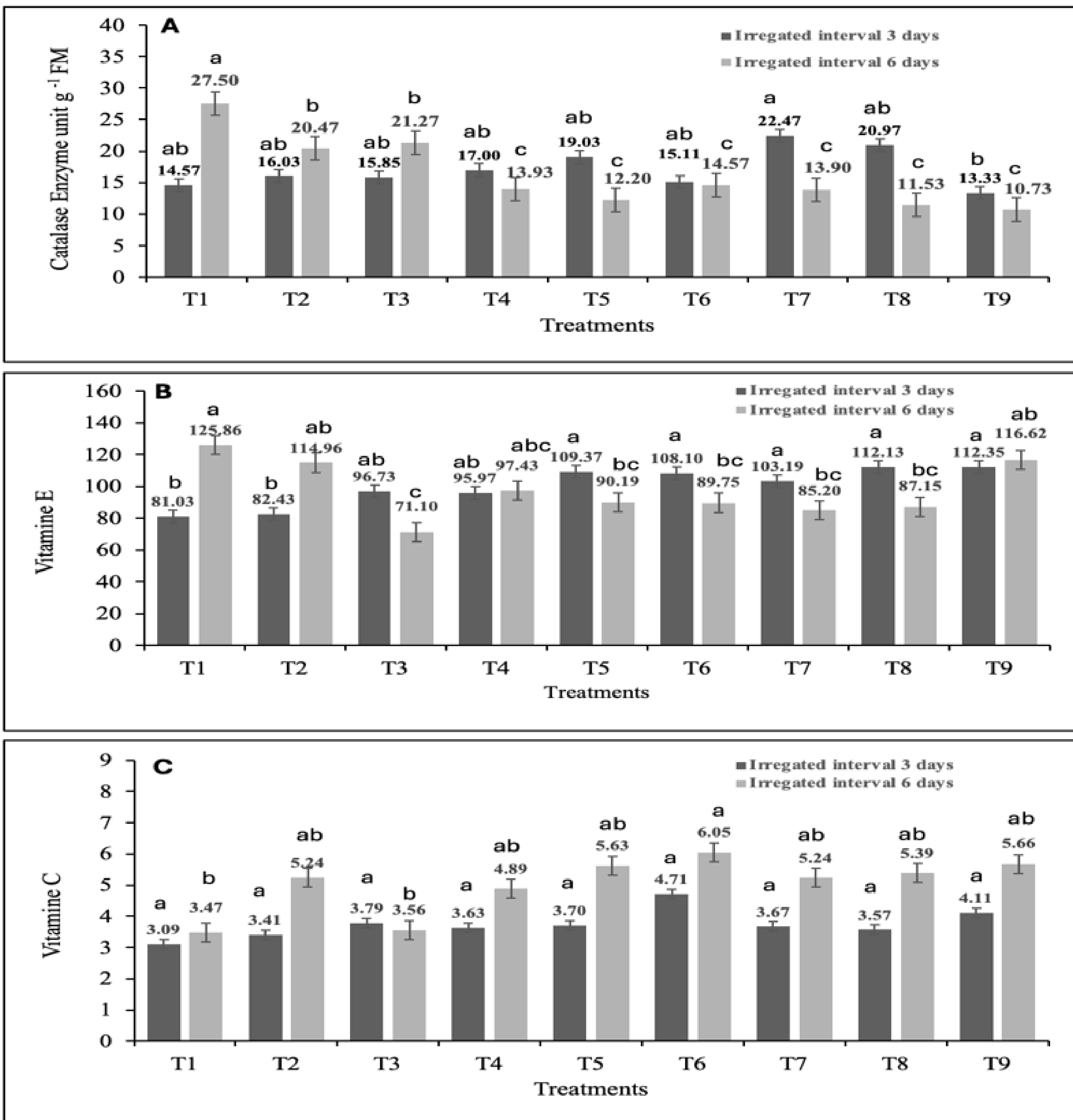

Figure 2: Effect of irrigation intervals, potassium, and boron on (A.) catalase enzyme, (B.) vitamin E, (C.) vitamin C. The different letters denote significant differences among the mean values of treatments.

than those obtained from irrigating at three-day intervals.

The peroxidase content is displayed in (Fig 1B). The minimum values were attained when the plants were irrigated every three days, as opposed to every six days. Extended irrigation periods lead to an increase in the peroxidase content of crops. The control treatment at six six-day irrigation intervals generated the highest peroxidase content value of 47.20 μg g^{-1} FM. When the irrigation interval was set at six days, the values declined as the applications of boron and potassium increased. Conversely, the peroxidase levels were lower when the intervals between irrigation were three days, as opposed to six days. The values of treatment applications increased somewhat, except for T7 and T9 at three-day irrigation intervals, which attained values of 12.17 μg g^{-1} FM and 12.13 μg g^{-1} FM respectively, compared to the other values. Nevertheless, no notable effect was observed in peroxidase content across all treatments with three-day irrigation intervals. When boron was applied every six days, it reduced the peroxidase content in plants, which means it reduced the water stress on plant tissue. The second highest value was obtained by applying 0 kg K ha^{-1} and 50 mg B l^{-1}, resulting in a decrease of approximately 10.02 % relative to the control treatment. The peroxidase concentration was lowest at T9, with a value of 14.90 μg g^{-1} FM. This was a 68.43 % decrease compared to the highest value of 47.20 μg g^{-1} FM T9 was reached with the application of 150 kg K ha^{-1} and 100 mg B l^{-1}.

Figure 1 C. shows substantial differences in the irrigated interval treatments at T1 and T2, with values of 0.16 mg g^{-1}FM and 0.47 mg g^{-1}FM, respectively, observed at three days. On the other hand, there was no substantial increase observed in the other treatments. When the irrigation is performed every six days, the findings show a notable increase in all the treatments. The control application, with a rate of 0 kg K ha^{-1} and 0 mg boron l^{-1}, exhibited the highest value of 0.35 mg g^{-1} FM, surpassing the values of the other treatments. The results indicate that the values fell as the administration of potassium and boron increased. The highest value was recorded at T6 (75 kg K ha^{-1} and 100 mg B l^{-1}), reaching a value of 0.30 mg g^{-1}FM, which did not show a significant increase compared to T1 and T2. Nevertheless, the T3 treatment, which involved the application of 0 kg K ha^{-1} and 100 mg B l^{-1}, resulted in the lowest value. This treatment exhibited a substantial percentage decrease of 34.28 % compared to T1. However, the carotene levels in the treatments with an irrigation interval of three days were lower than those with six days.

3.2 IMPACT OF BORON, POTASSIUM, AND IRRIGATION INTERVALS ON CATALYZE ENZYME, VITAMINE E, AND VITAMINE C

Exposing cabbage to water stress enhances the production of antioxidants by activating plant cells responsible for cabbage‘s defense mechanism (Fig 2 A). The results indicate that the catalase enzyme reached its greatest value during the T1 treatment (control with no potassium and boron applied) at six-day irrigation intervals (27.50 unit g^{-1} FM). The data indicate that the levels of catalase enzyme reduced with potassium and boron treatments, regardless of the irrigation intervals. Treatment T3, which involved an irrigation interval of six days, produced the second-highest value of 20.47 unit g^{-1} FM. This result represented a percentage drop of approximately 25.56 % compared to the T1 treatment. T3 treatment consisted of 0 kg K ha^{-1} and 100 mg B l^{-1}. The minimum values were achieved with three different irrigation interval treatments. Conversely, the greatest values were attained during the T7 and T8 treatments for three days of irrigation intervals. The treatments T7 and T8, which received 150 kg K ha^{-1} and 0 mg B l^{-1}, and 150 kg K ha^{-1} and 50 mg B l^{-1}, respectively, yielded values of 22.47 unit g^{-1} FM and 20.97 unit g^{-1} FM. According to the findings, the average values of the catalase enzyme showed a gradual increase when the watered interval treatments were set at three days. However, these values steadily declined when the irrigated interval treatments were imposed at six days

The impact of potassium and boron treatments on Vitamin E and Vitamin C was observed in both three-day and six-day irrigation intervals, as represented in (Fig 2 B and C). The results indicate that the highest concentrations of vitamin E and vitamin C were observed at T1 and T6, respectively, when employing a six-day irrigation interval. The values recorded were 125.86 for vitamin E and 6.05 for vitamin C. Vitamin E typically declined with increasing treatments, except for T9 (150 kg K ha^{-1} and 100 mg B l^{-1}) where it increased, at irrigation intervals of six days. However, when the irrigation interval was extended to three days, the levels of vitamin C gradually increased with the increase in treatments. Although there was no a significant difference observed between treatments, the increase in vitamin C levels was still noteworthy. The data on Vitamin E indicates that the mean values were significantly influenced by the application of potassium and boron to plants. This effect was observed in treatment T9, which had irrigation intervals of three days and achieved a value of 112.35. This value represents a percentage increase of about 38.35 % compared to the control treatment, which had no potassium (0 kg K ha^{-1}) and no boron (0 mg B l^{-1}) and attained a value of 81.03. The outcome of the T9 treatment (150 kg K ha^{-1} and 100 mg B l^{-1}) was significantly different from the control treatment. The data on Vitamin C indicates that the treatment T6, which involved irrigating every three days with 75 kg K ha-1 and 100 mg B l^{-1}, had the greatest value of 4.71. This value represents a percentage increase of 52.43 % compared to the control treatment, which had a value of 3.

3.3 IMPACT OF BORON, POTASSIUM, AND IRRIGATION INTERVALS ON TOTAL CHLOROPHYLL

The chlorophyll content level indicates the extent of photosynthetic activity in the cabbage plant. The data presented in Figure 3 demonstrates that a three-day interval of irrigation increased chlorophyll content. Furthermore, the use of potassium and boron further enhanced the elevation. The highest value of (236.33 mg 100 g^{-1} FM) occurred at T8, with a percentage increase of about 66.82 % compared to the control treatment, which yielded a value of 141.67 mg 100 g^{-1} FM. This observation was made at three-day intervals of irrigation. However, there was no substantial difference between T8 and T7 and T9. The lowest achievable value was attained at T1, representing the control treatment with no potassium (0 kg K ha^{-1}) and no boron (0 mg B l^{-1}). The T6 treatment, with irrigation intervals of three days, reached the greatest value of 181.33 mg 100 g^{-1} FM, which was a 34.00% increase compared to the control treatment's value of 135.33 mg 100 g^{-1} FM. The T6 treatment had a potassium concentration of 75 kg ha^{-1} and a boron concentration of 100 mg l^{-1}. Nevertheless, the chlorophyll content was influenced by the amount of irrigation over three days, as the application of potassium and boron led to a gradual increase in chlorophyll content. The irrigation interval of six days did not show a significant difference between the application of potassium and boron, and it resulted in lower values compared to the three-day irrigation interval.

3.4 RELATIONSHIP BETWEEN TOTAL CABBAGE YIELD AND SOIL NITROGEN, PHOSPHORUS, AND POTASSIUM, FOR THREE AND SIX IRRIGATION INTERVAL DAYS UTILIZING LINEAR REGRESSION

The primary aim of this study is to evaluate the potential influence of different parameters on yield productivity. This can be achieved by analyzing the correlation between the response variable, which is the total cabbage yield, and the explanatory variables or predictors, including soil nitrogen concentration (Total nitrogen NO_3^- and NH_4^+), soil phosphorus, and soil potassium (Fig 4 A-C). When soil nitrogen levels increased, the overall yield improved throughout a three-day irrigation cycle. Not-

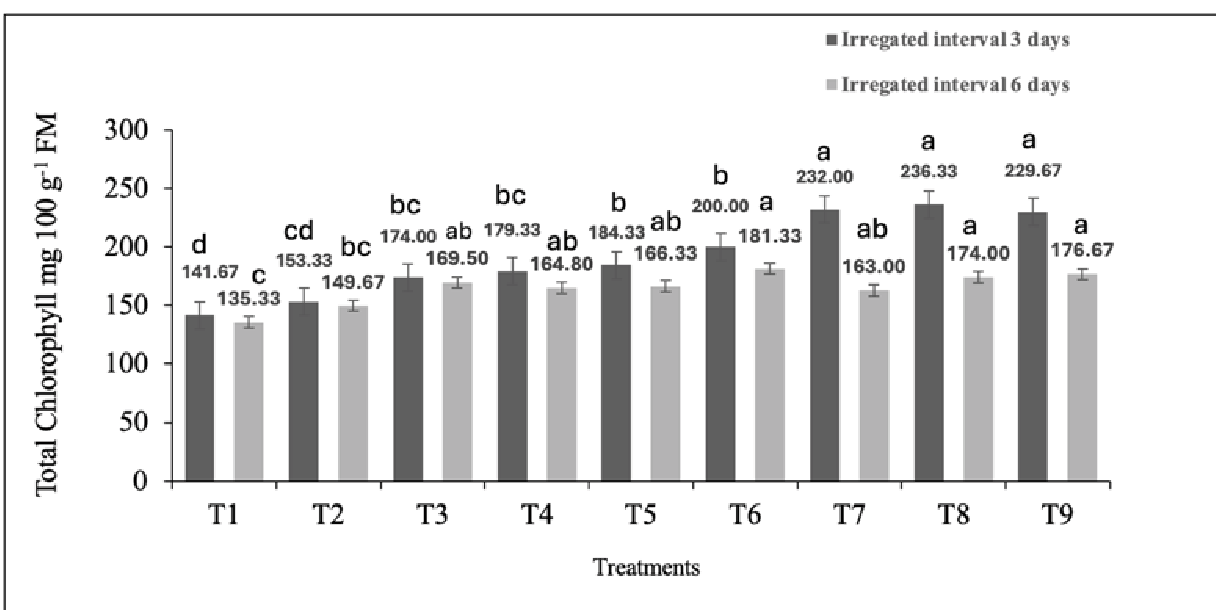

Figure 3: Effect of irrigation intervals, potassium, and boron on total chlorophyll. The different letters denote significant differences among the mean values of treatments.

withstanding the coefficient of determination (R^2) being relatively low at 0.23, there was still a strong correlation between the responsive variable and soil nitrogen. The results of the six-day irrigated interval indicate that the linearity was enhanced, as seen by the increase in R^2 to a value of 0.44. Figure 4.b. illustrates the correlation between the dependent variable (Total yield) and the independent variable (soil phosphorus concentration). The coefficient of determination (R^2) was found to be 0.50 for three-day irrigated intervals, while it was 0.59 for six-day irrigated intervals. The soil phosphorus and total yield showed improvement at a six-day irrigation interval, but there was no significant difference. In contrast, the results from a three-day irrigation interval did reveal a significant difference in the data. The linearity in (Fig 4 C) demonstrates a strong correlation between the response variable (Total yield) and the explanatory variable (soil potassium content). This correlation obtained an R^2 value of 0.62 at a three-day irrigation interval, and there was a significant difference noticed between the two variables. In contrast, the data did not show a significant difference when analyzed at six-day intervals. The coefficient of determination was calculated to be $R^2 = 0.58$. Nevertheless, the content of phosphate and potassium in the soil had a greater impact on the overall yield of cabbage, as indicated by the coefficient of determination for both irrigation intervals.

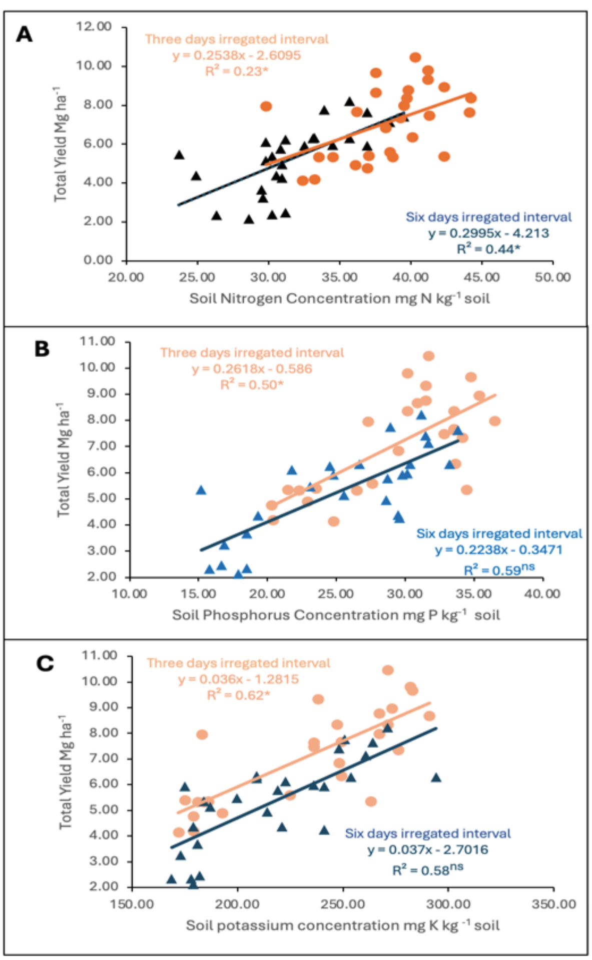

Figure 4: The relationship between cabbage total yield and (A.) soil nitrogen concentration. (B.) soil phosphorus concentration. (C.) soil potassium concentration for three and six irrigation interval days. The significant level is 0.05.

3.5 RELATIONSHIP BETWEEN TOTAL CABBAGE YIELD AND LEAF NITROGEN, PHOSPHORUS, POTASSIUM, AND BORON FOR THREE AND SIX IRRIGATION INTERVAL DAYS UTILIZING LINEAR REGRESSION

The (Figure 5 A-D) illustrates the correlation between leaf nitrogen, phosphorus, potassium, and boron and the total cabbage production. Figure 5a demonstrates a strong linear relationship between total yield and leaf nitrogen concentration. The coefficient of determination for the irrigated interval dates is 0.28 and 0.38, respectively. Although the coefficient of determination did not exhibit a significant disparity, it maintains value for irrigation intervals of three and six days. There was no significant difference in leaf phosphorus concentration when the plants were irrigated every three days. Additionally, the relationship between the total yield and leaf phosphorus concentration was not clear enough to provide significant details, on irrigation interval, as depicted in Figure 5b. The cabbage yield was strongly correlated with the concentration of potassium in the leaves at three and six intervals of irrigation ($R^2 = 0.36$ and $R^2 = 0.43$) (Figure 5 C). The coefficient of determination ($R^2 = 0.43$) showed a significant difference between the six irrigation interval days and the three three-day irrigation intervals, with the former having a higher coefficient of determination. The relationship between the total yield and leaf boron content is elucidated in (Fig. 5.D). The cabbage results were not significantly affected by the leaf boron content, regardless of the application of boron to the plants during both irrigated dates.

4 DISCUSSION

Numerous studies have consistently demonstrated the importance of irrigation schedules, along with the application of potassium and boron, for the long-term sustainability of crops (Al-Lami et al., 2023; Burhan and AL-Hassan, 2019; Saaseea and Al-a'amry, 2023; Sulaiman

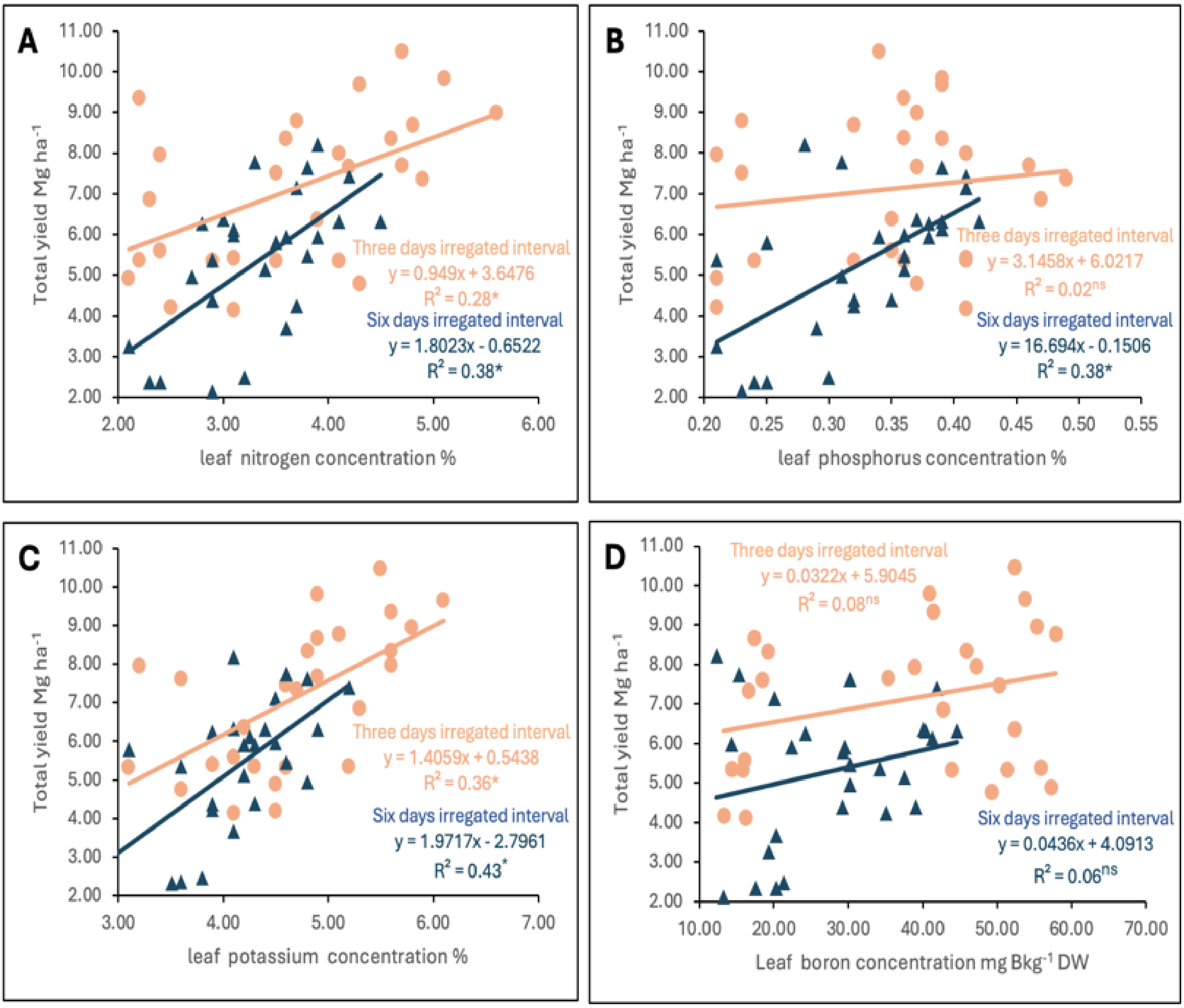

Figure 5: The relationship between (A.) leaf nitrogen concentration, (B.) leaf phosphorus concentration, (C.) leaf potassium concentration, and (D.)leaf boron concentration, and cabbage total yield. the significant level is 0.05.

and Sadiq, 2020). Effective soil-water-nutrient management may efficiently regulate crop needs uniformly across agricultural areas, without being influenced by spatial differences in soil-water-nutrient levels and crop water and nutrient demands (Dhannoon et al., 2020; Dizayee and Saleh, 2017; Khattab et al., 2019; Al-Ubaydi et al., 2017; Yadav et al., 2023. The present study found that the content of antioxidants such as proline, peroxidase, and carotene increased when the irrigation interval increased from 3 days to 6 days. The highest values were obtained at T1 for all antioxidants (Fig 1 A, B, and C), these results corresponded to the observations obtained by El-beltagi et al. (2020) who reported an elevation in antioxidant enzyme activity, non-enzymatic antioxidant levels, and proline in crops under stress. This phenomenon can occur as a result of crops producing enzymatic and non-enzymatic antioxidants that aid in reducing oxidative damage and improving crop yield and resilience to drought. Farrag et al. (2021) observed similar findings, attributing the increases in yield, vitamin C content, and total soluble solids % to sufficient water availability. They also suggested that this could enhance nutrient availability and therefore improve nutrient uptake. In contrast, deficit irrigation decreases cell formation, extension, and growth in plant elements such as roots, stems, and leaves. This can happen as a result of a decrease in the movement and absorption of nutrients, as well as a delayed uptake of nutrients through the roots due to the absence of the carrier factor, namely water (Al-Wasel and El-Rayes, 2024; Jasim et al., 2020). Applying high levels of potassium during a three-day irrigation period, which was the shorter interval, could result in a significant decrease in total crop production and a substantial increase in antioxidant content (Al-Wasel and El-Rayes, 2024). The greatest values of catalase enzyme, vitamin E, and vitamin C were obtained with T1, T1, and T6, where the values were 27.50 units g^{-1} FM, 125.86, and 6.05, respectively (Fig 2 A, B, and C). The application of potassium and boron can contribute to a drop in antioxidants, as the plant‘s roots can uptake more nutrients and water from the soil. Nevertheless, the limited supply of irrigated water can potentially lead to an increase in root length as a means to search for water and nutrients (Riaz et al., 2021). However, total chlorophyll increased with the longer irrigation interval. Furthermore, T7 treatment achieved the highest value at the six-day irrigation interval (236.33 mg 100 g^{-1} FM) (Fig 3). This phenomenon can be attributed to the application of potassium, which regulates the turgor pressure and perhaps enhances cell division, expansion, and the growth of meristematic tissues(Riaz et al., 2021). Adding boron has been found to increase root length, root hair, and development (Wang et al., 2021). Boron treatments can enhance the production of specific stress-related genes that are crucial for safeguarding crops. The study's findings are consistent with the research conducted by Akhtar et al. (2022). Optimal irrigation and nutrition play a crucial role in improving agricultural productivity, maintaining cell structures, and enhancing crop metabolisms (Anokye et al., 2021; Jasim et al., 2020; Maize et al., 2021). The correlation analysis revealed a significant impact of nitrogen on cabbage yield (Fig 4 A), particularly when using six-day irrigation intervals which attained a significant R^2 = 0.44, which outperformed three-day irrigation intervals. This finding corresponds to the study conducted by Terefa (2021), which indicated that the application of nitrogen fertilizer had a substantial impact on cabbage output. Specifically, the largest irrigation interval of 9 days resulted in the greatest effect. The improvement in nitrogen uptake can be attributed to the reduction in nitrogen losses caused by excessive watering, namely through the implementation of a more precise irrigation schedule. This allows for the restoration of soil nitrogen levels.

Contrary to expectations, there was no significant difference in cabbage production when the soil phosphorus content increased at a six-day watering interval (Fig 4 B). However, the results did demonstrate an increase in cabbage outcomes (Ahmed and Al-Tameemi, 2023). Soil phosphorus concentration obtained higher values at three-day irrigation interval compared with six-days interval. When the irrigation was performed every three days, there was a noticeable difference in the coefficient of determination, which led to significant crop production. This conclusion is similar to the findings reported by Farrag et al. (2021). The observed results could be attributed to the fact that phosphorus is usually considered a non-mobile or slow-moving nutrient in the soil. Consequently, the current watering schedule does not facilitate its absorption by plants, as reported by Onkoba et al. (2021).

A consistent correlation was observed between soil potassium levels and cabbage yield at both irrigation intervals (Fig 4 C). The linear regression between soil potassium concentration and total cabbage yield significantly differed at three days irrigation interval (R^2 = 0.62). The six-day irrigation interval did not present a significant coefficient of determination (R^2 = 0.58). This finding coincides with the research conducted by Yadav et al. (2023), which suggests that the increase in soil potassium availability can be ascribed to the increase in irrigation water.

Furthermore, this research denoted that the total yield at three-day irrigation interval was ultimately responsive to nitrogen, phosphorus, potassium, and boron concentrations in the leaves (Fig 5 A, B, C, and D). However, the nutrient content of cabbage leaves, specifically nitrogen, phosphorus, potassium, and boron, increased when the irrigation schedule was reduced. More specifically, when irrigation was applied once every three days, there was a valuable association, and a significant difference compared to irrigation performed every six days. This can be attributed to the increase in water availability, which would enhance the absorption of nutrients.

5 CONCLUSIONS

The current study exhibited that applying potassium and boron with a three-day irrigation interval positively impacted cabbage yield. The cabbage irrigation schedules, and potassium and boron applications impacted antioxidant enzymes and some vitamins, such as E and C. From the results, antioxidant enzymes increased with less frequent watering, this is one of the plant's defense systems against drought stress. Moreover, the data showed that total chlorophyll increased with a six-day irrigation interval, potassium application, and boron spray. In particular, the highest total chlorophyll was attained with T7 treatment at the six-day irrigation interval (236.33 mg 100 g^{-1} FM). The linear regression results revealed that soil test potassium achieved the highest significant R^2 = 0.62 at the three-day irrigation interval compared to the non-significant R^2 achieved at the six-day interval. The concentration of nitrogen, phosphorus, potassium, and boron in the cabbage leaves increased with the longer irrigation interval. However, boron concentration did not achieve a significant coefficient of determination or effect on total cabbage yield.

To recap the findings of this research, increasing the irrigation interval for a while with a drought-tolerant plant can mitigate the water needed in the arid and semi-arid areas that have water availability issues due to climate change, such as high ambient temperature, rain amount reduction, dry winds, etc. Thus, growers, large agrarian farmers, and investors must utilize these techniques with drip irrigation systems or other regimes, followed by applying appropriate fertilizer to increase the nutrient concentration, which is reflected in the crop yield. For further information, we encourage farmers and researchers to use various fertilizer applications with different irrigation schedules and crops.

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Mulching strategies and the significance of mulching in improving soil fertility and soil physical properties: A review

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Mulching strategies and the significance of mulching in improving soil fertility and soil physical properties: A review

Abstract: The population growth has increased the demands on food and resources like land and water, affecting crop productivity and yields in arid and semi-arid areas. Approximately 50 % of the land area on Earth is made up of arid and semi-arid areas. Optimizing agricultural field management techniques is crucial due to limited rainfall, but intensive cultural techniques like irrigation, weeding, fertilizers, and stress prevention make vegetable production expensive. This review has examined 118 recent published studies on mulching materials and procedures in vegetable farming, focusing on their impact on soil and environment. It suggests mulch can be a viable alternative to traditional methods, reducing irrigation and chemical inputs for weed control. The review also discusses the effects of organic mulching on soil temperature, moisture content, organic matter content, soil microorganisms, pest and insect control, and plant yield. The aim is to conduct a comparative analysis of the advantages and disadvantages of mulches in vegetable farming. Mulch techniques can enhance vegetable production by reducing cultivation costs, improving soil properties like temperature regulation, moisture, weed suppression, and structure, and providing habitat for insects and earthworms, thereby improving soil biological activities.

Key words: organic and inorganic mulches, soil properties, pros and cons of mulches, mulching and soil fertility

Načini mulčenja in pomen mulčenja pri izboljšanju rodovitnosti tal in njihovih fizikalnih lastnosti: pregled.

Izvleček: Rast človeške populacije je povečala zahteve po hrani in drugih virih kot sta obdelovalna zemlja in voda, kar je vplivalo na produktivnost gojenih rastlin in njihove pridelke v sušnih in polsušnih območjih. Na Zemlji je približno 50 % površin sušnih in polsušnih. Optimizacija tehnik upravljanja kmetijskih zemljišč je odločilna zaradi omejenih padavin, a intenzivni načini tehnik v kmetijstvu kot so namakanje, odstranjevanje plevelov, gnojenje in preprečevanje stresa delajo pridelavo zelenjave drago. V tem pregledu je bilo pregledanih 118 v zadnjem času objavljenih raziskav o načinih in pripomočkih mulčenja pri pridelavi zelenjave, ki se osredotočajo o vplivih na tla in okolje. Izsledki nakazujejo, da je mulčenje primerna alternativa tradicionalnim metodam, ker zmanjšuje potrebe po namakanju in vnosu kemikalij za zatiranje plevelov. Pregled komentira tudi učinke organskega mulčenja na temperaturo tal, vsebnost vode in organskih snovi v tleh, vpliv na mikroorganizme, uravnavanje škodljivcev in vpliv na pridelek. Namen je izvesti primerjalno analizo prednosti in slabostio mulčenaj pri pridelovanju zelenjave. Tehnike mulčenja lahko pospešijo pridelavo zelenjave z zmanševanjem stroškov pridelave, z izboljševanjem latnosti tal kot sta uravnavanje temperature in vlažnosti in z zatiranjem plevelnih združb. Tehnike mulčenja lahko pospešijo pridelavo zelenjave in zmanjšajo stroške, izboljšajo lastnosti tal kot so uravnavanje temperature in vlažnosti, zatiranje plevelov in zagotavljanje primernih habitatov za žuželke in deževnike in s tem izboljšanje biološke aktivnosti tal.

Ključne besede: organsko in anorgansko mulčenje, lastnosti tal, prednosti in slabosti mulčenja, mulčenje in rodovitnost tal

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1 BACKGROUND

Due to current globalization and health consciousness, there is a greater need than ever for upright horticultural crops, farmers are being forced to produce more and high-quality fruits and vegetables to survive in the global market due to the growing demand for these products, and market competition (Ranjan et al., 2017; Yaseen et al., 2023). The importance of managing soil as a nonrenewable resource to promote sustainable development is becoming more widely acknowledged the health of the soil determines the productivity of arable systems farm management techniques, such as the use of pesticides, fertilizers, mulching, and manure, can have an impact on soil health and sustainable development (Ngosong et al., 2019).

Additionally, rapid urbanization and industrialization have raised global temperatures, upsetting the balance of agro-ecological systems all over the world for sustainable food production (Iqbal et al., 2020). The new environmentally friendly agricultural techniques are therefore required by lowering soil evaporation, preserving moisture, regulating soil temperature, inhibiting weed growth, and enhancing microbial activity, also mulching can also be affective. Mulching is the process of applying different covering materials to the soil's surface to increase crop yield, reduce weed growth and moisture loss (Iqbal et al., 2020). The microclimate surrounding a plant is directly influenced by mulching materials, and this can have beneficial or detrimental effects on the physiological metabolism of the plant (Kader et al., 2017).

Various mulching materials can be used for different agricultural and horticultural crop in different climates. Mulch can be a ground cover made of live plants, loose organic and inorganic matter particles scattered over the soil, or sheets of natural or artificial materials applied to the soil's surface. The primary benefits of using organic mulches are the organic matter and nutrients they provide for soil organisms as well as plants. The primary functions of organic mulches are to enhance crop plant growth conditions and shield the soil's surface from adverse influences. Although organic mulches are biodegradable, a temporary decrease in soil mineral nitrogen may occur during the decomposition process (Ranjan et al., 2017; Yaseen and Takacs-Hajos, 2022).

The application of mulches improves soil structure, provides organic matter, lessens the effects of wind and, water erosion, and cools the soil during the summer, all of which increase crop yields (Kosterna, 2014a). Mulching increases yield more when it comes to species that are cultivated for early harvest (Qin et al., 2015). The main reasons for increased plant growth in response to mulching have been identified as soil moisture conservation, improving microbial activities, soil temperature regulation, and decreased competition from turf and other plants (Iqbal et al., 2020).

Conversely, synthetic mulches can be applied mechanically and offer a strong defence against the majority of weeds; however, they need to be taken out at the end of the growing season. Furthermore, crop root zone penetration of rainfall or overhead irrigation can be impeded by non-porous plastic mulches. While most of the water runs off the mulch into alleys and may not reach crop roots, some of it does run into planting holes. The labour-intensive end-of-season removal, the production of non-biodegradable waste, and the lack of organic matter or nutrients added to the soil are additional drawbacks of synthetic mulches (Kosterna, 2014b). In ecological farming, it is advised that soil be covered with composts, chopped straw, and other organic residues to supply crops with nutrients, particularly nitrogen (Bajoriene et al., 2013).

The purpose of this study is to look into how important is mulching in increasing crop productivity and improving soil. In particular, it compares organic and non-organic techniques in an effort to highlight the advantages of mulching in vegetable production.

2 MAIN TEXT

2.1 ADVANTAGES OF MULCHING PROCEDURE INTEGRATION WITH SOIL

In various mulching techniques, it was discovered that adding organic mulch to the soil increases its organic matter (Bajoriene et al., 2013). Liu et al. (2021b) stated that among the used materials for mulching (organic and inorganic materials), straw mulching and dual mulching of the ridge (film) and furrow (straw) improved soil organic carbon (SOC) storage due to straw decomposition in the soil. Using a variety of materials for mulching has the potential to increase soil moisture, decrease evaporation losses, and suppress weed growth. Various mulching techniques showed notable effects on the quantity, quality, and growth of different crops (Iqbal et al., 2020). There are inconsistencies regarding mulching's effectiveness because various scientists have reported negative effects from the practice. Plastic film mulching, for example, contributes to an increase in net global warming potential (GWP) and greenhouse gas emissions. (gu Lee et al., 2022). Caboň et al. (2021) also discovered that mulching has a negative impact on vascular plant diversity due to competition and changes in plant reproductivity. Additionally, mulching reduced fungal diversity of mac-

Table 1: Benefits of organic mulches for soil properties.

Types of mulch	Benefits	References
Straw mulch	Water productivity rises while water usage falls.	Jat et al. (2015)
	Decreases the Colorado potato beetle's insect pest invasion and late blight in potatoes	Finckh et al. (2015)
	There are improvements in rice yield, grain quality, and recovery.	Jabran et al. (2015)
	Less runoff or erosion, better soil water management, and temperature control	Montenegro et al. (2013)
Compost mulching	Mulching and organic composting workable ways to lessen the detrimental effects of water stress and save irrigation water by 15 %.	Abd El-Mageed et al. (2018)
Newspaper	Lower soil temperature keep moisture in soil	Haapala et al. (2014b)
Sawdust	Sawdust, whereas the control preserved moisture and generated comparatively less 'Savoy' baby cabbage.	Masarirambi et al. (2013)

Here are some advantages of mulching strategies on soil content and soil microbiological improvements:

ro-fungi collectively, known as CHEGD fungi, in semi-natural grasslands of Europe due to the decomposition of plant litter by saprotrophic organisms, which causes changes in soil properties. Despite the fact that mulches have many advantages over these drawbacks, the disadvantages reported by various scientists are not as risky in actual field conditions (Iqbal et al., 2020). Nonetheless, the literature suggests that mulches are an inexpensive way to control weed growth and maintain a significant level of soil moisture content (Iqbal et al., 2020). One of the mulch's biggest benefits is that it adds organic matter to the soil, particularly nutrient-rich mulch, which has a positive impact on the soil's chemical and physical characteristics and ultimately, crop productivity. The amount of chemical plant protection and mineral fertilisers applied can be decreased thanks to this management system. Numerous studies have shown that keeping organic matter on the soil's surface promotes plant growth and development and increases vegetable yield (Kosterna, 2014b; NING et al., 2017). From an ecological standpoint, mulching offers numerous benefits. These benefits are particularly significant in places where water is scarce. Mulching has many advantages as it is shown in Table 1.

2.1.1 Effect of mulch on soil moisture

Since the 20th century, conserving water and soil has become a significant issue to the human life (Mekonnen et al., 2015). The need for agricultural land is growing as the world's population continues to rise, and soil and water losses are getting worse. This is especially true in developing countries (Thomaz and Luiz, 2012). Based on the reports by FAO (2015); IPCC (2019), the quarter of the word's agricultural land has been degraded in the last few decades. Farmers can actively maintain the residual soil health by farming in a healthy manner; mulching is one such method; however, in order to preserve the ecosystem, this requires direct support from national and international governments.

By absorbing and using rainfall, mulching can significantly alter the growing conditions of crops and lower the likelihood of crop failure in the field, particularly in arid or semi-arid areas (Wang et al., 2021). The soil's conversion to barren land due to moisture loss is caused by numerous abiotic factors. The main purpose of mulches in the cultivation of vegetable crops is to protect the soil surface from the influence of unfavourable factors and to improve the growing conditions for the crop plants (Iqbal et al., 2020; van Donk et al., 2012). Mulches also help maintain stable soil temperature, increase soil porosity, and suppress weed growth, reducing evapora-

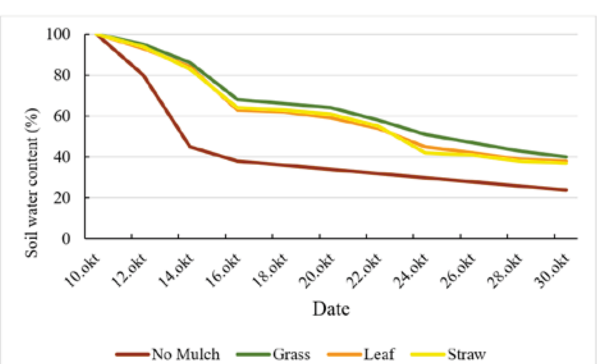

Figure 1: The effect of the three different mulch types on the soil water content. Source: McMillen (2013)

tion and conserve soil moisture and a significantly slow down soil water loss (Bajoriene et al., 2013; Kader et al., 2017). Consequently, a more consistent and elevated soil water regime is upheld, thereby decreasing the frequency of irrigation (Sajid et al., 2013). Narayan et al. (2017) have demonstrated that black double coated polythene mulch could significantly retain soil moisture by 16.74 % when compared to white double coated, black single coated inorganic mulch, and organic straw paddy mulch, this was because they increase retention and percolation while decreasing evaporation see (Figure 1).

Organic mulches have an impact on the soil water cycle. The exposed soil is more susceptible to the effects of rain, wind, and soil radiation. Mulches decrease rainfall water loses, which increases the soil's ability to absorb the precipitation water (Brunetti, 2014). Living mulch can grow alongside crops year-round, protect the soil layer over time with good coverage, and contribute significantly to soil and water conservation (Donjadee and Tingsanchali, 2016; Fracchiolla et al., 2020). Yin et al. (2016) have discovered that double mulching of organic and inorganic materials improves moisture retention in the soil by 4.6 %. Soil covered with plastic mulches can reduce evaporation and protect soil moisture in salt-affected reclamation soils. (Chen et al., 2023).

Table 2: Recent research works showing the effect the mulching types on soil temperature on different vegetables.

Mulch type	Materials	Effect on soil temperature	Reference
Organic	Biodegradable (eco-benign plastic)	Biodegradable mulches significantly reduced temperature by 2.04 °C-3.52 °C and 0.52 °C-0.88 °C ($p < 0.050$) throughout seedling and full growth periods, compared to plastic mulch.	Jia et al. (2020)
	Mushroom compost	maintained a higher temperature on the soil surface and a depth of 15 cm	Guo and Liu (2022), Yordanova and Gerasimova (2015)
	Barley straw	significantly increasing it in winter and then significantly decreasing it in spring and summer as well as regulating soil temperature	Ding et al. (2017), Song et al. (2024)
	Maize straw	lowest temperature was observed compared to plastic film, biodegradable film mulches	Li et al. (2013)
	Grass and pine needle mulch,	Helps regulating soil hydrothermal regimes	Sharma et al. (2024)
	Rice straw mulch	In comparison to without mulch (Mo), cover crops Arachis pintoi (Ma), rice straw mulch (Mj), and black silver plastic mulch (Mp), the rice straw mulch with a combination of organic and inorganic fertiliser produced the lowest soil temperature of 17.82 ^{0}C.	Nurzadeh Namaghi et al. (2018)
Inorganic	Black double coated, white double coated and black single coated polythene	Increased soil temperature was caused by the absorbing of solar radiation by plastic mulches and the transmission of heat into the soil deep of the first 20 cm.	Torres-Olivar et al. (2018), Gheshm and Brown (2020)
	Clear polyethylene mulch	The highest average temperatures were found for the clear mulch compared to blue plastic mulch.	Jia et al. (2020), Tesfaye et al. (2016), Pramanik et al. (2015)
	Coloured plastic mulches (silver, yellow and black)	Under greenhouse conditions, soil temperature at 20 cm depth decreased by 2-3 °C in summer and increased by 3–5 °Cin winter.	Laulina and Hasan (2018)
	Polythene film	increased the soil temperature by about 6 °C at 5 cm depth and by 4 °C and 1 °C at 10 cm depth	Gu et al. (2020), Yang et al. (2020)
	Orange plastic mulch (250 µm)	Soil temperature was increased compared to no mulch as the control, maize straw mulch, Grass mulch (Eragrostis lehmanniana Nees)	Malamba (2021)

2.1.2 Effect of mulch on soil temperature

Mulch is a type of soil cover that helps to keep the soil at a consistent temperature and humidity, prevent weed growth, and reduce soil erosion (Dvořák et al., 2012). Mulches are known to modify microclimatic conditions by influencing soil temperature through the transmission of light energy (Hu et al., 2011). Modifies the microclimate surrounding the grown plants, assisting it in adapting to climate change (Kasirajan and Ngouajio, 2012). Table 2 lists the most recent research publications on the effect of different mulch materials on soil temperature in growing plants.

2.1.3 Effect of mulch on improving soil organic matter

Due to its ability to restore degraded soils and provide the most basic energy substrate, soil organic matter (SOM) is essential to crop production (Guimarães et al., 2013). The primary benefits of organic mulches are their organic matter content and ability to supply nutrients to soil organisms as well as plants, the interplay of chemical, physical, and biological soil properties result in soil quality. According to a study by Hwang et al. (2020), inorganic mulch material (plastic film (PFM)) caused soil organic carbon (SOC) losses of 8–48 %, whereas organic mulch (green manure (GM)) could dramatically decrease SOC losses by 33-59 %. Additionally, after six years of applying organic mulch to the soil, SOM, soil fertility, and soil physicochemical properties improved. Cellulase, invertase, and dehydrogenase activities in the organic mulch-covered soil were 17.46 %, 78.98 %, and 283.19 % higher than those in the lawn, accordingly, at a depth of 0–20 cm (Liu et al., 2024).

The degree of soil alteration in both natural and agroecosystems can be assessed using specific soil properties, such as highlighted enzyme activities. Soil enzymes have the potential to function as early indicators of biological changes because they may react to modifications in soil management more quickly than other soil variables (Naorem et al., 2021). An essential function of soil enzymes is to catalyse processes related to the decomposition of organic matter and the cycling of nutrients (Jodaugienė et al., 2010). Additionally, mulch can enhance the quality of the soil. Short-term mulching can effectively reduce runoff and sedimentation, and long-term mulching can greatly increase the organic matter in the soil as a result of the mulching materials‘ deterioration, matter, and accessible nutrients (Jiménez et al., 2016). Fracchiolla et al. (2020) have reported that the mineral content in the soil covered with living mulch was significantly improved, such elements like Mg and Fe, whereas a reduction of Na, K, and Ca was observed. In addition to preventing weed growth, applying organic mulch to soils can improve soil fertility and supply plants with the necessary mineral elements (Mulumba and Lal, 2008).

Furthermore, a long-term nitrogen fertigation yield study by Ding et al. (2022) found that mulching with PFM reduced nitrogen fertiliser leaching, resulting in increased root biomass, SOC by 26 %, soil enzyme activities, and SOC mineralisation rates due to the diurnal internal water cycle within the mulch.

2.1.4 Effect of mulch on weed control suppression

Covering the soil with mulches helps prevent weed growth and weed competition. The mechanisms through which living mulches suppress weeds include competition for light and subsurface resources, and shading, which prevents weed seeds from germinating and allelopathy (Médiène et al., 2011; Petit et al., 2018). Mulching impact can be determined by a variety of parameters, including mulch material type, transparency, and thickness. van Donk et al. (2012) have measured a significant lower weed growth and number in the soils covered with wood chip mulch thicknesses of 5 and 10 cm compared to the same mulch but lower layer thickness at 0 and 2.5 cm. It is also found that when comparing mulch types, the best summer annual eclipta (*Eclipta prostrata* (L.) L.) weed control was achieved with hardwood at about 5 cm depth (Saha et al., 2019). Furthermore, Dragumilo et al. (2023) investigated organic mulching materials (sawdust of acacia and dry pine needles) and inorganic mulching materials (silver-brown and black „agrotextile" film) to determine their impact on weed biomass and dry mass. The results show that inorganic materials had a significant better reduction of weed biomass using silver-brown film by 96.3^{-1}00 %, followed by black „agrotextile" film at 74.6-95.9 %. Additionally, synthetic materials were able

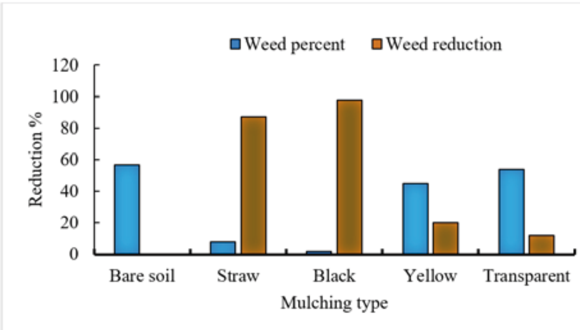

Figure 2: The typical percentage of weed growth for each treatment. **Source:** El-Shaih and Fouda (2008)

to substantially decrease total dry biomass by 84.6-100 %, while organic materials by 28.5-81.4 %.

In recent years, interest in living mulches has again increased because living mulch systems can contribute to agricultural sustainability through reducing herbicide inputs and improved soil health (Bartel et al., 2020). Sharma et al. (2024) have discovered that black mulch and mulch mat treatments provided 100 % weed control efficiency. Spreading mulch on the soil's surface penetration of light into soil, which slows the germination of small-seeded weed species. According to a research study by Mohtisham et al. (2013), there was no difference in the types of mulch used when comparing unmatched to mulched areas in fifteen districts; nevertheless, there was a noticeable difference in the number of weeds treated with bare soil. DeVetter et al. (2015) found straw and living mulches surpassed cultivation and herbicides in terms of weed control and reduction. Conversely, Dvorak et al. (2015) have found no consistent influence on weeds' aboveground biomass of potato field covered with grass mulch. In contrast, a research study discovered that prior to cabbage planting, oat and phacelia mulches drastically reduced fresh biomass and weed population (Franczuk et al., 2010). So that, many factors can influence of the effectiveness of mulches on weed control including mulch types, thickness, colour, the plant growth cycle and environmental factors. Narayan et al. (2017) noticed that mulching with hardwood chips recorded a better result in weed suppressing compared to pine bark and pine straw mulching. Figure 2 shows the percentage of weed growth for each treatment under black film, the percentage of weed was reduced to the lowest of 9 % and to the maximum of 98 % under transparent film. Because of the black film's significant reducing of light penetration into the soil, weed seedlings cannot grow under the mulch.

2.1.5 Influence of mulch on soil pest problem

Table 3: Different types of mulch effect of different insects and pathogens.

Type of mulch	Type of plant	Type of insect or pathogens	Effect	Source
Living mulch Red clover	Cabbage	Turnip root fly, *Delia floralis* (Fallén, 1824))	Decrease lying egg	Björkman et al. (2010)
Straw mulching	Kale (Brassicaceae)	*Myzus persicae* ( )	Decrease in population, and provide a better refuge for the natural enemies	Silva-Filho et al. (2014), Mochiah and Baidoo (2012)
plastic mulch	Okra (*Abelmoschus esculentus* (L.) Moench)	Beetle flea Podagrica species, roller of cotton leaves Cotton strainer with Notarcha derogate Whitefly, *Dysdercus* spp. *Oedaleus nigeriensis* Uvarov, 1926, the Nigerian grasshopper, and *Bemisia tabaci* (Gennadius, 1889)	Decreased insect pests and generated stronger yields	Ojiako et al. (2018)
Cowpea live mulch	Pepper (*Capsicum annuum* L.)	Aphids (*Aphis gossypii* Glover , 1877), thrips (*Thrips tabac*i Lindeman, 1889) and white flies (Bemisia tabaci (Genn., 1889)),	suppressing pest populations	Mochiah and Baidoo (2012)
Living mulch	Eggplant (*Solanum melongena* L.)	Colorado potato beetle, *Leptinotarsa decemlineata* Say, 1824 (Say): Flea beetles, Epitrix spp.	In comparison to the monoculture plots, fewer Colorado potato beetle adults, larvae, and eggs were discovered in the interplanted plots.	Hooks et al. (2013)
straw, walnut leaves, mixed leaves, compost	Potato tubers (*Solanum tuberosum* L.)	Potato tuber damage caused by soil-dwelling pests and soil-borne pathogens	mulch thickness (20 cm or more) can reduce damaging potato tubers caused by pathogens (e.g. Fusarium species).	Südiné Fehér and Zalai (2024)
Green plastic mulch increased in the planting and covered row	(tomato, cucumber, watermelon, strawberry, and vine)	the populations of fungevorous nematodes (*Aphelenchus* sp.) and Dorylaimida (omnivore nematodes) differed less between the soil mulching	Long-term growing of blueberries using green 100 % high-density polyethylene mulch did not significantly affect the presence of nematodes or the amount of Rhabditida (bacterial feeders).	Pedra et al. (2024)

Vegetables are extremely susceptible to pest and insect damage. It has been demonstrated that the distribution of pests in agriculture is significantly influenced by straw mulching (Phophi and Mafongoya, 2017). Organic and inorganic materials used as mulch have varying effects on pest populations in the soil. For instance, mulching straw increases the number of insects (Ma et al., 2021). Rice-straw mulching promotes natural enemies, while black polythene inhibits insect pest populations (Kumaratenna et al., 2022). In addition to other materials, organic mulches such peel and rice straw alter temperature and UV rays, which reduces the infestation of aphids (Silva-Filho et al., 2014). Plastic mulch is advised for optimal crop production and the management of *Abelmoschus esculentus* (L.) Moench insect pests (Ojiako et al., 2018). While straw mulch may offer a better haven for the natural enemies and is still a suggested choice for pest management in pepper production, cowpea mulch may be more successful at suppressing pest populations (Mochiah and Baidoo, 2012). Mulching can minimise pest population and crop damage by encouraging the activity of natural enemies in the soil and improving the micro-environment for pest disruption with natural enemy activity, and other reasons (Gill et al., 2011; Thomson and Hoffmann, 2007).

Table 3 shows some examples of how using various mulch types affects certain insects:

2.1.6 Influence of mulching on soil microorganisms

Mulching can change the biological content of the soil, reducing its sustainability and quality, and even causing soil alkalization, which can harm plants (Ni et al., 2016). Unlike other mulching methods, organic mulching is primarily made from plant residues, which have been shown to improve soil health (Kader et al., 2017). A research study by Sharma et al. (2024) indicated that grass and pine mulch had a substantial impact on total viable count, microbial activity, and microbial biomass carbon. Furthermore, mulching can increase the richness of the soil microecosystem (Kolota and Adamczewska-Sowinska, 2013). Tian et al. (2015) discovered that soil microorganisms greatly altered agroecosystem structure and nutrient cycling as soil fertility increased. Soil microorganisms are crucial to the soil‘s ability to maintain fertility and cycle nutrients, both of which are important for agroecosystem resilience and productivity (Liu et al., 2019; Yeboah et al., 2016).

Moreover, the movement of matter and energy within the soil ecosystem was facilitated by the diversity of soil microorganisms (Mori et al., 2018). The microbial community may change as a result of changes in the physicochemical characteristics of the soil during the mulching process (Qian et al., 2018). In addition to helping forecast changes in soil functions, soil microbial diversity and biomass showed sensitivity to changes in the physicochemical properties and management of the soil (Romaniuk et al., 2011). To take effective action to increase soil fertility and, productivity and guarantee the sustainable development of soil ecosystems, it would be beneficial to investigate the diversity and composition of microbial communities in soils that are being mulched.

Inorganic mulches may have a negative influence on soil microorganism activities. Pedra et al. (2024) have demonstrated that green plastic mulch was dramatically lower microbial activity in the planting row when compared to bare soil without mulching.

2.1.7 Influence of mulching on earthworm biomass

Earthworms have been identified as indications of soil biological health. Earthworms are typical ecosystem engineers because they significantly influence the physical qualities of soil in a variety of ways. Many research investigations indicate that mulching can boost biomass and population improvement, primarily with organic or biological mulches. Earthworms not only improve soil physical properties but also can improve soil water retention and reduce evaporation in lateritic red soil (Liu et al., 2021a). On the other hand, a 14-year study by Pelosi et al. (2015) examined organic and living mulch farming systems, and they discovered that organic and living mulch cropping systems had 1.5 and 2.3 times more earthworms than conventional systems. Radics et al. (2022) indicates that whereas irrigation greatly reduced earthworm biomass and abundance in the summer, organic mulching (made from wheat straw) significantly enhanced it.

Jodaugienė et al. (2010) have also indicate the activity of soil enzymes was not significantly affected by the thickness of the mulch layer, but the density and biomass

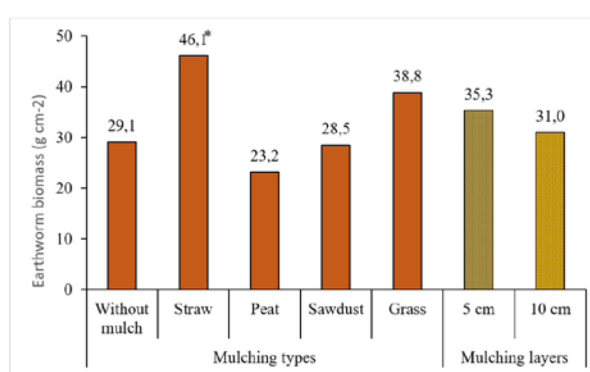

Figure 3: The influence of organic mulches and different thickness of mulch layer on the earthworm biomass. Source: Jodaugienė et al. (2010)

of earthworms were positively impacted by mulches made of grass and straw (Figure 3.)**.** According to the findings, grass or straw mulch creates and maintains favourable conditions for earthworms (Jodaugienė et al., 2010).

2.2 TYPE OF MULCHES

2.2.1 Organic mulch

Natural plant or animal matters are used to make organic mulches which can decompose naturally. Applying organic mulch as soon as the crop germinates or when the vegetable seedlings are transplanted will maximize its benefits on yield and ecological surrounding the plant (Iqbal et al., 2020). In addition to reducing nitrate leaching, organic mulches also improve the physical properties of soil, stimulate biological activity, maintain the nitrogen cycle in balance, supply organic matter, regulate temperature and water retention, and lessen erosion (El-Beltagi et al., 2022). Applying natural ingredients to crops requires a lot of work and is challenging. Due to financial and practical constraints, the use of organic mulch in horticultural crop production has been limited, with very little large-scale commercial application (Wang et al., 2014). Organic mulch has the potential to control and enhance the soil‘s chemical, biological, and physical characteristics (Xu et al., 2022). It has been discovered that mulching with organic materials has numerous benefits, including reducing soil erosion, boosting nutrient cycling, improving biological processes and crop yield (Korkanç and Şahin, 2021; Ranjan et al., 2017).

Millions of metric tons of organic materials are sadly wasted annually in the Kurdistan region of northern Iraq due to a lack of organic businesses and recycling companies. Mulching is done solely for research purposes, using very little organic material. After use, paper mulches decompose organically and become incorporated into the soil (Haapala et al., 2014b). The use of organic mulching in organic farming has several goals, including improving crop productivity, encouraging sustainable farming methods, and strengthening soil health. In brief, using organic mulching techniques in organic farming is a comprehensive and sustainable strategy. Long-term, it improves the resilience and general health of the farming ecosystem in addition to increasing crop productivity. Mulches can be alive, synthetic, dead, organic, non-organic, biodegradable, or neither. They can also take on many forms and contents (Gul et al., 2022). In this section we will focus more on organic and non-organic mulches.

2.2.2 The most commonly used ingredients or components for organic mulching

Straw or crop remnants are easily accessible after harvest. Straw mulch is an easily applied and lightweight material. These days, field mulch made of paddy straw is widely used because it enhances crop cultivation conditions. However, using straw as mulch can lead to several problems. Because straw mulches are highly flammable and contain grain seeds that may germinate and reduce soil nitrogen levels as they decompose, they must be replaced annually (Goodman, 2020).

Tree bark is another material that is widely used as mulch, particularly in gardens and small places. Bark mulches work well because they retain moisture longer and give the crop more time to get water. It is frequently applied to vegetation and landscaping. However, it shouldn‘t be used in vegetable fields due to its acidity (Iqbal et al., 2020). However, this mulch is suitable for covering the paths that run between the beds (Bantle et al., 2014). They are two types of bark clipping sources which can be used as mulch (hardwood and softwood). The paper and lumber industries produce hardwood bark clipping, which comes in a variety of sizes from chips to larger nuggets. It is primarily utilized close to trees and shrubs. Bark comes in both naturally occurring and coloured varieties. Typically, coloured varieties are made of a combination of non-natural peroxides and recycled wood waste. Although hardwood bark clippings are more nutrient-dense than soft wood, they are not always easily accessible (Prem et al., 2020). Whereas, a softwood bark is a byproduct of the paper and wood industries. Pine bark is a typical example, and it‘s often used beneath big trees and shrubs. These barks come in a variety of sizes and are typically applied to a depth of two to four inches (Ranjan et al., 2017).

Wood chips are made from a variety of tree species and reprocessed wood. Wood chip mulches may limit the amount of soil nitrogen available for plant absorption during their decomposition because of their high C: N ratio (Bantle et al., 2014).

Sawdust in areas where sawdust is easily accessible, it is widely used mulch. It is discovered while doing wood finishing processes. With only half the nutrients of straw, it has a lower nutritional value. Because of the high C: N ratio, the breakdown occurs slowly. Because of its breakdown, the soil faces the nitrogen deficiency which later requiring frequent fertilizer application. Low soil pH shouldn‘t be used because of its acidic nature. Having said that, it does retain moisture for a long time (Tan et al., 2016).

Compost easily made at home from a variety of waste materials, including leaves, grass, straw, and plant wastes, compost is a great mulch and soil conditioner. The availability and use of compost in agriculture have long been practiced. It increases the soil qualities and carbon content, which strengthens the soil‘s ability to hold water and promotes soil health. However, compost is not suggested to be used in vegetable fields due to its higher N content because of the increased likelihood of weed growth (Sofy et al., 2022).

Using newspaper mulch to suppress the germination of last season‘s fallen weed seeds is an inexpensive method of controlling weed growth. The layers of newspaper biodegrade into the soil rapidly. Newspaper breaks down over time, making it a better material Compare with organic mulch instead of plastic. It takes less time and is less costly (Haapala et al., 2014a).

Because leaves are abundant and easily accessible, they create excellent mulch ((Ranjan et al., 2017). The main issue with using leaves as mulch is that they are so light that they can be blown away even at low wind speeds. On the other hand, they are beneficial for keeping dormant plants warm and aiding in germination throughout the winter (Patil Shirish et al., 2013). In order to mitigate these issues, materials such as bark, stone, or any other substance that can lower wind speed should be used (Patil Shirish et al., 2013).

2.2.3 The most commonly used ingredients or components for inorganic mulching

Mulches composed of inorganic materials that are devoid of organic matter are called inorganic mulches. Stones and gravels, polyethylene films, landscaping materials, and rubbers are examples of inorganic mulches (Prem et al., 2020). Such as plastic mulch, make up the majority of mulch used in industrial crop cultivation. The plastics used as mulch are polyethylene films or polyvinyl chloride. Because of its increased permeability to long-wave radiation, it may cause an increase in temperature around the plants at night in the winter (El-Beltagi et al., 2022). Therefore, it is advised to use polyethylene film mulch as a mulching material when cultivating horticultural crops (Gosar and Baričevič, 2011). Using plastic as mulch in farming is a practice known as „ Plasticulture,“ which is gradually producing fresh vegetables (Serrano-Ruiz et al., 2021). Since they are synthetic, they cannot break down. According to the most recent update, mulching with synthetic materials specifically plastic has evolved into a cutting edge, sophisticated, and efficient method in field agriculture output today (Somanathan et al., 2022) and cased a severe issue to the most agricultural lands (Lal, 2023). Plastic mulching can treat plants, get rid of pests, and make the soil drier (Zhang et al., 2020). Every year, more than a million tons of plastic film mulch are utilized worldwide (Yu et al., 2018) .

And inorganic mulching has many disadvantages like when utilized as mulch, dry materials increase the chance of fire, which could harm trees (Petratou et al., 2023). Because thick mulches can serve as havens for mice and other rodents to breed and survive, they face the risk of damaging tree trunks and roots through their nibbling of bark and burrowing into the ground (Iqbal et al., 2020). Because inorganic mulches do not break down in the soil, seasonal crops should remove them from the field when their fruiting season is over (Adnan et al., 2020).

2.3 DISADVANTAGES OF MULCHING

Farm‘s unique circumstances and organizational structure will determine whether mulching is practical and cost-effective as well as how to implement it (Finckh et al., 2015). Additionally, other significant problems with some organic mulching materials, like straw and grass, are weed growth and acid leakage (Patil Shirish et al., 2013). There are certain disadvantages to mulching, including the need for more labour, higher transportation costs, and challenging removal and disposal (By and Rivera, 2023).

Because the plastic mulch is generating pieces that come into direct contact with the soil, the soil becomes contaminated (Wang et al., 2016). Plastic film wreckages are buried or disposed of by farmers using onsite burning or landfilling, which seriously contaminates the soil and hinders crop development (Gonzalez-Dugo et al., 2014). Mulching increases soil moisture retention, which limits the flow of oxygen near the roots due to poor soil drainage. If mulching is done in close proximity to the stem, many pests, diseases, and microorganisms may find refuge in the moisture surrounding the stem of the plant. Mulches made of straw, hay, or other seeds and grass clippings, can encourage weed growth (Prem et al., 2020). With the exception of biodegradable plastic mulches, inorganic mulches do not break down and do not contribute any nutrients to the soil. In certain cases, the sun will degrade inorganic mulch and cause it to deteriorate over time. If it is dispersed throughout a large area, it may increase the temperature of the earth. Rubber is non organic mulch that is potentially harmful to plants, poisonous and damaging to the ecosystem (Prem et al., 2020). Some very common disadvantages of mulches are shown in table 4.

Utilizing one waste material to reduce pollution caused by another is crucial for the development of sustainability and for improving environmental issues.

Table 4: Disadvantages of using mulches on plants, soil properties

Inorganic mulch	Disadvantage	Reference
Rock	Although soil is absorbent, it lacks organic matter, can get heated and impact the roots of plants with shallow roots, and cannot be tilled.	Hussain et al. (2023)
Gravel	absorbent, doesn't add organic matter to the soil; might get heated and damage shallow-rooted plants' roots	Datta and Meena (2021)
Black and plastic mulch	Not air- or water-permeable; capable of suffocating plants with shallow roots non-biodegradable; provides no nutrients; weeds thrive under clear plastic; have to be covered with more mulch to shield it from UV ray harm.	Iqbal et al. (2020)

In this regard, straw have both been utilized as natural environmental adsorbents as well as after undergoing a variety of treatments to increase their sorption capabilities (Goodman, 2020). Additional useful for the findings regarding microbial activity and functional diversity indicated that the weather had a significant impact on them across all mulching treatments (Brunetti, 2014).

In other studies, examining the effects of various mulching techniques, it was discovered that most mulching techniques generally raise soil temperature, which has an impact on the germination, growth, flowering, and harvesting stages of cucumber plants, generally speaking, the temperature rises with soil depth (Iqbal et al., 2020). It has been demonstrated that mulching raises the surface temperature while decreasing the temperature beneath the soil (El-Shaikh and Fouda, 2008). Furthermore, paper mulches have the advantage of not posing the disposal issue that plastic films and partially degradable bio-films frequently do.

3 CONCLUSIONS

In conclusion, mulching is an essential practical need for the arid and semi-arid environments since it provides many advantages to the soil, plant yield and water shortages. Because organic mulching has so many advantages over inorganic mulching, it is a better choice for farmers who want to engage in ecologically responsible and sustainable agriculture. Mulching has a significant effect on several soil and plant health factors. Natural materials such as bark or compost are used to create organic mulch, which can also improve soil moisture retention, balances soil temperature, and encourages seed germination. It increases the organic matter in the soil, which supports the growth of bacteria, fungi, and microorganisms. This natural cover also helps suppress weeds by reducing competition for resources. Organic mulch provides ecosystem support, which frequently reduces pest problems. As a result, there is a synergistic impact that favourably affects vegetable yield.

On the other hand, inorganic mulches, which are usually composed of chemical-based materials, do not offer the same organic advantages and can even interfere with the natural processes occurring in the soil. Although they suppress weeds, they have disadvantages such as decreased soil fertility and the possibility of heat accumulation. Organic mulches are superior for supporting sustainable agriculture since they increase soil health and ecological balance.

4 ABBREVIATIONS

Ca: Calcium
CPB: Colorado potato beetle
Fe: Iron
GWP: global warming potential
GM: Genetically modified
K: Potassium
Mg: Magnesium
Na: Sodium
N: Nitrogen
PFM: Plastic film mulch
SOC: Soil organic carbon
SOM: Soil organic matter

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6 AUTHOR CONTRIBUTIONS

AAY worked as MKA's MSc supervisor in the organic farming field. As part of her MSc study, MKA re-

ceived guidance from AAY on how to conduct research for and write a review article pertaining to her master's work. AAY examined and made improvements to all of the used data, figures, and references. AAY revised and enhanced certain sections and provided valuable recommendations to enhance other sections. Both authors proofread and examined the completed manuscript.

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There are no external funding sources for this review. The authors used their own resources to conduct the study.

8 DATA AVAILABILITY DECLARATION

No new data were created or analysed in this study. Data sharing is not applicable to this review article.

9 DECLARATIONS

9.1 ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable. Concerns or personal details were not included in the current investigation.

9.2 CONSENT FOR PUBLICATION

The document doesn't contain any recordings, interviews, or other personally identifiable information. Thus, giving agreement for topics of a personal nature is not appropriate. Moreover, there are no competing interests that would keep the authors from publishing this study.

9.3 COMPETING INTERESTS

The authors confirm that no conflicts of interest exist.

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