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ENHANCING QUALITY IN *Bidens ferulifolia*: INTERPLAY OF LIGHT EXTENSION AND GROWTH RETARDANTS IN GREENHOUSE CULTIVATION

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ABSTRACT

This study examined the effects of photoperiod extension (16-hour day) and growth retardant application during the liner stage (weeks 5–11) on nutrient uptake and plant quality in two *Bidens ferulifolia* cultivars (Fire&Spicy and Hot&Spicy). Treatments included supplemental lighting (L), growth retardant (R), both (L-R), and a control (NL-NR). Mature plants were assessed for plant architecture, flowering, nutrient status, photosynthetic pigments, and soluble sugars. All plants branched vigorously, but L-R produced the most commercially favourable structure, with fewer long shoots and more short ones. L-R also yielded the shortest shoots and highest dry mass, especially in Hot&Spicy alongside increased P and Zn. Retardants reduced fresh mass but increased levels of N, P, K, S, and Cu, while decreasing Fe and Mn. Light-treated plants have more fully open flowers but had similar bud numbers. Supplemental light improved nutrient accumulation, chlorophyll a, carotenoids, and sugar content, indicating better physiological efficiency. Cultivars responded differently to R, with Fire&Spicy showing greater micronutrient uptake. Combining light and retardant during the liner stage enhances visual quality and nutrient efficiency in *B. ferulifolia*, offering growers a strategy to improve crop performance while potentially reducing reliance on chemical growth regulation.

Keywords: balcony plants, macro- and microelements, ornamental plants, photosynthetic pigments, greenhouse plants production, vegetative traits

INTRODUCTION

Bidens ferulifolia, commonly known as Bidens or fern-leaved Bidens, is a vigorous, herbaceous ornamental species that serves as a summer bedding plant, widely appreciated for its bright yellow flowers, trailing habit, and adaptability in containers, hanging baskets, and landscaping. *B. ferulifolia*, a member of the

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Asteraceae family, is classified within the *Bidens* genus, which comprises approximately 280 species globally. The genus originated in Arizona, New Mexico and Northern Mexico, with subsequent diversification across North and South America, indicating a complex phylogenetic relationship with *Coreopsis* [Abdullah et al. 2025]. *B. ferulifolia* has been used as an ornamental plant for nearly three decades, particularly in gardens, containers, and mixed ornamental hanging baskets, despite being a perennial typically discarded after the end of the growing season [Nowak 2003, Walliser et al. 2022]. Its popularity among growers and consumers continues to grow due to its heat tolerance, prolonged flowering, and visual appeal [Dwyer 2022]. Progress in breeding programs has resulted in new cultivars with interesting new colour combinations, ranging from pure yellow to yellow-red, white-red, pure white, and purple, which contain natural pigments including flavonoids, anthochlors, and carotenoids [Walliser et al. 2022]. In commercial horticulture, bidens is propagated through vegetative cuttings, and the initial phase of plant development – liner production – is critical for ensuring uniformity and marketable quality at later stages [Nau et al. 2021].

In modern greenhouse cultivation, achieving compact, well-branched liners is a major production goal. However, cuttings of young balcony plants are prone to excessive elongation and internode extension, particularly under suboptimal light conditions during early spring or in high-density propagation settings [Munir and Alhajhoj 2017, Liebers and Pfannschmidt 2024]. This tendency for unwanted stem elongation not only affects the visual quality of the final product but also increases the need for manual pinching or chemical interventions, both of which add to production costs and labour inputs [Faust et al. 2016].

Light is one of the most potent environmental cues in regulating plant growth, with photoperiod and light intensity influencing both vegetative and reproductive development [Vyavahare et al. 2024, Kowalczyk et al. 2024]. Extending the day length using supplemental lighting to achieve a 16-hour photoperiod has been shown to reduce stem elongation, branching, and biomass accumulation in many bedding plant species that belong to long-day or neutral-day plants [Adams and Langton 2005, Meng and Runkle 2014]. In addition to morphological changes, light can impact physiological processes such as nutrient uptake and assimilation. However, while the role of extended photoperiods in promoting compact growth is well-documented in genus like *Petunia*, *Viola* [Collado and Hernández 2022] the specific effects on *Bidens ferulifolia* – particularly in interaction with growth regulators – remain underexplored.

Chemical growth retardants are widely used in ornamental production to manage plant height and habit. Compounds such as daminozide, paclobutrazol, or chlormequat chloride reduce internode elongation by inhibiting gibberellin biosynthesis, leading to more compact and marketable plants [Pobudkiewicz 2008, Whipker 2019]. In addition to modifying shoot architecture, growth retardants alter physiological processes by reducing water consumption, enhancing root formation, and modifying plant morphology, which affects the root-shoot ratio [Gent and McAvoy 2024]. Despite their routine use, the influence of these compounds on the accumulation of macro- and microelements in liner-stage bidens has not been clearly established.

While both photoperiod manipulation and growth retardants are common strategies in liner production, little is known about their potential interaction effects on nutrient dynamics and overall plant quality. Most existing studies examine these factors in isolation, and few address their combined impact under realistic commercial greenhouse conditions [Munir and Alhajhoj 2017, Collado and Hernández 2022]. Furthermore, growers seek sustainable approaches that minimise chemical inputs while maintaining high production standards, highlighting the need for trials that integrate lighting strategies with moderate plant growth regulator (PGR) use.

We hypothesised that extending the photoperiod to 16 hours and applying growth retardants during the liner stage would not only regulate shoot elongation and branching but also affect nutrient uptake, pigment accumulation, and overall quality of *Bidens ferulifolia*. In particular, we expected that the combined treatment would enhance commercial value by producing compact, well-nourished plants with desirable architecture. Therefore, the objective of this study was to assess the effects of photoperiod extension and growth retardant application during the rooting stage on nutrient status, plant architecture, flowering, and the

concentration of photosynthetic pigments. By testing these factors under commercial production conditions, we aimed to provide practical recommendations for optimising young plant quality while reducing reliance on chemical growth regulators.

MATERIAL AND METHODS

The study was carried out in early 2023 at the Plantpol nursery (Jezioro Street, Zaborze, Poland), using two cultivars of *Bidens ferulifolia* Fire&Spicy and Hot&Spicy. Unrooted cuttings were collected from mother plants on February 1, 2023 (week 5) and immediately placed to the greenhouse for the propagation stage of commercial production. The experiment consisted of two successive cultivation phases: young plant production – liners production (where researched treatments were applied) and subsequent finishing to produce market-ready plants (following the observations and tests) [Szewczyk-Taranek et al. 2025].

The cultivation stages are described in detail below.

Stage 1: Liner Production. Cuttings (approx. 2.5 cm) of two cultivars (Fire&Spicy and Hot&Spicy) were planted singly into paper pots filled with a substrate containing 30% coconut fiber, 40% fine peat, 15% polystyrene, and 15% perlite (substrate SoMi 537, producer Hawita, Vechta, Germany). These were arranged in 104-cell trays (HerkuPlant, Germany) and placed on greenhouse benches. During the initial week, the fogging maintained a relative humidity of 70–80%, which was gradually reduced to 55–65%. The temperature was held at 20 °C/18 °C (day/night, ±2 °C) for the first 3 weeks, then lowered to 18 °C/16 °C. After rooting, the cuttings were pinched (week 4), spaced at half density (52 per tray), and grown for an additional 3 weeks.

Stage 2: Finishing. In week 11, rooted liners were transplanted into 12 cm pots (1 dm^3), filled with a growing medium of 70% white peat, 15% coconut fiber, and 15% clay (substrate EP 340, pH 5.8, producer Hawita, Vechta, Germany). Plants were cultivated for 3 weeks until they reached commercial maturity. During both phases, an ebb-and-flow irrigation system was employed with fertigation, using a 0.03–0.05% solution of the soluble fertilizer Granusol 10-30-20 + MgO + TE (Mivena BV, The Netherlands). Environmental conditions were managed via a SERCOM SC800 climate control system (Regeltechniek BV, The Netherlands).

The study followed a 2 × 2 factorial design assessing: (1) photoperiod: natural light only (NL) vs. 16-hour day with supplemental lighting (L) and (2) growth retardants: untreated (NR) vs. chemically treated (R). This yielded four treatment groups: NL-NR: no light, no PGR (Plant Growth Retardants); NL-R: no light, with PGR; L-NR: light only; L-R: light and PGR. Each treatment included four replicates: one tray of 104 plants per replicate in Stage 1 and 52 pots per replicate in Stage 2. Plants remained assigned to the same treatment group throughout both stages.

Extended photoperiod treatment was achieved with the use of supplemental lighting applied from week 5 to 11, extending the day to 16 hours (5–21) using 600W HPS lamps (OSRAM Plantastar, 87,000 lumens, 2000K, OSRAM GmbH, Germany), delivering ~100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD at 1 m height. Lamps were adjusted to avoid overlap with natural daylight – activated 20 minutes before sunset and deactivated 20 minutes after sunrise. Daylength during the experiment naturally ranged from 8 to 11.5 hours.

Growth retardant application involved weekly sprays of daminozide (Dazide Enhance 85 SG, Fine Agrochemicals The Netherlands), 0.2% solution from week 2 to 11. Additionally, one application of paclobutrazol (BONZI, Syngenta, Switzerland, 0.3%) was applied as a foliar spray. Sprays ensured full shoot coverage without runoff. The application volume was 100 dm^3 per 1,000 m^2. At the end of Stage 2, plant quality traits were assessed, including the number and length of shoots (>5 cm), the number of flowers and buds, and biomass of the aboveground plant part (without flowers and flower buds). The data was subjected to a three-factor analysis of variance.

Biochemical analyses included assessment of photosynthetic pigments and soluble carbohydrate content. Leaf samples from 30 plants per treatment were lyophilised and homogenised. Chlorophyll a, chlorophyll b, and carotenoid contents were determined spectrophotometrically after extraction in 96% ethanol, using absorbance values at 470, 649, and 664 nm, and calculated according to Sumanta et al. [2014]. For the determination of sugars, leaf samples (1 g of tissue) were homogenised for 2 minutes at 25 strokes per second using a steel ball. This was followed by an

additional 1-minute homogenization at 20 strokes per second in the presence of 1 mL of deionised water. The resulting mixtures were centrifuged at 15 °C for 10 minutes at 12,800 rpm (15.2 · g). Soluble sugar content was then determined using the anthrone method, as described by Dische [1962]. Absorbance was measured at 620 nm using a spectrophotometer. A standard calibration curve was prepared using glucose solutions of known concentrations, which was then used to calculate the glucose content in the samples.

Matured plants were also tested for nutrient content. For this analysis, above-ground tissues (excluding flowers) from 30 plants per replicate were dried at 75 °C, ground, and analysed following the PN-ISO 6496:2002 and PN-EN-ISO 712:2012 methods. Total nitrogen was measured via the Kjeldahl method [Sáez-Plaza et al. 2013]. Macro- and micronutrient content was quantified by ICP-OES (Prodigy Plus instrument, Teledyne Leemans Labs, USA) following microwave digestion in concentrated HNO_3 (MARS 2 system, CEM Corp., USA). The elemental concentration in plant tissue was expressed on a dry mass basis as a percentage (%) for the macronutrients, N, P, K, Ca, Mg, S. The micronutrients are expressed in parts per million (mg kg^{-1} dry mass) for B, Cu, Fe, Mo, Mn, Zn.

Data were subjected to tree-way ANOVA using Statistica 13.3 (TIBCO Software Inc., USA). Differences among means were assessed via the Duncan's test at $p \leq 0.05$ or Tukey's HSD test at $p \leq 0.01$ (for mineral status).

RESULTS AND DISCUSSION

This study assessed the impact of supplemental lighting (extending the day to 16 hours) and growth retardants on the quality and nutrient status of *Bidens ferulifolia* during greenhouse cultivation. Evaluations included morphological, physiological, and biochemical parameters, with a focus on commercially relevant traits.

Table 1. The effect of supplemental light and retardants on morpho-physiological traits of matured *Bidens ferulifolia* plants; L – prolonged light period 16 h, R – retardant treatment, NL – no supplemental light, NR – no retardant, C – cultivar

Treatment	Short shoots number	Long shoots number	Shoots number (mean)	Shoots length (cm)	Upper part weight (g)
Fire&Spicy:					
NL-NR	18.50 ab*	24.94 c	43.44 ab	14.35 bc	37.48 b
NL-R	27.00 cde	17.50 ab	44.50 b	10.02 a	31.72 a
L-NR	23.38 bcd	17.75 ab	41.13 ab	13.15 bc	47.44 d
L-R	32.00 e	15.25 a	47.25 b	9.52 a	40.83 b
Hot&Spicy:					
NL-NR	13.13 a	21.88 bc	35.00 a	15.09 d	39.87 b
NL-R	20.75 bc	20.63 abc	41.38 ab	9.69 a	29.53 a
L-NR	19.50 ab	24.25 c	43.75 b	12.45 b	46.98 cd
L-R	28.13 de	18.13 c	46.25 b	8.20 a	41.35 bc
Main effects:					
L	***	**	**	***	***
R	***	***	***	***	***
C	***	ns	ns	ns	ns
L × R	ns	ns	ns	ns	ns
L × C	ns	**	**	ns	ns
R × C	ns	ns	ns	ns	ns
L × R × C	ns	***	ns	ns	

* means in columns followed by the same letter do not differ significantly at α = 0.05 according to Duncan's multiple range test. Significant effect: **, $p \leq 0.05$; ***, $p \leq 0.01$; ns – not significant

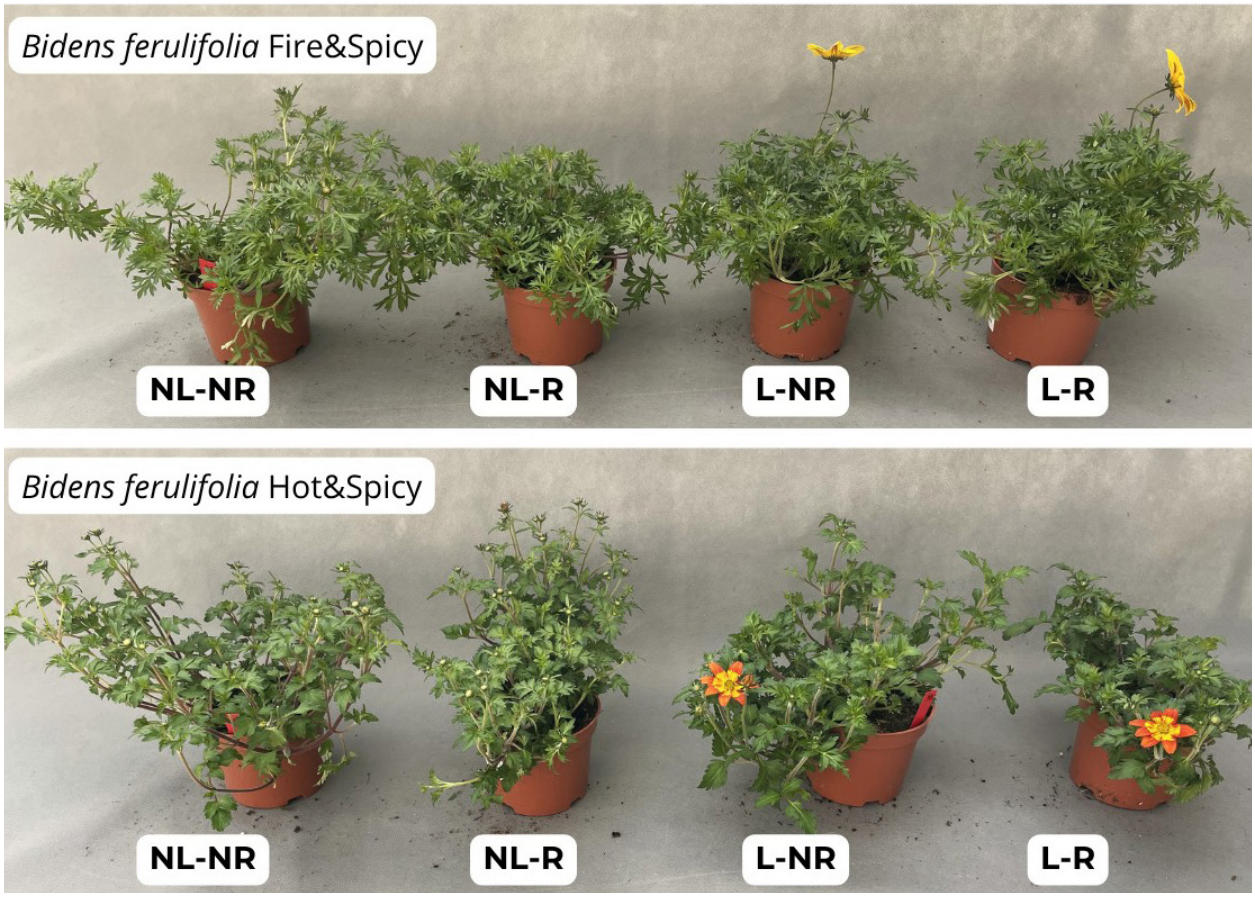


Fig. 1. Mature plants of two cultivars of *Bidens ferulifolia* – final commercial product at the end of stage 2, treated with extended lighting and retardants in stage 1 (liner production). Treatments: L – extended lighting to 16 h, R – retardant treatment, NL – no extended lighting, NR – no retardant

Plant architecture and biomass

Both tested cultivars exhibited prolific branching, with over 40 shoots per plant at maturity (ranging from 41–47), except for Hot&Spicy in the NL-NR treatment, which averaged 35 shoots. The branching pattern was not significantly affected by cultivar (Table 1, Fig. 1). The most compact habit – marked by fewer long shoots – was recorded in the L-R treatment, while the highest number of short shoots occurred in Fire&Spicy under NL-R conditions. From a commercial standpoint, a high number of short, evenly distributed shoots is desirable, as it enhances the plant's visual density and marketability. Both cultivars responded to growth retardants with significantly shorter shoot lengths, regardless of light treatment, suggesting that retardants effectively controlled vegetative elongation across genotypes. No significant interactions between factors were observed in this trait.

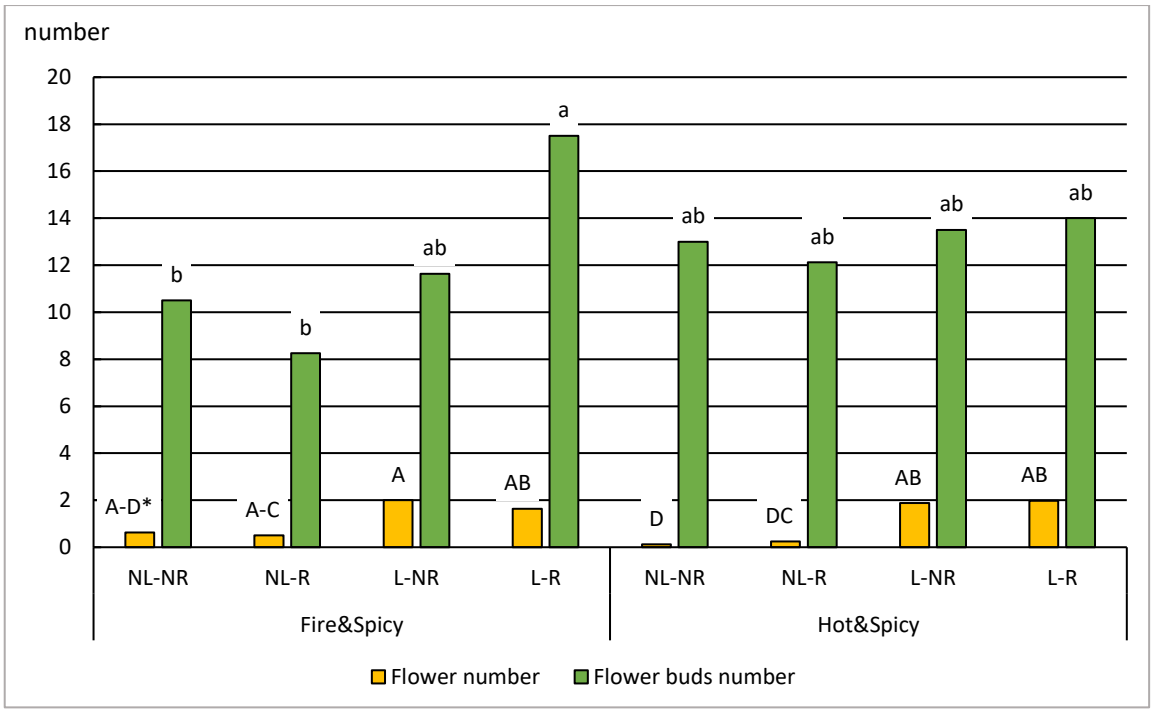
Growth retardants are widely used in bedding plant production to regulate plant height, prevent unwanted elongation, and improve visual compactness. Their mode of action typically involves inhibition of gibberellin biosynthesis, reducing internode extension and promoting lateral branching. However, the final response depends on species sensitivity, environmental conditions, and retardant formulation, concentration, and application schedule [Pobudkiewicz 2008, Bergstrand 2017]. In our study, combining retardant application with supplemental light led to more compact and commercially attractive plants.

Fresh weight measurements indicated that the L-NR treatment resulted in the greatest biomass accumulation in both cultivars (Table 1, Fig. 1). This aligns with previous reports that higher light exposure increases dry mass accumulation by boosting photosynthetic activity [Llewellyn et al. 2020]. Interestingly, while retardants reduced shoot elongation, they also slightly decreased biomass, likely due to restricted growth and reduced leaf area.

Number of flowers and flower buds

At market maturity, plants originating from liners grown under extended photoperiod (L) had a higher number of fully open flowers – typically around two per plant – making them more appealing to potential customers. In contrast, plants from the other treatments flowered only sporadically. Regarding flower buds, Fire&Spicy showed greater responsiveness to treatments, particularly under L-R conditions, where over 17 buds were counted. In comparison, Hot&Spicy exhibited less variation in flower bud number across treatments.

Although the number of flower buds did not differ significantly, supplemental light accelerated flower opening, particularly in Fire&Spicy. This suggests that while bud initiation remained unaffected, the light treatment advanced floral development and phenological progression. These findings are consistent with the behaviour of other bedding plants, such as *Petunia* and *Pelargonium*, where supplemental light or higher daily light integrals (DLI) were shown to accelerate the number of flowers and improve ornamental quality [Runkle et al. 2011, Wollaeger and Runkle 2014]. Prolonged light exposure enhances the total number of shoots, flower buds, and flowers in *Petunia* and *Calibrachoa*. Species classified as long-day plants exhibited the highest total numbers of flowers and flower buds under prolonged lighting [Szewczyk-Taranek et



*means followed by the same letter do not differ significantly at α = 0.05 according to Duncan's multiple range test; uppercase letters indicate differences in flower number; lowercase letters indicate differences in flower buds

Fig. 2. Effect of extended lighting and retardant treatment in stage 1 (liner production) on the number of flowers and flower buds of two cultivars of mature *Bidens ferulifolia* plants. Treatments: L – extended lighting to 16 h, R – retardant treatment, NL – no extended lighting, NR – no retardant

al. 2025]. Although *B. ferulifolia* is considered photoperiod-neutral, our findings imply that light supplementation can positively influence flowering time and floral display, even without altering bud initiation. This highlights the value of manipulating light environments in photoperiod-insensitive species to enhance market quality and shelf appeal.

Photosynthetic pigments and soluble sugars

Supplemental light significantly increased chlorophyll a, chlorophyll b, and carotenoid levels ($p \leq 0.001$), with the strongest effect observed for chlorophyll a under L treatments (Table 2). Retardants slightly enhanced pigment content as well, though not significantly in all cases. Hot&Spicy showed a higher baseline carotenoid content, which was further enhanced by light exposure. Interestingly, while chlorophyll a increased under light in both cultivars, chlorophyll b in Hot&Spicy was higher under natural light, suggesting a genotype-specific light response.

Elevated pigment content under extended photoperiods is typical of plants grown under high light intensity or prolonged photoperiod, as increased DLI promotes chloroplast development and enhances photosynthetic efficiency [Bergstrand and Schüssler 2012, Llewellyn et al. 2020]. The rise in pigment levels also reflects improved plant vigour and a higher capacity for carbohydrate synthesis.

Total soluble sugar content was significantly affected by all factors and their interactions, with the highest concentrations recorded in plants exposed to supplemental light during the liner stage (Table 2). This supports the hypothesis that supplemental light improves assimilate production, contributing to enhanced plant vitality and postharvest performance. High sugar levels may also serve as osmoprotectants, improving stress resilience and post-transplant recovery [Morrow 2008, Runkle 2007].

Table 2. The effect of supplemental light and retardants on photosynthetic pigments and sugar levels in leaves of *Bidens ferulifolia* plants; L – prolonged light 16 h, R – retardant treatment, NL – no supplemental light, NR – no retardant, C – cultivar

Treatment	Chlorophyll a (µg/g d.m.)	Chlorophyll b (µg/g d.m.)	Carotenoids (µg/g d.m.)	Soluble sugars (mg/g d.m.)
Fire&Spicy:				
NL-NR	33.68 a*	18.34 a	6.14 a	86.57 b
NL-R	35.98 b	20.40 abc	6.70 b	76.17 a
L-NR	36.49 bc	19.50 ab	6.44 ab	93.87 c
L-R	37.27 c	19.64 ab	5.92 a	100.00 c
Hot&Spicy:				
NL-NR	36.31 b	27.88 d	8.63 c	75.81 a
NL-R	41.05 d	32.74 e	8.33 c	85.23 b
L-NR	46.90 e	22.41 bc	9.93 d	100.47 c
L-R	48.45 f	22.90 bc	10.21 d	110.87 d
Main effects:				
L	***	***	***	***
R	ns	***	ns	***
C	ns	***	***	***
L × R	ns	***	ns	***
L × C	ns	***	***	***
R × C	ns	ns	ns	***
L × R × C	***	ns	***	***

* explanations as in Table 1

The combined use of supplemental lighting and growth retardants clearly influenced the physiological and morphological traits of *B. ferulifolia*, with differences observed between cultivars. The L-R treatment consistently led to more compact, well-branched plants with better flower display and increased pigment and sugar content – features that enhance commercial value. Particularly favourable responses were recorded in Hot&Spicy under L-R conditions.

Our findings reinforce the importance of managing environmental conditions during the early stages of production. Interventions during the liner phase (weeks 5–11) had lasting effects through to week 15, supporting earlier studies showing that early environmental signals have long-term impacts on development and market performance [Wollaeger and Runkle 2014]. Even though bidens is not classified as photoperiod-sensitive, our data demonstrate that

Table 3. The effect of supplemental light (L) and retardants (R) on mineral profile (macroelements content expressed as % of dry mass) of the plants of two cultivars (C) of *Bidens ferulifolia* in the mature stage

Treatments				d.m.	N	Ca	K	Mg	P	S
Light		L		9.87 b*	5.82 b	1.45 b	5.26 a	0.35 b	0.94 b	0.37 b
		NL		9.53 a	5.50 a	1.32 a	5.32 a	0.33 a	0.86 a	0.30 a
Growth retardation (R)		NR		9.74 a	5.51 a	1.38 a	5.19 a	0.35 b	0.89 a	0.32 a
		R		9.66 a	5.81 b	1.39 a	5.39 b	0.33 a	0.91 b	0.34 b
Cultivar (C)		Fire&Spicy		9.98 b	5.85 b	1.25 a	5.09 a	0.33 a	0.89 a	0.32 a
		Hot&Spicy		9.41 a	5.46 a	1.52 b	5.49 b	0.36 b	0.90 a	0.34 b
L × R	L	NR		9.64 b	5.78 a	1.47 c	5.22 a	0.36 c	0.92 b	0.35 a
		R		10.1 c	5.86 a	1.44 c	5.29 a	0.34 b	0.96 c	0.38 a
	NL	NR		9.83 bc	5.25 a	1.29 a	5.16a	0.33 ab	0.86 a	0.29 a
		R		9.23 a	5.75 a	1.35 b	5.48 b	0.32 a	0.86 a	0.31 a
L × C	L	Fire&Spicy		10.0 c	5.85 b	1.31 a	5.15 a	0.33 a	0.94 c	0.35 b
		Hot&Spicy		9.69 b	5.78 b	1.59 a	5.36 b	0.37 a	0.93 c	0.39 c
	NL	Fire&Spicy		9.92 bc	5.85 b	1.18 a	5.03 a	0.32 a	0.84 a	0.29 a
		Hot&Spicy		9.13 a	5.15 a	1.45 a	5.61 c	0.34 a	0.87 b	0.30 a
R × C	NR	Fire&Spicy		10.0 a	5.78 a	1.24 a	4.95 a	0.34 b	0.87 a	0.30 a
		Hot&Spicy		9.43 a	5.25 a	1.51 a	5.43 a	0.36 d	0.91 b	0.34 b
	R	Fire&Spicy		9.93 a	5.93 a	1.25 a	5.23 a	0.32 a	0.92 b	0.34 b
		Hot&Spicy		9.39 a	5.68 a	1.53 a	5.55 a	0.35 c	0.90 b	0.35 b
L × R × C	L	NR	Fire&Spicy	9.88 a	5.88 a	1.33 a	4.97 a	0.35 bc	0.91 a	0.33 a
			Hot&Spicy	9.40 a	5.68 a	1.60 a	5.47 c	0.38 d	0.93 a	0.38 a
		R	Fire&Spicy	10.2 a	5.82 a	1.29 a	5.33 bc	0.32 a	0.97 a	0.36 a
			Hot&Spicy	9.99 a	5.89 a	1.59 a	5.26 bc	0.36 cd	0.94 a	0.39 a
	NL	NR	Fire&Spicy	10.2 a	5.68 a	1.15 a	4.93 a	0.32 a	0.83 a	0.27 a
			Hot&Spicy	9.46 a	4.82 a	1.42 a	5.39 bc	0.34 b	0.88 a	0.31 a
		R	Fire&Spicy	9.65 a	6.03 a	1.22 a	5.13 ab	0.32 a	0.86 a	0.31 a
			Hot&Spicy	8.80 a	5.48 a	1.47 a	5.83 d	0.34 b	0.86 a	0.30 a

*means in columns for each factor followed by the same letter do not differ significantly at α = 0.01 according to Tukey's test

light management during propagation can positively affect flowering behaviour, foliage colouration, and biomass accumulation – traits directly linked to consumer preferences and retail success. These results align with findings in other balcony plants where light and growth regulators were used to optimise aesthetic quality [Llewellyn et al. 2020, Bergstrand and Schüssler 2012].

Mineral nutrient status

Understanding how plants accumulate and store elements is a research topic of current interest, particularly in the context of improving plant nutrition and enhancing plant quality. Our investigations are determining and helping to understand how treatments affect nutrient absorption and accumulation in bidens plants. Though general mineral nutrient guidelines exist for plants, specific sufficiency ranges for macro and micronutrients that define the boundaries of healthy plant growth are often limited and vary by plant species, growth stage, and environmental conditions. There is no data available to establish precise nutrient uptake guidelines and nutrition for interpreting plant analyses of the bidens plant.

Assessment of the two cultivars used in the experiment revealed that Fire&Spicy bidens plants had significantly higher dry mass, nitrogen (N), and boron (B) content in their aboveground biomass (Tables 3 and 4). On the other hand, the Hot&Spicy was distinguished by significantly higher calcium (Ca), potassium (K), magnesium (Mg), and sulfur (S), as well as all micronutrients, regardless of B and molybdenum (Mo) content.

The physiological age of a plant is the main factor influencing the mineral nutrient content in the plant's dry mass. Mineral nutrient concentrations are generally higher in young plants or actively growing tissues than in older ones. As plants develop, the nutrient content per unit of dry mass tends to decline, a phenomenon often described as a dilution effect within the tissue [Bryson and Mills 2014, Barker and Pilbeam 2015].

For most herbaceous plants, the optimal nitrogen content for proper growth typically ranges between 2% and 5% of the plant's dry mass [Barker and Pilbeam 2015]. In our study, the N content in bidens plants ranged from 4.82% (Hot&Spicy NL-NR) to 6.03% dry mass (Fire&Spicy NL-R, the lowest biomass was obtained for this treatment), see Table 3. Herbaceous fertilised plants commonly have a concentration of nitrogen that exceeds 3% of the dry mass in mature leaves. Though in the early stages of growth, concentration is high throughout the plant. The supply of N determines a plant's growth, vigour, colour, and yield [Barker and Pilbeam 2015, Bryson and Mills 2014]. Correspondingly, an enhancement of N assimilation and protein synthesis leads to an increase in chloroplast constituents such as chlorophyll [Marschner 2012]. Chloroplast growth and function were influenced by interactions between light and phytohormones [Brini et al. 2022]. This was confirmed by our study, which showed that the dry mass and chlorophyll content (Tables 2 and 3) increased with the nitrogen content in plants supplemented with additional light. However, excessive N can cause delayed flowering and ripening and increase water content in tissues [Zhang et al. 2023]. Other effects on development (shortening stem length, flowering, etc.) are involved, as would be expected, from the application of growth regulators that interfere with the phytohormone balance in plants by inhibiting the biosynthesis of gibberellins [McLoughlin 2000, Zheng et al. 2012].

The aboveground biomass of bidens plants contained from 4.93% of K (Fire&Spicy NL-NR) to 5.83% of K (Hot&Spicy NL-R) of dry mass (Table 3). Potassium is an important quality agent, both through a direct effect on crop quality and because it strengthens stress resistance. Jiang et al. [2024] revealed that the flower size and yield of chrysanthemum under high potassium treatment were significantly increased compared to using standard fertilisation practice. The K requirement for optimal nutrient status in plants is in the range of 2–5% of the plant's dry mass of vegetative parts. Highest concentrations of K are present in the new leaves, petioles, and stems [Bryson and Mills 2014]. When potassium supply is limited, interference with the uptake and physiological availability of magnesium and calcium can occur. In the presented study, in plants treated with NL-R, the more potassium was determined, the less calcium and magnesium the Hot&Spicy of plants contained (Table 3). In our study, the aboveground bidens biomass concentration of magnesium ranged from 0.32% Mg to 0.38% Mg, and calcium from 1.15% Ca to 1.60% Ca in the dry

plant mass (Table 3). The Mg requirement for growth and plant development falls within range in the range of 0.15–0.35% of the dry mass of the vegetative stage [Bryson and Mills 2014]. The optimal calcium content of plants varies between 0.1 and >5% Ca of dry mass, depending on the growing conditions. Its rate of uptake can be strongly depressed by other cations, such as potassium, ammonium, and manganese. Mg deficiency induced by antagonistic cations is a widespread phenomenon [Bryson and Mills 2014].

The phosphorus content in plants sampled for chemical analysis after 2 months of vegetation was high and, depending on the experimental factors, ranged from 0.83% P (Fire&Spicy NL-NR) to 0.97% P (Fire&Spicy L-R) in the dry mass (Table 3). For optimal growth, plants require only 0.3–0.5% of dry mass

Table 4. The effect of supplemental light (L) and retardants (R) on mineral profile (microelements content expressed as mg kg^{-1} of dry mass) of the plants of two cultivars (C) of *Bidens ferulifolia* in the mature stage

Treatments				B	Cu	Fe	Mn	Mo	Zn
Light		L		55.8 b*	12.5 b	175 b	93.4 b	4.69 b	54.0 b
		NL		49.3 a	9.0 a	120 a	64.9 a	1.90 a	45.0 a
Growth retardation (R)		NR		53.0 a	10.3 a	151 b	81.2 b	3.45 a	48.9 a
		R		52.1 a	11.2 b	143 a	77.1 a	3.14 a	50.1 a
Cultivar (C)		Fire&Spicy		54.3 b	9.8 a	123 a	77.0 a	3.41 a	43.3 a
		Hot&Spicy		50.8 a	11.6 b	171 b	81.3 b	3.18 a	55.7 b
L × R	L	NR		56.7 a	12.1 a	190 d	95.7 a	4.90 a	52.4 b
		R		55.0 a	12.9 a	159 c	91.2 a	4.47 a	55.6 c
	NL	NR		49.3 a	8.4 a	112 a	66.7 a	2.00 a	45.5 a
		R		49.3 a	9.6 a	127 b	63.0 a	1.81 a	44.5 a
L × C	L	Fire&Spicy		57.6 a	11.3 c	132 c	90.1 a	5.03 c	46.4 b
		Hot&Spicy		54.1 a	13.7 d	217 d	96.8 a	4.35 b	61.6 d
	NL	Fire&Spicy		51.0 a	8.4 a	115 a	63.8 a	1.79 a	40.2 a
		Hot&Spicy		47.6 a	9.6 b	125 b	65.9 a	2.02 a	49.8 c
R × C	NR	Fire&Spicy		53.8 bc	8.9 a	118 a	77.2 a	3.03 a	41.7 a
		Hot&Spicy		52.3 b	11.7 c	185 d	85.3 b	3.87 b	56.2 c
	R	Fire&Spicy		54.9 c	10.8 b	129 b	76.8 a	3.80 b	44.9 b
		Hot&Spicy		49.4 a	11.6 c	157 c	77.3 a	2.50 a	55.2 c
L × R × C	L	NR	Fire&Spicy	58.1 d	10.2 b	124 b	88.8 a	3.80 c	43.6 a
			Hot&Spicy	55.3 cd	14.0 d	256 e	103 a	6.00 d	61.2 a
		R	Fire&Spicy	57.0 cd	12.5 c	139 c	91.5 a	6.25 d	49.3 a
			Hot&Spicy	52.9 bc	13.3 cd	178 d	90.9 a	2.70 b	61.9 a
	NL	NR	Fire&Spicy	49.4 ab	7.5 a	111 a	65.6 a	2.26 ab	39.9 a
			Hot&Spicy	49.3 ab	9.3 b	113 a	67.9 a	1.73 ab	51.1 a
		R	Fire&Spicy	52.7 bc	9.2 b	119 ab	62.1 a	1.31 a	40.6 a
			Hot&Spicy	45.8 a	9.9 b	136 c	63.8 a	2.30 ab	48.5 a

* explanations as in Table 3

during the vegetative stage of growth [Barker and Pilbeam 2015]. The critical deficiency level of P in the whole plant decreases drastically with age in plants, but remains relatively constant at approximately 1% in young plant tissues [Barker and Pilbeam 2015].

In the studies, the sulphur content in bidens plants, depending on the cultivar, lighting and growth retarding, ranged from 0.27% (Fire&Spicy NL-NR) to 0.39% dry mass (Hot&Spicy L-R), see Table 3. Sulphur is a constituent of the amino acids cysteine and methionine, and hence of protein. Sulfur assimilation also shares many standard features with nitrate assimilation [Barker and Pilbeam 2015]. Plant S requirement varied between 0.1% to 0.5% of the dry mass. Sulphate reduction in leaves is a reaction strongly stimulated by light because ferredoxin is a reductant for the carrier-bound sulfite, and this light enhancement is to be respected. Rapid sulphate reduction stimulated probably S uptake by roots [Marschner 2012].

Not surprisingly, bidens plants exposed to additional light (L), regardless of the cultivar used, had significantly higher dry mass, N, Ca, Mg, P, S, and all micronutrient content in their aboveground biomass (Tables 3 and 4). Light, its intensity and quality affect all life processes of the plant, but mainly photosynthetic production and distribution of assimilates, and consequently, the growth intensity of its specific organs. Maximising the photosynthetic potential of plants is one method for improving yield [Brandon et al. 2018]. Plants receiving additional light assimilate more carbon and use proportionally less assimilates for respiration [Marschner 2012]. This favours the increased growth of shoots and leaves, which, as expected, was confirmed by the study conducted. In such conditions, N-use is also economical, which improves plant productivity. Bueno and Vendrame [2024] revealed that under white light, plants tend to have higher concentrations of K, Mg, and Ca in their biomass due to the increased dry mass, which was also observed in our research.

Chemical growth retardants used in our studies can induce several physiological responses, including reduced hormone biosynthesis, increased chlorophyll content, altered carbohydrate status, delayed flowering and senescence, and increased tolerance to environmental stresses [McLoughlin 2000, Zheng et al. 2012]. Growth retarding (R) treatment, which can decrease gibberellin (GA) synthesis in the subapical meristems of shoot tips [Rademacher 2000], as a consequence, reduced internode extension and altered plant morphology, increased the content of N, K, P, S, and copper (Cu) in bidens plants (Tables 3 and 4). On the other hand, growth retardants significantly reduced the iron (Fe) and manganese (Mn) content in biomass. The effects of paclobutrazol and other plant growth retardants on reproductive growth vary considerably among species, dose rates, and timing of application [McLoughlin 2000]. Plants retarded by exogenously introduced growth regulators or grown under limited light must adopt a survival strategy. The most significant activity in mobilising photosynthetic products and reserve substances is exerted by leaves and shoots, potential donors of assimilates [Epstein and Bloom 2005]. Plants strive to integrate the processes of nutrient synthesis and distribution in a way that ensure maximum plant growth under these stress conditions. Flowers/fruits are then not the primary acceptors of assimilates [Marschner 2012]. The application of growth retardants without light supplementation decreased flower number, flower buds, and upper part weight (Fig. 2, Tables 3 and 4). GA also influences the expression of flowering-initiation genes in both the leaf and shoot apical meristem. GA biosynthesis is activated rapidly after a transition from short to long days [Brini et al. 2022]. Plants constantly adapt to changing environmental conditions, exhibiting a large scale of metabolic and morphological plasticity [Epstein and Bloom 2005, Marschner 2012]. Adaptive responses are regulated by many factors, including sugar content and hormones that regulate gene expression. In the presented research, we detected an increase in sugar content in two cultivars of bidens plants treated with supplemental light and chemically retarded (L-R), see Table 3. The potassium and GA act synergistically. Cell expansion in leaves controlled by GA is closely related to their potassium content. The enhancement of stem elongation by GA is also dependent on the K^+ supply. The highest potassium content was detected in bidens plants underexposed to light and chemically treated (NL-R).

The number of flowers and fruits per plant can be directly affected by the supply of mineral nutrients. This is particularly true for several micronutrients [Epstein and Bloom 2005]. For example, copper deficiency af-

fects the reproductive phase. When the copper supply is adequate, the generative organs have the highest Cu content in the flowers and also the highest Cu demand [Jun et al. 2023]. Similar results are observed for zinc (Zn) and manganese deficiency. Also, low boron supply inhibits flowering and seed development. The review by Jun et al. [2023] discussed the role of micronutrients, such as boron, zinc, iron, and copper, in enzymatic reactions and hormone biosynthesis, which affect flower development and reproduction. The critical deficiency level of copper in vegetative parts is generally in the range 1–5 mg Cu kg^{-1} d.m. [Bryson and Mills 2014]. In our study, copper content in bidens plants was high and varied between 7.5 mg Cu kg^{-1} (Fire&Spicy NL-NR) and 14.0 mg Cu kg^{-1} dry mass (Hot&Spicy L-NR), see Table 4.

In the presented study, the boron content in bidens plants ranged from 45.8 mg B kg^{-1} dry mass (Hot&Spicy NL-R) to 58.1 mg B kg^{-1} dry mass (Fire&Spicy L-NR), see Table 4. A range of values that describe the nutrient status of different bedding plants varies from 15 to 80 mg B kg^{-1} dry mass [Bryson and Mills 2014]. However, the ratio of toxic to adequate boron levels is smaller than for most other nutrient elements. Growth retarding reduces the boron content in Hot&Spicy plants, while it is contrary to the Fire&Spicy plants. Similar relationships were found for Fe and Mo (Table 4). The critical manganese deficiency contents in bidens plants are similar, varying between 10–20 mg Mn kg^{-1} dry mass in fully expanded leaves [Barker and Pilbeam 2015]. In the shown studies, the Mn content was high, ranging from 62.1 mg Mn kg^{-1} in dry mass (Fire&Spicy NL-R) to 103 mg Mn kg^{-1} dry mass (Hot&Spicy L-NR), see Table 4. In general, supplemented light increased the Mn content in bidens plants, and the growth retarding treatment significantly reduced it. Depending on plant species, the critical molybdenum deficiency varies between 0.1 and 1.0 mg kg^{-1} leaf dry mass. The function of molybdenum in plants is closely related to nitrogen metabolism, and the Mo requirement strongly depends on the manner of N supply [Marschner 2012, Jun et al. 2023]. The studies showed that the range of Mo content in bidens plants was between 1.31 mg Mo kg^{-1} dry mass (Fire&Spicy NL-R) and 6.25 mg Mo kg^{-1} dry mass (Fire&Spicy L-R), see Table 4.

A significant interaction between the applied factors, lighting and growth retarding (L × R), was demonstrated on the dry mass content and macro- and micronutrients in the biomass of bidens (Tables 3 and 4). Plant growth and development are strongly influenced by various light signals, as well as plant hormones, which play crucial roles in regulating these responses to light [Brini et al. 2022]. Our study showed that the lowest dry mass (9.23% d.m.) was observed in plants with non-supplemental lighting and retarding treatments (NL-R), while the highest (10.1% d.m.) was found in plants under supplemental light with retarding (L-R). Plants with a 16-hour photoperiod (L), regardless of the growth retardant treatment, had significantly higher Ca, P, and Zn content in their biomass. Conversely, plants without light supplementation (NL) showed the lowest Mg, P, and Fe content. Phosphorus is vital during the development of reproductive organs [Jun et al. 2023]. A lack of P at this growth stage can delay flower initiation and reduce the number of flowers [Zhang et al. 2023]. The highest phosphorus and zinc content was found in light-supplemented and retarded (L-R) bidens plants. This was related to the high number of flower buds and flowers, especially in the Fire&Spicy (Table 3, Fig. 2).

In leaves, the critical deficiency levels are below 15–20 mg Zn kg^{-1} dry mass. In the shoot meristems, a zinc content of at least 100 mg Zn kg^{-1} dry mass is essential for maintaining protein synthesis [Barker and Pilbeam 2015]. Suppression of stem elongation due to Zn-deficiency also reduces flowering because of poor bud development [Marschner 2012]. In the presented research, the microelement content ranged between 39.9 mg Zn kg^{-1} dry mass (Fire&Spicy NL-NR) and 61.2 mg Zn kg^{-1} dry mass (Hot&Spicy L-NR), see Table 4.

The highest iron content was determined in plants with additional light and no chemical growth control (L-NR). This was particularly visible in the case of the Hot&Spicy, which contained significantly more Fe in the L-NR (256 mg Fe kg^{-1} in d.m.) treatment than in the NL-NR (113 mg Fe kg^{-1} in d.m.), see Table 4. The same was proved for chlorophyll a, carotenoids, and sugars (Table 3).

Mitochondria and chloroplasts have a high requirement for iron, and the chloroplasts may be the site of storage of Fe. Transport into chloroplasts is stimulated by light. In Fe-deficient leaves, the content of chlorophyll and carotene declines. The lower CO_2 fixation

rate per unit of chlorophyll is also possible [Marschner 2012]. There is a close positive correlation between the total Fe content in leaves and the chlorophyll content when the supply of iron is optimal [Jun et al. 2023]. The critical deficiency content of iron in leaves is in the range of 50–150 mg Fe kg^{-1} dry mass [Barker and Pilbeam 2021].

The genetic background for plant nutrition is an area which research interest is still expanding. Different responses of the used cultivars to the light factor (C × L) were noted (Tables 3 and 4). Non-supplemented with light (NL) Hot&Spicy plants had the lowest dry mass (9.13% dry mass), contained the least nitrogen (5.15% N dry mass), phosphorus (0.87% P dry mass), and sulphur (0.30% dry mass), as well as significantly more potassium (5.61% K dry mass), Cu, Fe, Mo, and Zn. On the other hand, Fire&Spicy plants with NL treatment were distinguished by the lowest content of P, S, Cu, Fe, Mo, and Zn. Chemically treated (R) bidens Fire&Spicy resulted in a significant increase in the content of P, S, Cu, Fe, Mo and Zn in plants. On the other hand, retarded plants of the Hot&Spicy showed significantly lower content of Mg, B, Fe, Mn and Mo in biomass (Tables 3 and 4).

CONCLUSION

The results of this study demonstrate that both supplemental lighting and growth retardant application significantly influence the architecture, nutritional status, and physiological quality of *Bidens ferulifolia* plants during greenhouse cultivation. Both tested cultivars, Fire&Spicy and Hot&Spicy, exhibited strong branching, typically producing around 40 shoots per plant in the final stage. Plant architecture, characterised by a high number of short shoots and reduced elongation, was achieved when liners were produced under extended day conditions combined with growth retardant application (L-R treatment). Plants from this treatment not only exhibited the most compact and logistically suitable form but also had the shortest shoots and the fewest long ones. Furthermore, these plants recorded the highest dry mass as well as elevated phosphorus and zinc content, indicating a favourable balance between biomass production and mineral nutrient accumulation. In the absence of growth retardants, plants accumulated more fresh mass, regardless of lighting conditions. However, chemical growth regulation enhanced the concentrations of nitrogen, potassium, phosphorus, sulfur, and copper, while reducing the levels of iron and manganese. These findings suggest that retardants may modify nutrient uptake patterns by altering plant morphology and metabolic activity.

Flower development was strongly influenced by supplemental lighting during the liner stage. Only plants exposed to light during early propagation had already opened flowers by the end of production – typically two per plant – whereas others exhibited mostly buds. Importantly, the number of buds did not significantly differ among treatments, indicating that light accelerates flower opening rather than initiation. Regardless of cultivar, plants grown under extended daylength also had greater dry mass and significantly higher concentrations of macronutrients (N, Ca, Mg, P, S) and all tested micronutrients, confirming the positive effect of light on nutrient acquisition and assimilation. Moreover, light supplementation during the early growth phase led to an increase in chlorophyll a and soluble sugar content in the final plants. This suggests enhanced photosynthetic capacity and carbohydrate accumulation – traits that contribute to improved overall plant vigour and post-transplant performance. The findings support the conclusion that nutrient availability and plant architecture are tightly linked to environmental regulation and growth control strategies. Supplemental lighting, particularly in combination with growth retardants, can optimise nutrient use efficiency, coordinate flowering, and promote compact, high-quality ornamental plants. While both cultivars responded to the treatments, their reactions to growth retardants differed. Fire&Spicy tended to accumulate higher levels of minerals – especially micronutrients – under growth regulation, whereas Hot&Spicy showed the opposite trend. These cultivar-specific differences highlight the importance of tailoring production strategies to individual genetic responses.

In summary, integrating supplemental lighting and chemical growth regulation during the liner phase offers a practical approach to enhance the commercial quality of *Bidens ferulifolia*, influencing not only plant form and flowering behaviour but also internal nutrient composition and physiological condition.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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DUAL ROLE OF HYDROGEN SULFIDE IN MODULATING PHOTOSYNTHESIS, ANTIOXIDANT DEFENSE, AND MEMBRANE INTEGRITY IN *Cucurbita pepo*

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ABSTRACT

Although traditionally regarded as a toxic environmental gas, hydrogen sulfide (H_2S) has recently been recognized as a gasotransmitter involved in regulating various physiological processes in both plants and animals. This study aimed to investigate the stage- and concentration-dependent effects of exogenous H_2S on the growth, photosynthetic capacity, and antioxidant performance of squash (*Cucurbita pepo*) plants. Fifteen-day-old seedlings were subjected to foliar application of H_2S at different concentrations (0, 25, 50, 75, 100, 200, and 300 µM) and monitored until the end of the experiment. Plant samples were collected at two distinct intervals following H_2S treatment – 24 hours and 15 days to comprehensively assess growth, physiological, and biochemical parameters. The results revealed a biphasic response to H_2S treatments. Application of 100 µM H_2S significantly improved growth traits (including shoot and root length, dry biomass, and leaf area), photosynthetic performance, carbonic anhydrase activity, antioxidant enzyme activities, and proline accumulation, while reducing electrolyte leakage and lipid peroxidation compared to untreated controls. In contrast, higher concentrations (200 and 300 µM) adversely affected these parameters and caused increased cellular damage. These findings suggest that 100 µM H_2S is the optimal concentration for enhancing physiological and biochemical traits in squash and may serve as a promising tool for improving crop productivity via improved photosynthetic and stress-response mechanisms.

Keywords: antioxidants, hydrogen sulfide, photosynthesis, proline

INTRODUCTION

Historically, hydrogen sulfide (H_2S) has been regarded as a toxic environmental gas characterized by its pungent odor resembling rotten eggs. It is an inorganic, water-soluble gas that, at high concentrations, poses toxicity risks to living organisms. However, over the past two decades, H_2S has emerged as a crucial gasotransmitter, a signaling molecule analogous to nitric oxide (NO), carbon monoxide (CO), and hydrogen peroxide (H_2O_2) that regulates a wide range of physiological processes in both plants and animals [Vandiver and Snyder 2012, Kimura 2014, Aroca et al. 2018]. In plants, H_2S is biosynthesized via two main

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pathways: (i) the sulfate assimilation route, where sulfate is reduced to sulfide through a series of enzymatic reactions involving ATP sulfurylase, APS reductase, and sulfite reductase, and (ii) the cysteine-dependent pathway, where enzymes such as L-cysteine desulfhydrase (LCD) and D-cysteine desulfhydrase (DCD) catalyze the conversion of cysteine to H_2S [Alvarez et al. 2010, Li et al. 2016, Hancock and Whiteman 2016]. These biosynthetic processes occur in subcellular compartments including chloroplasts, mitochondria, and the cytosol [Corpas et al. 2019].

Recent studies have established the pivotal roles of H_2S in regulating seed germination, root architecture, photosynthesis, and organogenesis, both under optimal and stress conditions [Arif et al. 2021, Zhou et al. 2021]. At low concentrations, H_2S enhances chlorophyll synthesis, boosts the activity of photosynthetic enzymes like ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), and promotes the formation of functional chloroplasts [Xie et al. 2014, Ye et al. 2020]. In contrast, high concentrations of H_2S have been shown to exert inhibitory effects, leading to growth retardation and oxidative stress, thereby confirming its concentration-dependent duality [Khan et al. 2017, Liu et al. 2019, Corpas and Palma 2020]. Additionally, H_2S plays an active role in activating both enzymatic and non-enzymatic antioxidant defense mechanisms, reduces lipid peroxidation, and facilitates osmoprotection by enhancing proline accumulation and carbonic anhydrase activity under abiotic stress [Khan et al. 2017, Zhang et al. 2022]. H_2S is also a key player in the complex signaling network of plants, exhibiting crosstalk with various phytohormones and signaling molecules. It interacts synergistically or antagonistically with abscisic acid (ABA), gibberellic acid (GA), and ethylene to regulate stomatal movement, senescence, and stress adaptation [Liu et al. 2012, Scuffi et al. 2014, Xie et al. 2014]. Furthermore, H_2S is known to modulate NO and H_2O_2 signaling, thus influencing redox balance and cellular homeostasis [Zhang et al. 2010, Lisjak et al. 2011]. Despite these advances, many mechanistic aspects surrounding H_2S signaling, including its precise biochemical regulation and interactions with other pathways, remain underexplored and are active areas of research [Aroca et al. 2018, Corpas et al. 2019].

Given these multifaceted roles, H_2S has gained attention as a promising agent for enhancing plant resilience and productivity. The present study was therefore designed to investigate the stage- and concentration-dependent effects of exogenously applied H_2S on squash (*Cucurbita pepo*) plants. Specifically, the research aims to explore how different H_2S concentrations influence plant growth, photosynthetic performance, and the modulation of enzymatic and non-enzymatic antioxidant defense systems, thereby identifying optimal conditions for potential agricultural application.

MATERIALS AND METHODS

Biological material

Squash (*Cucurbita*) seeds were procured from a local seed market, Al Ain, UAE. Healthy and uniformly sized seeds were washed 2–3 times with deionized water and then sterilized with 0.01% mercuric chloride ($HgCl_2$) aqueous solution followed by repeated washing with deionized water.

Preparation of hydrogen sulfide

Sodium hydrosulfide (NaHS) was used as the donor compound for H_2S. A 1 mM stock solution was prepared by dissolving the appropriate amount of NaHS in a small volume of deionized water and adjusting the final volume to 100 mL with deionized water. Working solutions with final concentrations of 0, 25, 50, 75, 100, 200, and 300 µM H_2S were prepared by serial dilution of the stock solution. Prior to foliar application, a surfactant (Tween-20) was added to the solutions to enhance leaf surface absorption.

Experimental set up and treatment patterns

Thirty-five plastic pots (23 cm in diameter) were filled with commercial potting soil and placed in a plant growth chamber under controlled environmental conditions (temp: 25 °C day /20 °C night; light: 14−16 h photoperiod, 400–600 PPFD; RH: 60–70%; airflow: constant gentle circulation). Five replicate pots were assigned to each of the seven treatments and arranged in a completely randomized block design. Surface-sterilized squash seeds were sown in each pot and allowed to germinate. When germination began, the seedlings were thinned to maintain three plants per pot. At 15 days after sowing, the plants were subjected to a foliar application

of H_2S at concentrations of 0 (deionized water, control), 25, 50, 75, 100, 200, or 300 µM. Each plant was sprayed three times with its respective H_2S solution using a sprayer nozzle adjusted to deliver approximately 1 mL per spray. The plants were grown until 30 days after sowing. Plant samples were collected at two time points: 16 days after sowing (24 hours after H_2S treatment) and 30 days after sowing. These samples were analyzed for various growth, physiological, and biochemical parameters.

Assessment of plant growth biomarkers

The plants from each replicate were gently uprooted and thoroughly washed with tap water to remove adhering soil particles. After washing, roots and shoots were separated using a scalpel, and their respective lengths were measured. The separated roots and shoots were then placed in a hot air oven at 70 °C for dehydration. Upon complete drying, the dry weights of both roots and shoots were recorded.

Leaf area was determined using a gravimetric method. Leaves were randomly selected from each treatment group, and their outlines were traced onto graph paper to calculate the area.

Determination of leaf relative water content (LRWC)

To determine leaf relative water content (RWC) we weighed fresh leaf discs of 2 cm diameter, excluding midrib, and floated discs on deionized water in Petri dishes for 24 hours in the dark, where they remained saturated with water. The adhering water on the discs was blotted out, and the turgor mass was measured. The discs were dehydrated at 60 °C for 72 h, and the LRWC was calculated as:

$$LRWC = (FM – DM) / (TM – DM) \times 100$$

where: FM = fresh mass; DM = dry mass; TM = turgor mass.

Chlorophyll content

Using the method of Arnon (1949), 1 g of fresh leaves were homogenized in a mortar with sufficient amounts of 80% acetone. The chlorophyll extract was placed in a centrifuge tube and diluted with 80% acetone to make 10 mL. The supernatant was collected in a cuvette and absorbance was measured at 645 nm and 663 nm on a spectrophotometer.

Determination of net photosynthetic rate and stomatal conductance

An infrared gas analyzer (IRGA) portable photosynthetic system (LI-COR 6400, LI-COR, Lincoln, NE, USA) was used to measure gas net photosynthetic rate and stomatal conductance on the third fully expanded leaves between 11.00 and 12.00 h. Air temperatures, relative humidity, CO_2 concentration, and PPFD were adjusted to 25 °C, 85%, 600 µmol mol^{-1} and 800 µmol mol^{-2} s^{-1} to measure net photosynthetic rate and stomatal conductance, respectively.

Measurement of carbonic anhydrase (CA) activity

Carbonic anhydrase (CA) activity in fresh leaves was assessed using the method described by Dwivedi and Randhawa [1974]. Fresh leaves from each replicate were cut into small pieces, and 200 mg of these pieces were weighed and transferred to Petri plates containing 10 mL of cysteine hydrochloride solution. The samples were incubated at 4 °C for 20 minutes. After incubation, the leaf pieces were blotted dry and transferred to fresh tubes containing phosphate buffer (pH 6.8). Each tube was then supplemented with 4 mL of sodium bicarbonate solution and 0.2 mL of bromothymol blue. The tubes were shaken and incubated again at 4 °C for 20 min to allow the catalytic action of carbonic anhydrase on $NaHCO_3$, resulting in the liberation of CO_2. The amount of CO_2 released was estimated by titrating the reaction mixture against 0.05 N HCl using methyl red as an indicator. A blank, containing all components of the reaction mixture except the leaf sample, was run in parallel with each set of samples.

Determination of leaf electrolyte leakage and lipid peroxidation

Leaf electrolyte leakage was calculated from the total inorganic ions leaked out from the leaves by the method described by Sullivan and Ross [1979].

According to Hodges et al. [1999], the malondialdehyde equivalents of the leaf were determined by homogenizing the leaves in 80% ethanol and centrifuging them at 3000 g for 10 min at 4 °C.

The pellet obtained after centrifugation was extracted twice with the same solvent, and the resulting supernatants were pooled in a test tube. In the same test tube, butylated hydroxytoluene (0.15%) and thiobarbituric acid (0.65%) were simultaneously added along

with supernatants. The reaction mixture was prepared by combining equal volumes of the supernatant, 20% trichloroacetic acid, 0.01% butylated hydroxytoluene, and 0.65% thiobarbituric acid . The mixture was then heated at 95 °C for 25 min and subsequently cooled to room temperature. Optical density of sample was measured at 440, 532, and 600 nm and using these values, rate of lipid peroxidation was calculated according to the formula given by Hodges et al. [1999].

Analysis of antioxidant enzymes (catalase, peroxidase, and superoxide dismutase)

Fresh leaves (1 g) were homogenized with cold lysis buffer (70 mM phosphate buffer; pH 7.0, 1 mM EDTA, 1 mM PMSF, 0.5% Triton X-100 and 2% PVP) and homogenate was centrifuged at 12000 × g for 20 min at 4 °C. Supernatant was collected and stored at –20 °C for the analysis of catalase, peroxidase, and superoxide dismutase activities.

Catalase activity was determined utilizing the method of Aebi [1984]. Reaction mixture (50 mM phosphate buffer – pH 7.0, 15 mM H_2O_2 and 100 µL enzyme extract) was prepared to measure the loss of H_2O_2 during the start of the reaction with the help of a spectrophotometer at 240 nm for 2 min.

For the analysis of peroxidase activity, method of Sánchez et al. [1995] with slight changes was performed. In this method, reaction mixture with 50 mM phosphate buffer (pH 7.0), 20 mM guaiacol, 1.5 mM H_2O_2 and 100 µL enzyme extract was prepared. The enzyme activity in reaction mixture was measured as absorbance at 436 nm for 1 min at 25 °C.

During the estimation of superoxide dismutase activity, a reaction mixture was prepared using 50 mM phosphate buffer (pH 7.8), 9.9 mM L-methionine, 55 µM nitroblue tetrazolium (NBT), 2 mM EDTA, 0.02% Triton X-100, 40 µL enzyme extract and 1 mM riboflavin (added at last). The absorbance of sample prepared was measured at 560 nm for 2 min at 25 °C.

Determination of proline content

Using the Bates et al. [1973] method, fresh leaf samples were extracted in sulphosalicylic acid, followed by additions of acetic acid and ninhydrin solutions. A 5 mL solution of toluene was subsequently added to the extract, at 100 °C, together with equal volumes of glacial acetic acid and ninhydrin solutions. The reaction was terminated in ice bath and absorbance of chromophore (toluene layer) recorded at 528 nm on a spectrophotometer.

Statistical analysis

Graphs presented in manuscript are the mean of five replicates (n = 5) ±standard error. Data were statistically analyzed with the help of ANOVA and Tukey's HSD test was used to determine significant different between means at $p \leq 0.05$ level using Minitab 17, Statistical software, UK.

RESULTS

Growth performance

As shown in Figure 1, the application of H_2S at various concentrations (25–300 µM) had differential effects on key growth parameters such as shoot and root length, dry mass, and leaf area at two different growth stages i.e., 1- and 10-day post-treatment. No significant changes were observed at 1 day after treatment. However, at 15 days post-treatment (30 days after sowing), 100 µM H_2S significantly enhanced all growth indices compared to the control and other concentrations. In contrast, higher concentrations (200 and 300 µM) exhibited phytotoxic effects and significantly reduced growth parameters relative to untreated plants.

Leaf relative water content (RWC)

H_2S treatments elicited notable shifts in leaf RWC at both 15 and 30 days after sowing (Fig. 2A). While lower concentrations (≤100 µM) either maintained or improved RWC, the 100 µM treatment showed the most pronounced effect, increasing RWC by 21.5% at 30 days post-sowing compared to control plants. Conversely, high concentrations (200 and 300 µM) significantly reduced RWC, with more severe effects at the later growth stage.

Chlorophyll content

The chlorophyll content responded positively to 100 µM H_2S, which induced significant increases of 37.3% and 29% at 16 and 30 days after sowing, respectively (Fig. 2B). Lower concentrations (25 and

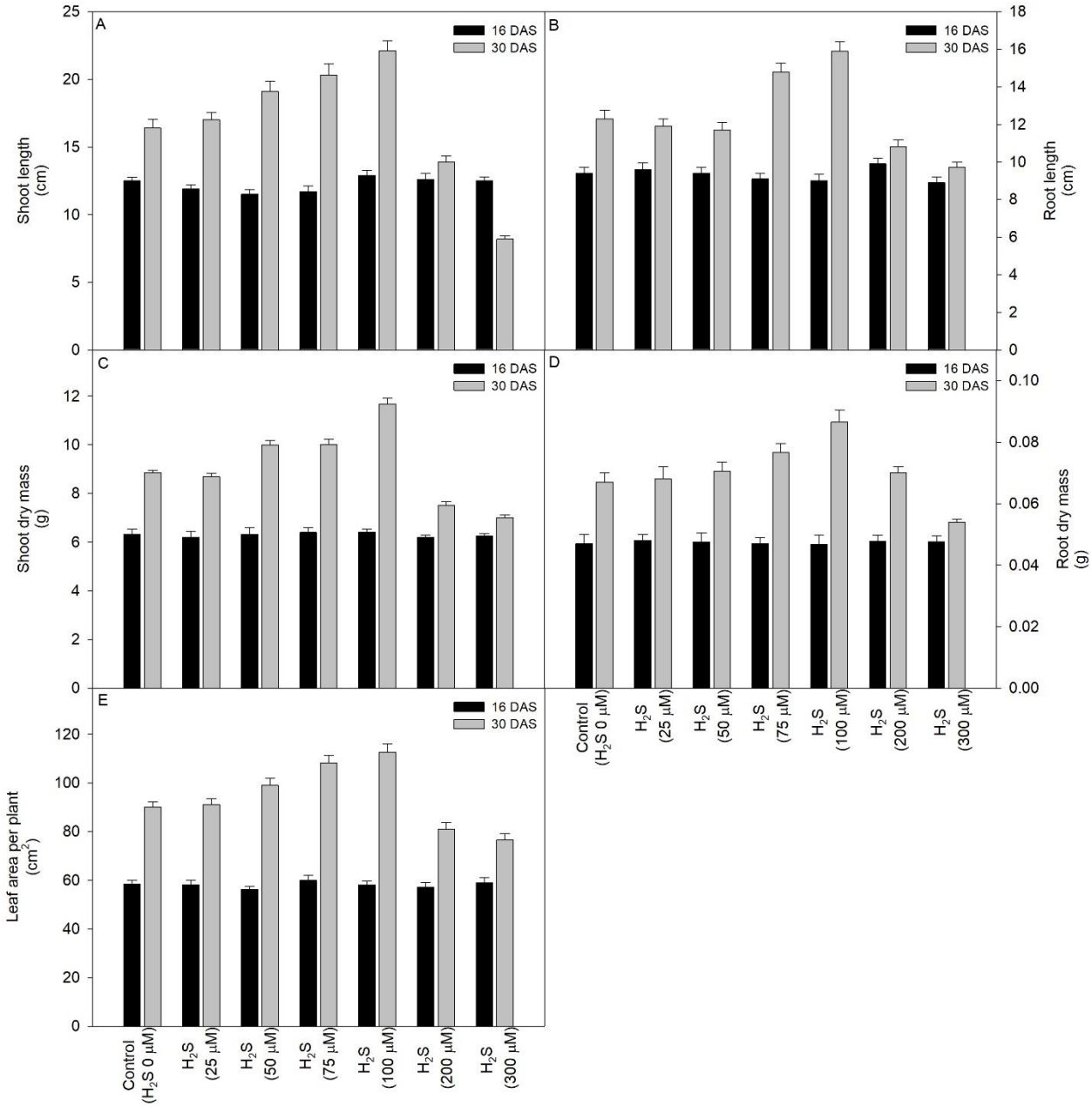

Fig 1. Hydrogen sulfide (H_2S) at concentrations of 0, 25, 50, 75, 100, 200, and 300 µM induced changes in (A) shoot length, (B) root length, (C) shoot dry mass, (D) root dry mass, and (E) leaf area of *Cucurbita* (squash) plants, measured at 16 and 30 days after sowing (DAS). Data represents the mean of five replicates (n = 5), and vertical bars indicate standard errors (±SE)

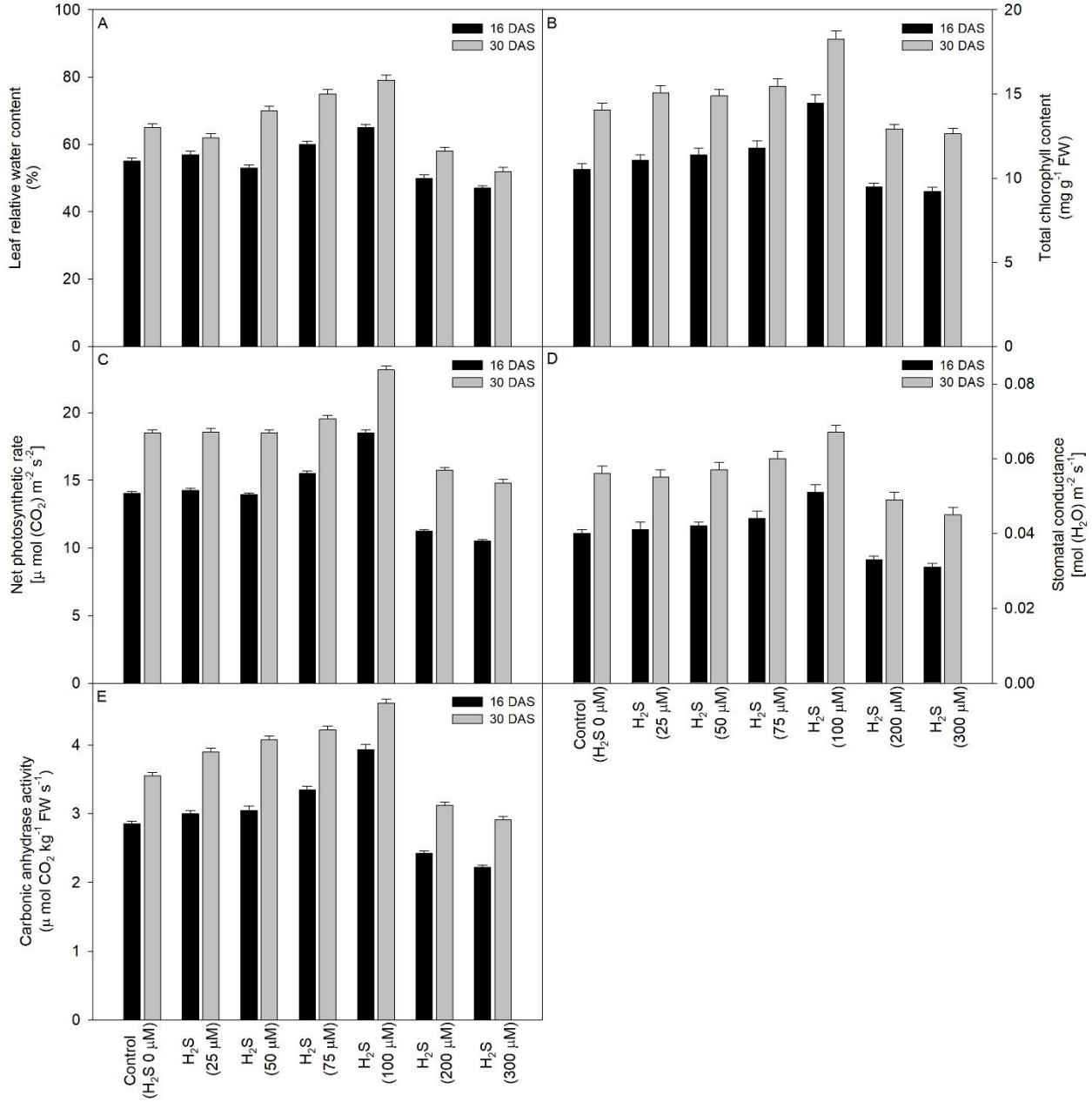

Fig 2. Hydrogen sulfide (H_2S) at concentrations of 0, 25, 50, 75, 100, 200, and 300 µM induced changes in (A) leaf relative water content, (B) total chlorophyll content, (C) net photosynthetic rate, (D) stomatal conductance, and (E) carbonic anhydrase activity of *Cucurbita* (squash) plants, measured at 16 and 30 days after sowing (DAS). Data represents the mean of five replicates (n = 5), and vertical bars indicate standard errors (±SE)

50 μM) had negligible effects, while 200 and 300 μM treatments led to marked chlorophyll degradation, indicating potential oxidative or metabolic stress at higher doses.

Net photosynthetic rate and stomatal conductance

Photosynthetic rate and stomatal conductance (Figs 2C and 2D) increased significantly in response to increasing H_2S concentrations up to 100 μM. Maximum enhancement was observed at 100 μM, beyond which (at 200 and 300 μM), both parameters declined sharply. Notably, the 300 μM treatment led to reductions of 25% and 20% at 16 and 30 days after sowing, respectively, underscoring a dose-dependent biphasic response.

Carbonic anhydrase activity

Carbonic anhydrase activity exhibited a similar biphasic pattern (Fig. 2E). The 100 μM H_2S treatment enhanced enzyme activity by 37.8% and 29.5% at 16 and 30 days after sowing, respectively, relative to control plants. While low concentrations had no notable effect, higher doses (200 and 300 μM) significantly suppressed enzyme activity, with the impact more pronounced at the earlier stage.

Membrane integrity: electrolyte leakage and lipid peroxidation. Membrane stability, assessed via electrolyte leakage and lipid peroxidation, was adversely affected at high H_2S concentrations (200 and 300 μM), indicating oxidative stress (Fig. 3). In contrast, 100 μM H_2S minimized both parameters at both growth stages, suggesting a protective effect at this concentration. Lower concentrations had a negligible influence.

Antioxidant enzyme activities (CAT, POX, and SOD). Treatment with H_2S modulated the activities of key antioxidant enzymes in a concentration-dependent manner. CAT activity increased with concentration up to 100 μM, peaking with enhancements of 62.2% and 50% at 16 and 30 days post-sowing, respectively (Fig. 4A). Higher concentrations reduced CAT activity, indicating enzyme inhibition under stress.

POD activity progressively increased with concentration (Fig. 4B), with maximum elevations of 74.1% and 47.2% at 100 μM at 16 and 30 days after sowing, respectively.

SOD activity revealed that 100 μM H_2S consistently promoted SOD activity at both time points, while other concentrations showed variable trends (Fig. 4C), reflecting differential redox modulation.

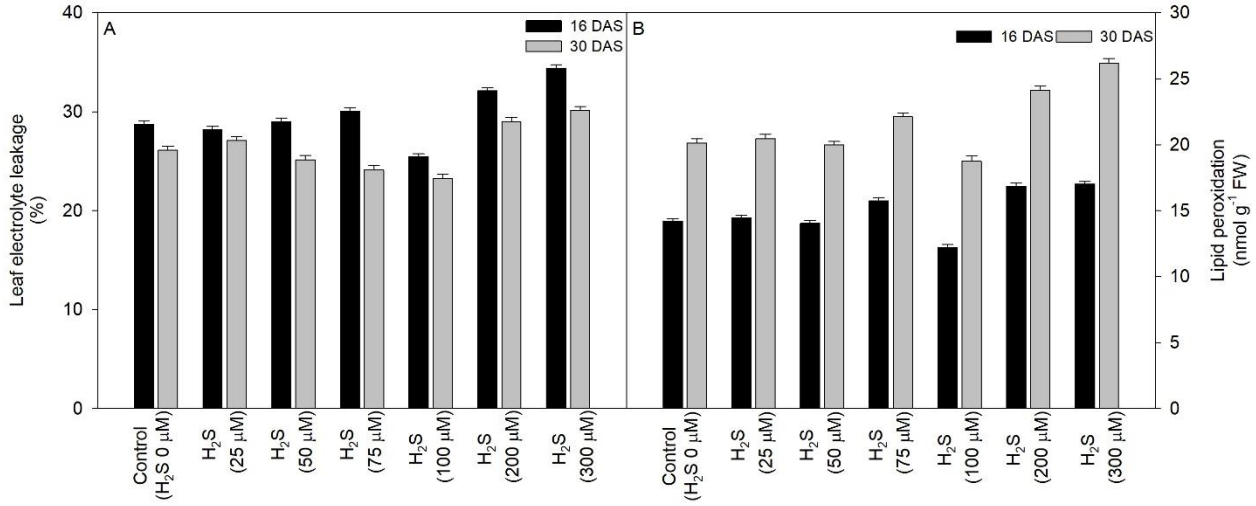

Fig 3. Hydrogen sulfide (H_2S) at concentrations of 0, 25, 50, 75, 100, 200, and 300 μM induced changes in (A) leaf electrolyte leakage, and (B) lipid peroxidation of *Cucurbita* (squash) plants, measured at 16 and 30 days after sowing (DAS). Data represents the mean of five replicates (n = 5), and vertical bars indicate standard errors (±SE)

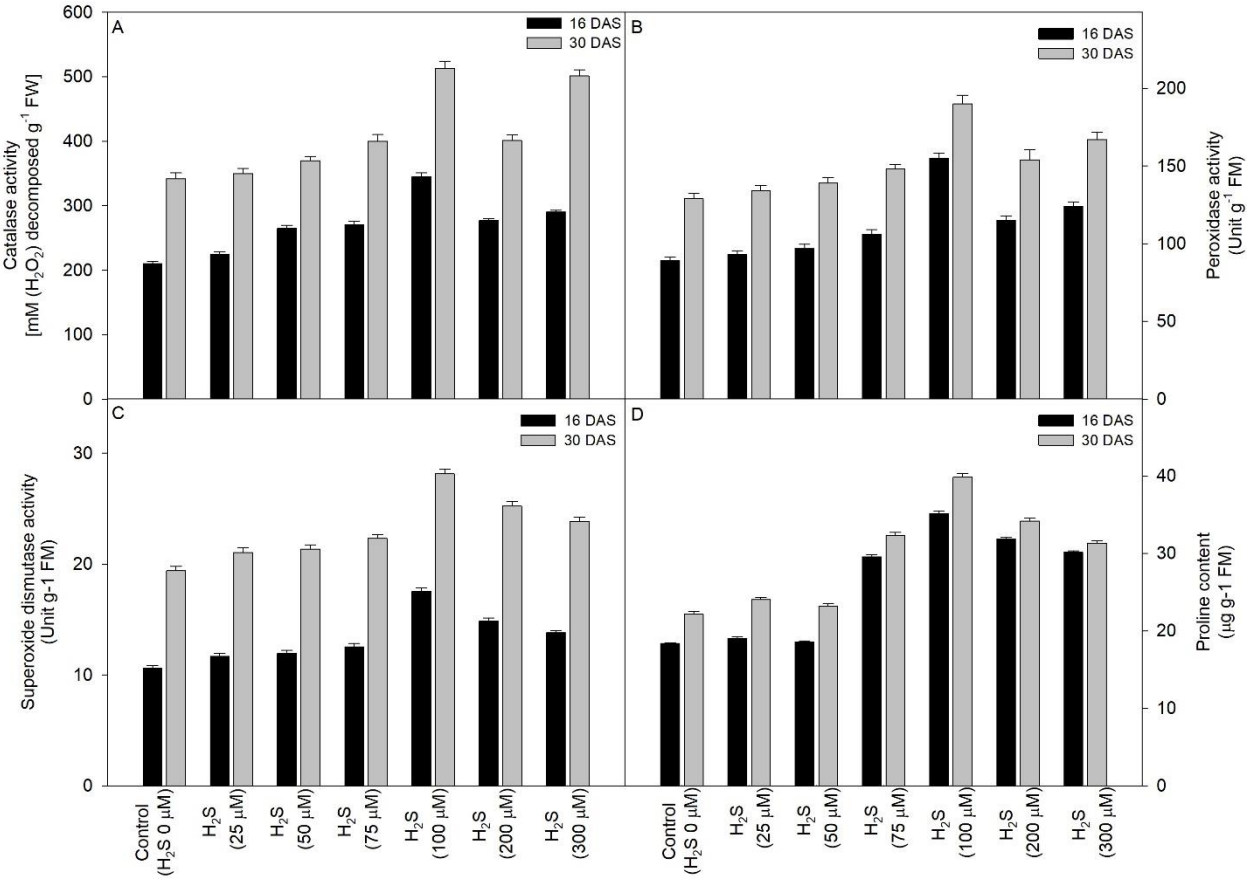

Fig 4. Hydrogen sulfide (H_2S) at concentrations of 0, 25, 50, 75, 100, 200, and 300 µM induced changes in activities of (A) catalase, (B) peroxidase, (C) superoxide dismutase, and (D) proline content of *Cucurbita* (squash) plants, measured at 16 and 30 days after sowing (DAS). Data represents the mean of five replicates (n = 5), and vertical bars indicate standard errors (±SE)

Proline accumulation. Proline content, a marker of osmotic stress response, was significantly elevated by 100 µM H_2S (Fig. 4D), with increases of 64.9% and 44.9% at 16 and 30 days after sowing, respectively, over control. Lower concentrations had minimal effects, while higher doses were less effective, suggesting an optimal concentration window for osmoprotection.

DISCUSSION

The present study demonstrates the concentration-dependent effects of exogenous H_2S on squash (*Cucurbita pepo*) seedlings, revealing its dual role as both a growth promoter and a potential phytotoxin and shows involvement in the production of ROS in plants [Zhang et al. 2017]. The application of 100 µM H_2S emerged as the most effective treatment, significantly enhancing growth attributes such as shoot and root length, dry biomass, and leaf area. These findings are consistent with recent reports indicating that low concentrations of H_2S can stimulate plant growth through improved nutrient assimilation, hormonal regulation, and photosynthetic performance [Alamer 2023, Guo et al. 2023]. Notably, 100 µM H_2S also enhanced chlorophyll content and photosynthetic rate, supporting the role of H_2S in modulating chloroplast function and carbon assimilation under optimal concentrations [Chen et al. 2011].

Improvement in physiological parameters such as RWC and reduced membrane damage as indicated by lower electrolyte leakage and lipid peroxidation under 100 μM H_2S treatment suggests enhanced water retention and membrane stability, likely through modulation of osmotic and oxidative stress pathways. This aligns with previous findings where H_2S treatment improved drought and salinity tolerance by enhancing cell turgor and reducing oxidative damage [Christou et al. 2014, Mostofa et al. 2015]. The significant accumulation of proline in treated plants further supports the hypothesis that H_2S mediates osmoprotection by modulating osmolyte biosynthesis and stress signaling [Hao et al. 2020, Mingjian et al. 2025]. At the enzymatic level, the upregulation of antioxidant enzymes such as catalase, peroxidase, and superoxide dismutase under 100 μM H_2S indicates a robust antioxidative defense mechanism activated by H_2S to scavenge excess ROS. This response reflects the protective role of H_2S in maintaining redox homeostasis under environmental stress conditions, as previously reported in wheat, rice, and *Arabidopsis* models [Wang et al. 2022, Du et al. 2021, Jurado-Flores et al. 2023]. Moreover, recent mechanistic insights suggest that H_2S may regulate these antioxidant pathways through post-translational modifications such as protein persulfidation, thereby altering the activity and stability of redox-related proteins [Aroca et al. 2018, Huang et al. 2021].

However, the adverse effects observed at higher concentrations (200 and 300 μM), including suppressed growth, reduced chlorophyll content, and impaired antioxidant responses, underline the potential toxicity of excessive H_2S. This biphasic effect emphasizes the importance of dosage, as high levels of H_2S can trigger oxidative stress rather than alleviate it, possibly due to overaccumulation of sulfur-containing metabolites or disruption of homeostasis [Li et al. 2013, Huang and Xie 2023]. Such findings are in agreement with earlier reports that highlighted the hormonic behavior of gaseous signaling molecules like H_2S, where a narrow concentration window determines beneficial versus detrimental outcomes [Daneshvand et al. 2024].

CONCLUSION

It is concluded that squash plants exhibit stage-specific and concentration-dependent responses to exogenous H_2S application. Among the tested concentrations (25, 50, 75, 100, 200, and 300 μM), the higher doses (200 and 300 μM) induced deleterious effects at both developmental stages, with more pronounced damage observed at the early growth stage. In contrast, 100 μM H_2S consistently enhanced photosynthetic efficiency and antioxidant activity at both stages, while significant improvements in growth-related traits were particularly evident at 30 days after sowing. Therefore, 100 μM H_2S appears to be the optimal concentration for improving physiological and biochemical performance in squash, offering potential for enhancing crop productivity through improved photosynthetic capacity. However, caution must be exercised in its application, as supra-optimal doses can reverse these benefits. These insights contribute to a growing body of evidence advocating for the strategic use of gasotransmitters like H_2S in sustainable agriculture to bolster crop resilience under abiotic stress conditions.

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CONFLICT OF INTEREST

The author declares that they have no conflict of interest.

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MOLECULAR ANALYSIS OF SOME DISEASES AND REPRODUCTION CHARACTERISTICS IN APPLES FROM CENTRAL ANATOLIA (NIĞDE PROVINCE)

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ABSTRACT

Apple scab and fire blight are among the main diseases in apple production. Researchers are conducting studies to tackle these diseases as well as endeavoring to provide apple producers with disease-resistant plant materials. Self-incompatibility in apples engenders problems in pollination and yield. Molecular studies are crucial for revealing the potential of plant materials in this aspect. In this study, 48 genotypes among Niğde Misket Apple were investigated regarding apple scabs and fire blight resistance as well as self-incompatibility with respective markers and genes. Results showed genotypes had resistance alleles of *Rvi6* and QTL *FB_Mar12,* as well as the presence of S_{26} and S_9 alleles of the *S* gene. These results highlight new hypotheses for further research, particularly regarding disease resistance related to these genes, as well as the relationships among genotypes, cultivars, and species carrying these alleles.

Keywords: *Malus domestica*, plant genetic resources, *Venturia inaequalis*, *Erwinia amylovora*, DNA analysis, *S* alleles

INTRODUCTION

The cultivation of apples (*Malus domestica* Borhk.) spreads around the globe, where a temperate climate prevails. The center of origin of the apple is Central Asia, the Caucasus, and Anatolia (Türkiye) [Brite 2021]. Production of apple reached 95 835 965 tons globally, and Türkiye is one of the most important apple producers, ranked second with 4 817 500 tons [FAO 2024]. Niğde province is an important apple producer in Türkiye, and third in apple production with 581 304 tons [TÜİK 2024].

Disease of Apple scab is one of the most important fungal diseases that requires several fungicide applications. *Venturia inaequalis* is the fungus causing this disease. In most cases, up to 15 fungicide sprays are required to tackle the disease [MacHardy 1996]. Still, when cultivating vulnerable cultivars in some regions with heavy disease pressure and rainfall, 20 to 30 sprays would be necessary to decrease the spread and damage of the fungus [Ayer et al. 2019]. Integrated pest management techniques may help to reduce fungicide inputs for disease control. In addition to that, it is crucial to utilize supplementary disease management techniques to establish fungus-free orchards [Van Den Bosch et al. 2018]. Therefore, lowering the risk of fungicide breakdown and disease pressure is aided by leaf litter reduction [Porsche et al. 2017].

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If scab disease spreads and causes damage despite all efforts, cultivating scab-resistant apple varieties is inevitable as a last resort in apple production.

Most scab-resistant apple cultivars developed to date rely predominantly on a single resistance gene, *Rvi6* (*Vf*). The durability of this resistance is compromised, as virulent isolates (*avrRvi6*) have already been reported across Europe and in the USA [Vinatzer et al. 2004]. Moreover, for many of the scab resistance genes used in apple breeding, corresponding virulent isolates have been identified, with some isolates exhibiting multiple virulences [Peil et al. 2018]. Although current cultivars are still feasible for apple production to some degree in terms of this manner, these observations underscore the necessity of developing new cultivars with more durable and long-lasting scab resistance.

One of the main factor that limits apple output is fire blight, which is brought on by the necrotrophic bacteria *Erwinia amylovora* Burr. [Sobiczewski et al. 2017]. The polyphagous bacteria infest all aboveground organs of multiple host plant species, mostly those in the *Rosaceae* family [Zwet et al. 2012]. Infection frequently results in the rapid death of the infected parts or the whole plant. Environmental factors, plant susceptibility, and the size and appearance of the infection site all affect how severe the illness is. Apple trees are mostly protected from fire blight by combining chemical treatments with cultural applications; however, this does not always ensure complete efficacy. The majority of restrictions are linked to the pathogen's ability to survive on host plants, changes in environmental conditions, and insufficient bactericides [Peil et al. 2009]. Utilizing cultivars that are tolerant or resistant increases the likelihood that the disease will be less detrimental to the orchard's establishment [Sobiczewski et al. 2021].

It takes 13 to 17 years of study to create new apple cultivars using traditional breeding techniques [Sedov 2014]. In order to choose new cultivars and their pollinizers, the procedure starts with the selection of parents who possess desirable features. The gametophytic self-incompatibility (GSI) mechanism in *Malus* limits the range of potential parental pairings [Pereira-Lorenzo et al. 2018]. The incapacity of a fertile plant to generate zygotes following self-pollination or pollination with pollen who share S-alleles is known as its GSI. Located on the 17th chromosome of the apple genome, the *S*-locus is in charge of determining self-incompatibility [Janssens et al. 1995, Sakurai et al. 2000]. To enhance genetic diversity in plant populations, genetically compatible crosses should be performed, including those involving different species when feasible. For this purpose, identifying the *S*-alleles of the genotypes within the population is essential when planning crosses [Karataş et al. 2023].

In the past, pollination and pollen tube growth tests were used to indirectly determine the presence of *S*-alleles. However, this approach is highly sensitive to environmental factors in various vegetative and generative periods in seasons to guarantee the accuracy of this determination [Muñoz-Sanz et al. 2020]. Breeders can design crosses between compatible genotypes by using genetic markers, such as allele-specific primers, to discover *S*-alleles and learn about their distribution among apple genotypes.

Niğde Misket Apple is the local apple landrace that produced since the early years of the Türkiye Republic around the Niğde province in Central Anatolia. According to morphological, pomological, and genetic analysis, Niğde Misket Apple shows a genetic diversity. Genetic analysis indicated similarity rates differentiate 0.61–1.00 [Gencer and Serçe 2022]. According to observations in the region, these local apple genotypes have also not been severely affected by apple scab as well as fire blight diseases compared to known cultivars in the same region, in spite of cultural practices not being carried out in a proper way and time mostly for orchards where Niğde Misket Apple produce.

Molecular studies are an important aspect of conducting modern studies on apples [Cieślińska and Borisova 2019] on many related topics, such as rootstocks [Stachowiak and Świerczyński 2012] and artificial intelligence [Ropelewska and Lewandowski 2024].

One of the biggest QTLs governing resistance to fire blight in apple is QTL *FBF7*. It was located on the Fiesta variety's 7th chromosome [Papp et al. 2015]. Previous research indicated the 210 bp allele of the CH-F7-Fb1 marker is linked to QTL *FBF7* fire blight resistance, and QTL *FBF7* is present in various apple cultivars such as Gala (174 bp allele that is not related to resistance) and Fuji (210 bp allele that resistant resistant-related) [Lyzhin and Saveleva 2021].

QTL *Fb_MR5* identified in *Malus* × *robusta* 5 on LG 3 [Peil et al. 2007]. The Ch03g07 molecular mark-

er was previously utilized to detect the presence of QTL *Fb_MR5* in a study, a 145 bp allele affiliated with resistance to fire blight of *QTL Fb_MR5* [Fahrentrapp et al. 2013].

QTL *FB_Mar12*, that a major effect on fire blight resistance, colocalized on the distal end of LG12 (linkage group 12) in *M. floribunda*, *M. Evereste,* and *M.* × *arnoldiana*, and findings of studies indicated QTL *FB_Mar12* potentially present genes with domains definitive to resistance of the disease [Durel et al. 2009, Emeriewen et al. 2017, 2021]. Although there is evidence of hybridization between these two species, it is still unknown if the resistance of fire blight QTL is independent or shared by both species [Emeriewen et al. 2021, Tegtmeier et al. 2023]. Previous studies detected QTL *FB_Mar12* with ChFbE01 in various species. ChFbE01 is affiliated only 266 bp size allele [Parravicini et al. 2011] for resistance.

QTL *Fb_Mfu10* is an important QTL for resistance to fire blight, which was identified on LG10 of *Malus fusca*, accession MAL0045 [Emeriewen et al. 2014, 2018, 2020, 2022, Mansfeld et al. 2023]. FRM4 molecular marker utilized to detect a resistance-related allele (156 bp) on this QTL [Emeriewen et al. 2014, 2018].

Rvi6 was the most prevalent *R* gene when considering all other scab-resistant cultivars from the gene bank and the first scab-resistance gene discovered from a wild cousin of apples (*M. floribunda* Siebold ex Van Houtte). This gene, which is found on LG1, is still widely researched and described as a gene for resistance to scab in apples [Vinatzer et al. 2004]. The resistance allele of the Ch-Vf1 marker showed a linkage with the Vf2ARD 527-bp fragment [Boudichevskaia et al. 2009] that is a resistant candidate gene.

The gene of *Rvi11* was discovered in *M. baccata* and was mapped to LG2 [Dayton and Williams 1968, Gessler et al. 2006]. CH02c06 marker was utilized previously, amplified various alleles (230, 236, 240, 248 bp), and the 248 bp allele were indicated as resistance-related [Gianfranceschi et al. 1998, Gygax et al. 2004]. A resistance allele of the gene was previously reported in the Modi cultivar [Madenova et al. 2024].

Rvi4 (*Rvi15*) was mapped on the top of LG 2 using Idared × GMAL 2473 population [Patocchi et al. 2004]. A recent study found that *Rvi4* and *Rvi15* are identical genes, and they suggested the use *Rvi4* name instead of *Rvi15* for future studies [Peil et al. 2023]. CH02f06 was utilized in studies to detect alleles of *Rvi4* (*Rvi15*) related to scab resistance at 146 bp [Galli et al. 2010, Patocchi et al. 2004], 152 bp [Patocchi et al. 2009], and 155 bp [Peil et al. 2023] on GMAL 2473. So, it's a compound repeat-type marker [Gianfranceschi et al. 1998]. Studies conducted on *Rvi4* (*Rvi15*) indicated that some known apple cultivars such as Gala, Granny Smith, Fuji, SuperChief, Modi, Pink Lady, Granny Smith, Honeycrisp and Jeromine were the carriers of *Rvi4* (*Rvi15*) resistance [Khankishiyeva 2020, Madenova et al. 2024].

The *Rvi5* gene was indicated to play a role in resistance in *Malus micromalus* Mak. and *Malus atrosanguinea* 804 [Dayton and Williams 1970]. A study on *Rvi5* developed the Hi07h02 (SSR marker), which is tightly linked with *Rvi5* on LG-17 [Patocchi et al. 2005]. A 228 kb area that most likely contains the *Rvi5* gene was recently discovered using the genome of the apple as a basis [Bandara et al. 2013]. Previous studies showed some apple cultivars indicated as carriers of the *Rvi5* gene resistance, such as Jeromine and SuperChief [Madenova et al. 2024]. Hi07h02 marker previously utilized and amplified 224 bp [Cova et al. 2015] and 230 bp [Patocchi et al., 2009] alleles coupling with the resistance in the *Rvi5* gene.

The *Rvi12* locus of scab resistance was identified from *M. baccata* Hansen's baccata #2 (HB2) [Dayton and Williams 1968]. *Rvi12* was mapped to LG 12 of the apple genome [Erdin et al. 2006]. Then a study on a fine map of the *Rvi12* locus of scab resistance indicated the scab resistance gene *Rvi12* from HB2 was reported on LG12 in the cross Gala × HB2, as mapping to the apple [Padmarasu et al. 2014].

Apple has a multi-allelic gametophytic incompatibility system controlled by a single *S* gene. Many alleles of the *S* gene are identified with different molecular markers [Janssens et al. 1995, Sakurai et al. 2000]. The MalusS_{26} marker can detect the S_{26} allele of the *S* gene on a 193 bp size [Sakurai et al. 2000]. This allele is a rare presence in known apple cultivars. Some examples of known S_{26} allele carriers are crabapple cultivar Baskatong and *Malus floribunda* 821, which has an apple scab resistance [Broothaerts et al. 2004], as well as local apple genotypes in Türkiye [Karataş et al. 2023], and Marubakaido apple rootstock (*Malus prunifolia* Borkh) [Brancher et al. 2020].

The MalusS$_9$ marker can detect the S_9 allele of the *S* gene on a 343 bp size [Janssens et al. 1995]. This allele is present among known apple cultivars like Starking Delicious, Fuji, and Jonagold [Broothaerts et al. 2004].

This study aimed to investigate apple scab and fire blight resistance/tolerance as well as the self-incompatibility status of Niğde Misket Apple genotypes with molecular markers. Central Anatolia, Türkiye, is already one of the major locations for apple production and the center of origin of apples in the world. Niğde Misket Apple has the potential for a future breeding program. This potential will be evaluated in terms of apple scab and fire blight resistance/tolerance and self-incompatibility, and shed light on future studies.

MATERIAL AND METHOD

Plant material. In this study, 48 different apple genotypes were used. These genotypes were collected from different areas in Niğde (Fig. 1, Table 1) [Gencer and Serçe 2022] and preserved in Niğde Ömer Halisdemir University Faculty of Agricultural Science and Technologies Faculty, as a collection orchard. All of them were grafted on MM106 apple rootstock with bud graft in 2019 and planted in the orchard in 2020. Leaves are collected from the collection orchard.

Fuji, Amasya, G41, Modi, Gala, SuperChief, and Golden Delicious cultivars were used as control groups for molecular analysis.

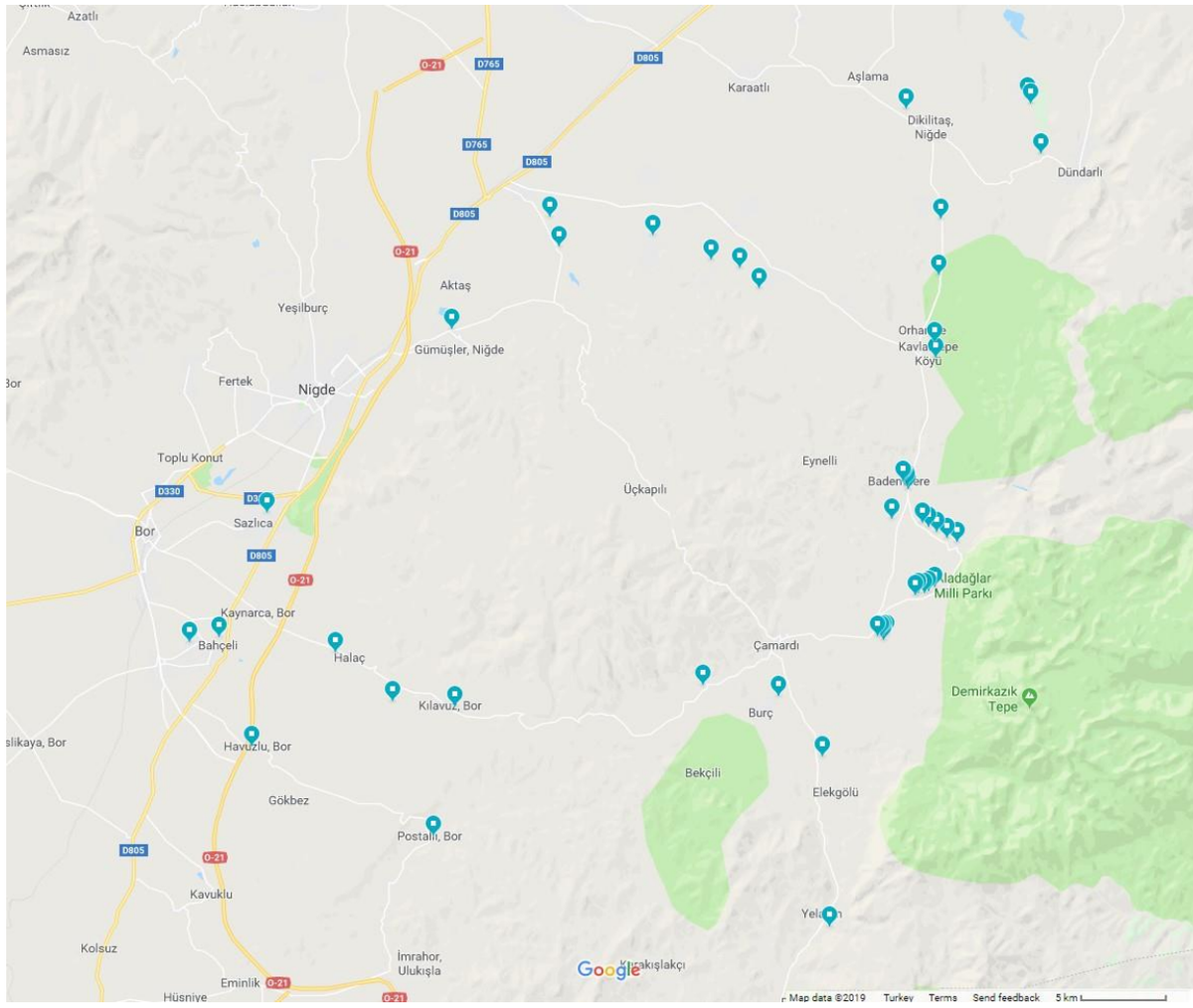

Fig. 1. Map of the collected genotypes. Blue markers indicated the GPS data [Gencer and Serçe 2022]

Table 1. Information related to the plant material [Gencer and Serçe 2022]

Tree codes	Name of locations	GPS data	Elevation (meter)
KMR	Kemerhisar	37°49'56.9"N 34°35'29.3"E	1125
BHC	Bahçeli	37°50'06.7"N 34°36'39.5"E	1147
SZL	Sazlıca	37°54'04.3"N 34°38'34.8"E	1211
HLC	Halaç	37°49'39.0"N 34°41'19.3"E	1297
KRC	Karacaören	37°48'04.1"N 34°43'36.9"E	1487
KLV	Kılavuz	37°47'53.8"N 34°46'06.7"E	1571
HVZ	Havuzlu	37°46'38.0"N 34°37'59.1"E	1213
PST	Postallı	37°43'46.9"N 34°45'17.0"E	1394
DGR	Değirmenli	38°02'54.4"N 34°54'06.4"E	1494
DND	Dündarlı	38°05'28.7"N 35°09'54.4"E	1326
CKR1	Çukurbağ	37°50'09.6"N 35°03'25.8"E	1484
CKR2	Çukurbağ	37°50'08.7"N 35°03'33.2"E	1493
CKR3	Çukurbağ	37°49'60.0"N 35°03'27.7"E	1499
CKR4	Çukurbağ	37°50'07.1"N 35°03'21.4"E	1480
CKR5	Çukurbağ	37°50'07.2"N 35°03'10.9"E	1455
BDM1	Bademdere	37°55'04.7"N 35°04'14.8"E	1601
BDM2	Bademdere	37°55'01.5"N 35°04'18.1"E	1595
BDM3	Bademdere	37°54'58.9"N 35°04'24.5"E	1586
BDM4	Bademdere	37°54'53.9"N 35°04'24.2"E	1582
BDM5	Bademdere	37°54'47.8"N 35°04'26.2"E	1576
PNR1	Pınarbaşı	37°53'43.7"N 35°05'00.8"E	1574
PNR2	Pınarbaşı	37°53'36.7"N 35°05'15.9"E	1569
PNR3	Pınarbaşı	37°53'26.4"N 35°05'35.5"E	1572
PNR4	Pınarbaşı	37°53'15.0"N 35°06'02.0"E	1562
DMR1	Demirkazık	37°51'41.0"N 35°05'31.5"E	1577
PNR5	Pınarbaşı	37°53'06.4"N 35°06'24.2"E	1598
DMR2	Demirkazık	37°51'32.2"N 35°05'16.6"E	1558
DMR3	Demirkazık	37°51'28.7"N 35°05'04.8"E	1545
DMR4	Demirkazık	37°51'28.4"N 35°04'50.9"E	1556
DMR5	Demirkazık	37°51'25.4"N 35°04'43.4"E	1560
CLL	Celaller	37°48'34.6"N 34°56'09.5"E	1687
BRC	Burç	37°48'12.9"N 34°59'11.4"E	1445
ELG	Elekgölü	37°46'18.5"N 35°00'59.3"E	1365
KVL1	Kavlaktepe	37°59'29.8"N 35°05'34.0"E	1671
KVL2	Kavlaktepe	37°59'00.8"N 35°05'34.9"E	1726
HCB1	Hacıbeyli	38°07'17.7"N 35°09'19.9"E	1280
HCB2	Hacıbeyli	38°07'05.3"N 35°09'28.9"E	1283
DKL	Dikilitaş	38°06'56.9"N 35°04'25.3"E	1435
YSL	Yeşilova	38°03'31.3"N 34°49'58.3"E	1388
ULG	Uluağaç	38°02'34.6"N 34°50'20.2"E	1435
GMS	Gümüşler	37°59'56.2"N 34°45'59.7"E	1344
HMM	Himmetli	38°02'08.8"N 34°56'32.7"E	1552
ELM1	Elmalı	38°01'52.1"N 34°57'41.6"E	1603
ELM2	Elmalı	38°01'12.8"N 34°58'29.0"E	1605
KCP	Kocapınar	38°01'37.2"N 35°05'43.2"E	1571
EYN	Eynelli	37°53'51.3"N 35°03'46.9"E	1531
ICM	İçmeli	38°03'24.2"N 35°05'49.6"E	1519
YLT	Yelatan	37°40'51.6"N 35°01'14.0"E	1320

Table 2. Information about genes / QTLs and markers

No	Markers	Sequence 5' to 3'	Gene/QTL	Chromo-some	Disease/Trait	Positive control	Negative control	Ta °C	Expected allele size*	References
1	CH-F7-Fb1_F	AGCCAGATCACATGTTTTCATC	QTL *FbF7*	7	fire blight	G41	Gala	57	174–**210** bp	[Lyzhin and Saveleva 2021, Papp et al. 2015]
	CH-F7-Fb1_R	ACAACGGCCACCAGTTTATC								
2	CH-Vf1_F	ATCACCACCAGCAGCAAAG	*Rvi6*	1	apple scab	Modi	Gala	60	129–180 bp 139, 166, **159** bp	[Boudichevskaia et al. 2009, Höfer et al. 2021, Madenova et al. 2024, Vinatzer et al. 2004]
	CH-Vf1_R	CATACAAATCAAAGCACAACCC								
3	CH02c06_F	TGACGAAATCCACTACTAATGCA	*Rvi11*	2	apple scab	Modi	Fuji	60	216–254 bp **248** bp	[Dayton and Williams 1968, Gessler et al. 2006, Gianfranceschi et al. 1998, Madenova et al. 2024]
	CH02c06_R	GATTGCGCGCTTTTTAACAT								
4	CH02f06_F	CCCTCTTCAGACCTGCATATG	*Rvi4* (*Rvi15*)	2	apple scab	Modi	Golden Delicious	60	**146**–158 bp **152**, **155**	[Galli et al. 2010, Gianfranceschi et al. 1998, Khankishiyeva 2020, Madenova et al. 2024, Patocchi et al. 2004, 2009, Peil et al. 2023]
	CH02f06_R	ACTGTTTCCAAGCGCTCAGG								
5	Ch03g07_F	AATAAGCATTCAAAGCAATCCG	QTL *Fb_MR5*	3	fire blight	G41	Fuji	60	119–171 bp **145 bp**	[Fahrentrapp et al. 2013, Peil et al. 2007]
	Ch03g07_R	TTTTTCCAAATCGAGTTTCGTT								
6	ChFbE01_F	TTCAAGTCCCTGCATTTCAC	QTL *FB_Mar12*	12	fire blight	G41	Golden Delicious	60	**266** bp	[Durel et al. 2009, Emeriewen et al. 2017, 2021, Parravicini et al. 2011, Tegtmeier et al. 2023]
	ChFbE01_R	CAAGCTCATTGACCAGTTCG								
7	FRM4_F	GGGTTTGGTGGAGTGTCAAT	QTL *Fb_Mfu10*	10	fire blight	G41 (not have resistance allele)	Fuji	60	**156**–166 bp	[Emeriewen et al. 2014, 2018, 2020, 2022, Mansfeld et al. 2023]
	FRM4_R	AAAGGCAGATCTGGTGATGC								
8	Hi07h02_F	ATTTGGGGTTTCAACAATGG	*Rvi5*	17	apple scab	SuperChief	Fuji	60	220–280 bp **224**, **230** bp	[Bandara et al. 2013, Cova et al. 2015, Dayton and Williams 1970, Madenova et al. 2024, Patocchi et al. 2005, 2009]
	Hi07h02_R	GTTTCGGACATCAAACAAATGTGC								
9	MalusS26_F	GAAGATGCCATACGCAATGG	*S*	17	self-incom-patibility	Amasya	Golden Delicious	55	193 bp	[Brancher et al. 2020, Broothaerts et al. 2004, Karataş et al. 2023, Sakurai et al. 2000]
	MalusS26_R	ATGAATTCTTAATACCGAATATTGGCC								
10	MalusS9_F	CAGCCGGCTGTCTGCCACTT	*S*	17	self-incom-patibility	Fuji	Golden Delicious	62	343 bp	[Brancher et al. 2020, Broothaerts et al. 2004, Janssens et al. 1995, Karataş et al. 2023]
	MalusS9_R	CGGTTCGATCGAGTACGTTG								
11	SSR-MDC005174.220_F	GTAGTAATCCACCCCCATGC	*Rvi12*	12	apple scab	Gala (not have resistance allele)	Fuji	60	209–223 bp **216** bp	[Dayton and Williams 1968, Erdin et al. 2006, Padmarasu et al. 2014]
	SSR-MDC005174.220_R	TGTATGACTCGTCGCTCACG								

* Bolded allele sizes are related to disease resistance according to references.

Investigation of fire blight resistance. For the investigation of fire blight resistance, the CH-F7-Fb1 marker targeted QTL *FbF7*, the Ch03g07 marker targeted to QTL *Fb_MR5*, the ChFbE01 marker targeted QTL *FB_Mar12*, and the FRM4 marker targeted to QTL *Fb_Mfu10* utilized in this study. These markers were selected because they have been validated in previous studies, and their expected allele sizes are suitable for detection using agarose gel. Details for markers (sequences, controls, Ta °C, expected allele sizes, and references) are given in Table 2.

Investigation of apple scab resistance. For the investigation of apple scab resistance, the CH-Vf1 marker targeted the *Rvi6* gene, the CH02c06 marker targeted the *Rvi11* gene, the CH02f06 marker targeted the *Rvi4* (*Rvi15*) gene, the Hi07h02 marker targeted the *Rvi5* gene, and the SSR-MDC005174.220 marker targeted the *Rvi12* gene utilized in this study. These markers were selected because they have been validated in previous studies, and their expected allele sizes are suitable for detection using agarose gel. Details for markers (sequences, controls, Ta °C, expected allele sizes, and references) are given in Table 2.

Investigation of self-incompatibility. For the investigation of self-incompatibility, MalusS_{26} and MalusS_9 markers targeted the *S* gene utilized in this study. MalusS_{26} and MalusS_9 markers were utilized due to the amplified alleles (S_{26} and S_9 alleles), with these markers being common among apples local to Anatolia [Karataş et al. 2023]. Details for markers (sequences, controls, Ta °C, expected allele sizes, and references) are given in Table 2.

DNA extraction. Extraction processes of DNA from healthy and young leaves were performed by the CTAB method [Dellaborta et al. 1983]. DNA concentrations were then analyzed by the Quawell Q5000 UV-Vis Spectrophotometer then diluted to 5 ng/µL.

Polymerase Chain Reaction steps. PCR was performed in 25 µL total volume with 5 ng DNA, 2.5 µL 10x buffer, 0.2 mM dNTPs, 0.5 µM of each primer, 2.0 mM $MgCl_2$, 1 U Taq DNA polymerase, and dH_2O. Thermal cycler protocol was performed following initial denaturation at 94 °C for 2 min, 35 cycles (1 min 94 °C, 1 min annealing temperature of primer, 1 min 30 s 72 °C), and final extension at 72 °C for 1 min.

Agarose gel conditions and evaluation. Agarose gel (2.5%) electrophoresis (110 volts, 2 hours 30 min) was used to separate the DNA fragments of different sizes in the PCR products. TriTrack DNA Loading Dye (6x) and The GeneRuler 50 bp DNA ladder (Thermo Fisher Scientific) were used to identify the sizes of DNA samples on gels. Gels are imaged utilizing an agarose gel imaging system (DNR MiniLumi Bio Imaging System). Positive controls always load on the gel as the first sample after the ladder. Negative controls always load on the gel last after all samples are loaded. A binary number file was created from the scored gel images (0 or 1 depending on the presence of bands on the gel).

Fragment analysis. Fragment analysis was conducted with primers (CH-Vf1 and ChFbE01) that showed resistance-related allele presence according to agarose gel results. PCRs were performed again with these primers and M13 primer (5′-CACGACGTTGTAAAACGAC-3′) to forward primers. Genotypes ICM and KMR are utilized in fragment analysis as plant materials because these two genotypes have clear and the same bands as the other genotypes that have resistance-related allele presence. 6-FAM and HEX fluorescent dyes are utilized to label M13 primers. Products are loaded to the Applied Biosystems (ABI) 3500 Series Genetic Analyzer with GeneScan™ 500 LIZ™ dye as a size standard, and results are evaluated in GeneScan® Analysis Software.

RESULTS

With fire blight-related markers. The results of the CH-F7-Fb1 marker, targeted to QTL FBF7, indicated no disease-related allele among the genotypes.

The results of the Ch03g07 marker, targeted to QTL Fb_MR5, indicated no disease-related allele among the genotypes.

The result of the ChFbE01 marker (Figs 4 and 5) targeted to QTL *FB_Mar12* indicated that 47 of the 48 genotypes (except BDM4) have a disease resistance-related allele (266 bp).

The result of the FRM 4 marker targeted to QTL *Fb_Mfu10* indicated no disease-related allele among genotypes.

With apple scab-related markers. The results of the CH-Vf1 marker targeted to the *Rvi6* gene (Figs 2 and 3) indicated that all genotypes have a disease resistance-related allele (159 bp). Except for genotype

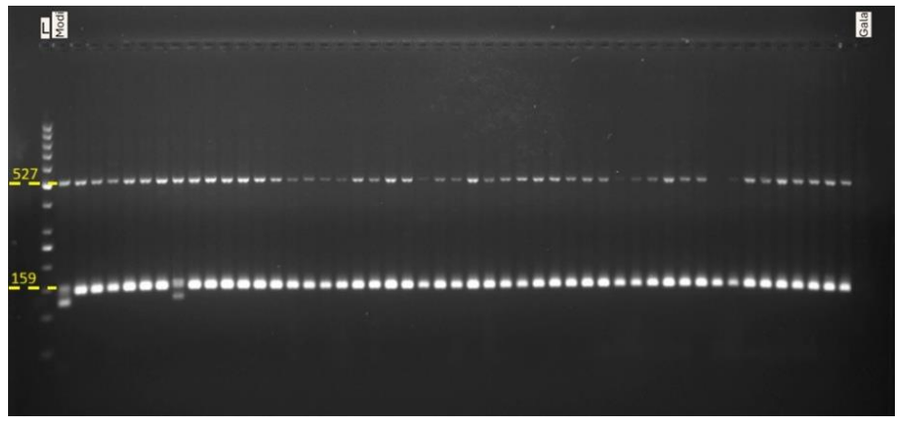

Fig. 2. Gel image of CH-Vf1 marker, yellow lines, and labels represent bp size of interest

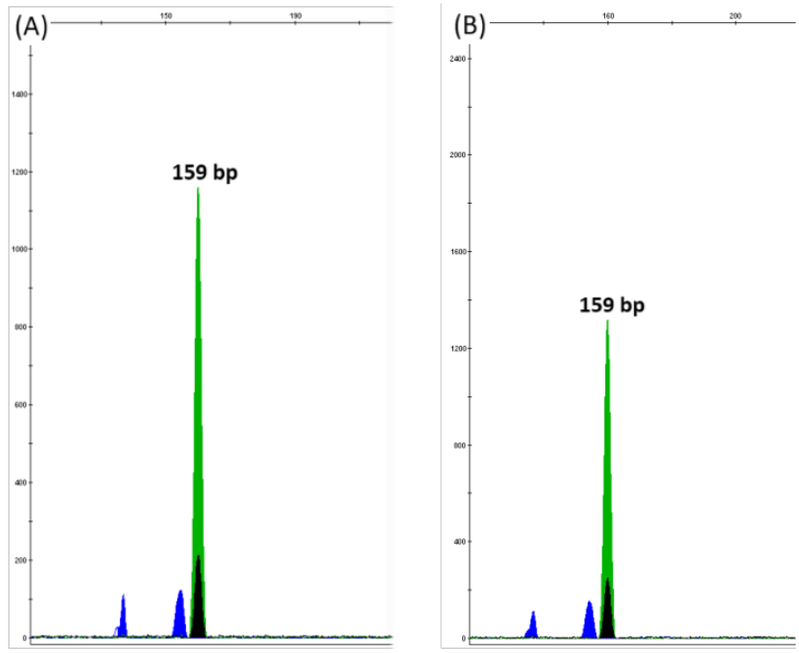

Fig. 3. Results of fragment analysis of CH-Vf1 marker (*Rvi6*). A) genotype ICM, B) genotype KMR

DMR4, other genotypes also have a 527-bp allele, which shows linkage to the 159 bp allele.

The result of the CH02c06 marker targeted to the *Rvi11* gene indicated no disease-related allele among genotypes.

The results of the CH02f06 marker targeted to the *Rvi4* (*Rvi15*) gene indicated no disease-related allele among the genotypes.

The results of the Hi07h02 marker targeted to the *Rvi5* gene indicated no disease-related allele among the genotypes.

The result of the SSR-MDC005174.220 marker targeted to the *Rvi12* indicated no disease-related allele among genotypes. The PIC value of the SSR--MDC005174.220 marker is 0.369.

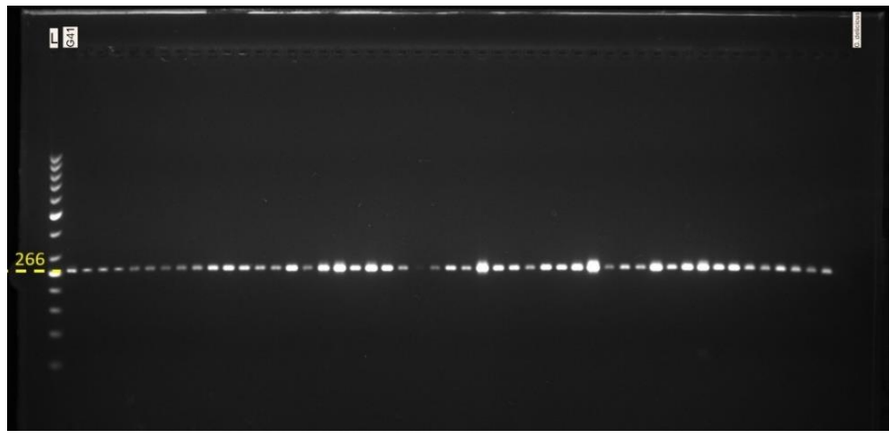

Fig. 4. Gel image of ChFbE01 marker, yellow line, and label represent bp size of interest

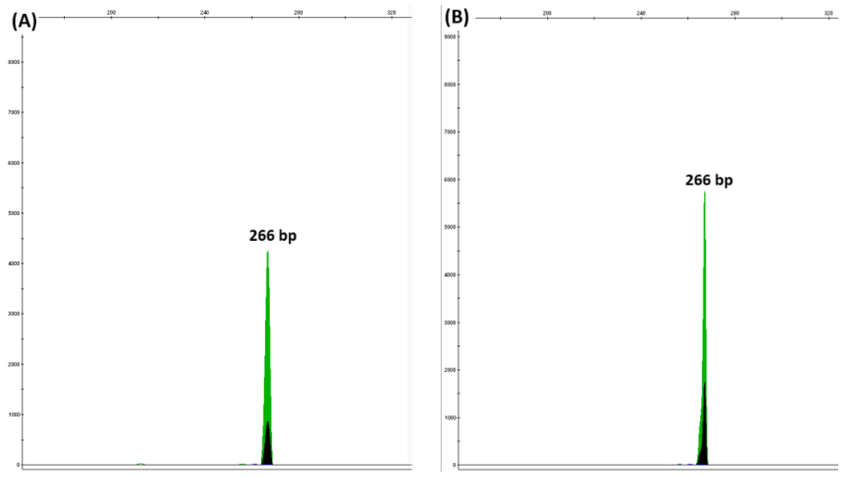

Fig. 5. Results of fragment analysis ChFbE01 marker (QTL *Fb_Mar12*). A) genotype ICM, B) genotype KMR

With self-incompatibility-related markers. The result of the MalusS_{26} marker targeted to the *S* gene (Fig. 6) indicated that 47 of the 48 genotypes (except the genotype PNR2) have S_{26} allele (193 bp) of the *S* gene.

The result of the MalusS_9 marker targeted to the *S* gene (Fig. 7) indicated that all genotypes have S_9 allele (343 bp) of the *S* gene.

DISCUSSION

According to the results, no disease resistance-related alleles presence detected with the utilized molecular markers (Table 2) for QTL *FBF7*, *Rvi11*, *Rvi4* (*Rvi15*), QTL *Fb_MR5*, QTL *Fb_Mfu10,* and *Rvi5* among the genotypes. Although other alleles were detected with some of these markers, the genetic variations already presented in the previous study with IPBS markers [Gencer and Serçe 2022]. Due to that fact, no further analysis was conducted for that purpose.

Results indicated resistance allele presence among the Niğde Misket Apple genotypes for *Rvi6* and QTL *FB_Mar12* with their respective molecular markers (Table 2).

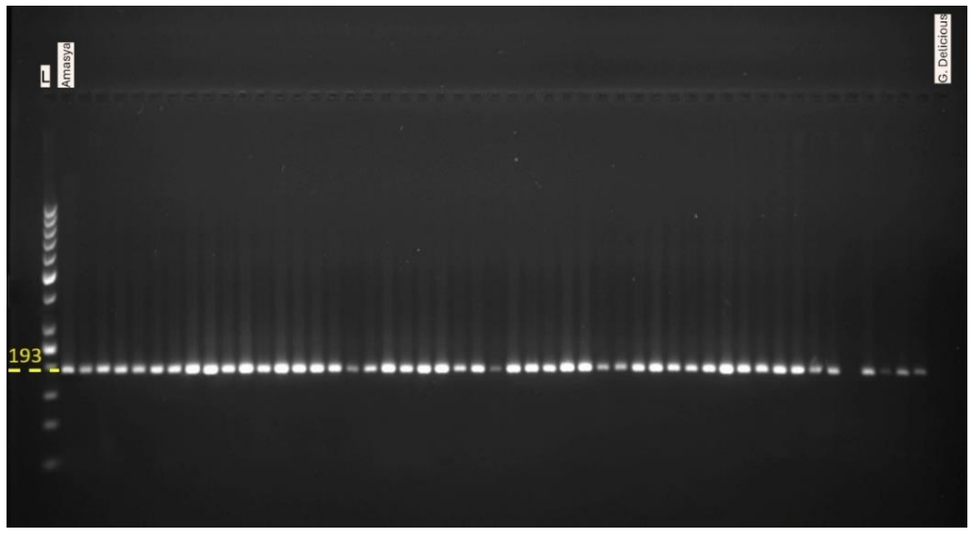

Fig. 6. Gel image of MalusS26 marker, yellow line, and label represent bp size of interest

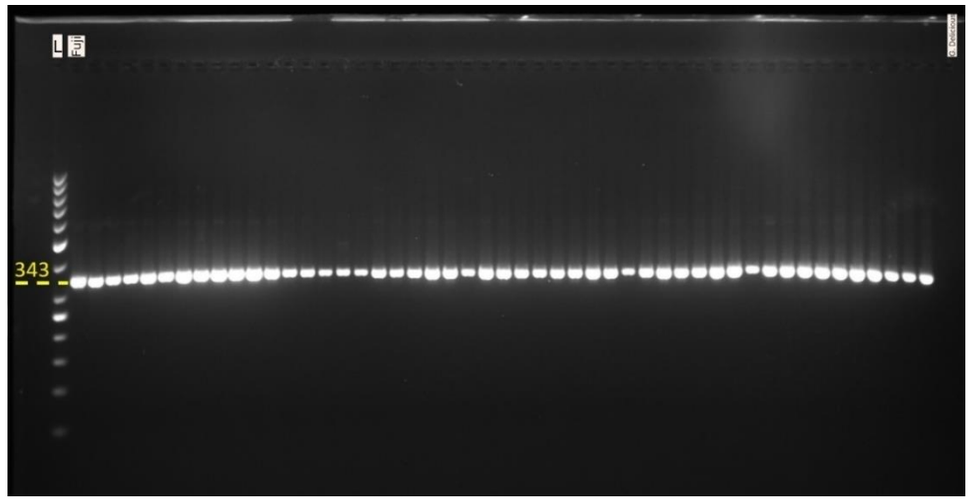

Fig. 7. Gel image of MalusS_9 marker, yellow line, and label represent bp size of interest

These results may fitted in due to *Rvi6* being the most abundant gene among *R* genes for scab resistance [Vinatzer et al. 2004] but given the limited scope of this study, it's not an exact proof. Also, the presence of the *Rvi6* has already been reported in different cultivars [Höfer et al. 2021, Madenova et al. 2024]. Therefore, this result unearths a new question for further studies to test the relations among Niğde Misket Apple genotypes and the parental lineage of these cultivars. Although *Rvi6* is one of the most common *R* genes, studies indicated that the same origin of *V. inaequalis* populations has an infection on the *Rvi6* resistance allele carried and *M. floribunda* originated cultivars in Europe [Lemaire et al. 2016] and *Rvi6* resistance is suppressed and virulent progeny scatter, the plantation of *Rvi6* resistance allele-carrying cultivars is still a substantial factor for apple production to meet with sustainability requirements against apple scab [Peil et

al. 2018]. In terms of this aspect, Niğde Misket Apple may have an important advantage, and further studies related to the scab resistance can establish their basis for those results.

The results of QTL *FB_Mar12* can be an indication of the relationship between *M. floribunda*, *M. Evereste*, and *M.* × *arnoldiana* species to Niğde Misket Apple as well as the potential of the Niğde Misket Apple in terms of sustainable apple production against fire blight. But similar to the *Rvi6* results, additional research needs to be conducted in that manner for the results of QTL *FB_Mar12*. In terms of this aspect, Niğde Misket Apple may have an important advantage, and further studies related to the fire blight resistance can establish their basis for those results.

The presence of the S_{26} and S_9 alleles of the *S* gene shows the two different heritages of the Niğde Misket Apple genotypes. Because of the rare presence of the S_{26} allele, which is mostly reported on crabapples like Baskatong, *Malus floribunda* 821 [Broothaerts et al. 2004] and Marubakaido apple rootstock (*Malus prunifolia* Borkh.) [Brancher et al. 2020], Niğde Misket Apple probably had some relation to crabapples in its heritage. However, given the limited scope of this study, this hypothesis requires further investigation. Also, some of the apple genotypes from Türkiye have the presence of the S_{26} allele [Karataş et al. 2023], Niğde Misket Apple genotypes have this fundamental basis of Türkiye apple genotypes. Although Niğde Misket Apple genotypes share the same basis with crabapples in terms of the S_{26} allele, they also have the presence of the S_9 allele. From this point of view, they also have a relation to the parental lineage of known apple cultivars like Starking Delicious, Fuji, and Jonagold [Broothaerts et al. 2004], in terms of the S_9 allele. For future breeding efforts, those results can be utilized for self-incompatibility aspects.

CONCLUSION

This study evaluated Niğde Misket Apple genotypes regarding fire blight and apple scab resistance as well as self-incompatibility with some known molecular markers. Results provided genotypes that had resistance alleles of *Rvi6* and QTL *FB_Mar12* with respective molecular markers. Those results can be the basis for further scab and fire blight resistance-related research to enhance and test in line with the results. Genetic diversity of genotypes has already been indicated in previous studies; no further analysis has been conducted in that manner. From a self-incompatibility point of view, genotypes have relations to crabapple as well as the parental lineage of known apple cultivars for a specific allele of the *S* gene. These relationships should be further investigated to determine whether they are limited to *S* genes or involve additional genetic similarities that could be leveraged in plant breeding. New hypotheses emerged from this study. With future studies, more interpretation for the potential of the Niğde Misket Apple for plant breeding can be revealed.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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DIFFERENTIAL EFFECTS OF PLANT GROWTH REGULATORS AND CARBOHYDRATES ON *in vitro* PROPAGATION OF *Scutellaria barbata* D. DON

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ABSTRACT

Experiments were conducted to establish the procedure for sterilizing single-node explants from the mother plant of barbed skullcap *Scutellaria barbata* L7 line (characterized by high scutellarin content) grown in the greenhouse and to induce organogenesis. The effect of different PGRs and carbohydrates on shoot number and shoot length was investigated. The largest number of shoots per explant (16.4) was formed after treatment with 3 mg·dm^{-3} BAP. Shoot multiplication occurred most intensively on medium with the combination of 0.09 M sucrose and 1 mg·dm^{-3} KIN (9.8 shoots per explant), and their elongation on the medium with 1 mg·dm^{-3} GA_3 (10.0 cm). The rhizogenesis process was intensified by using 2 mg·dm^{-3} IBA (87%). Regenerated, rooted plants were acclimatized to *ex vitro* conditions, planted in pots, and placed in a greenhouse.

Keywords: micropropagation, shoot multiplication, explant, organogenesis, barbed skullcap, PGR

INTRODUCTION

Scutellaria L. is a genus that includes over 350 species distributed worldwide [Wang et al. 2012]. The most famous species of this genus include *Scutellaria baicalensis* Georgi and *Scutellaria lateriflora* L. This genus also includes the barbed skullcap (*Scutellaria barbata* D. Don), which is valuable for its adaptogenic and healing properties. *Scutellaria barbata* naturally occurs mainly in northern China, Korea, and Japan. This perennial plant in its natural habitats can grow up to 50 cm. The leaves are lanceolate or triangular in shape and are about 3 cm long. The flower is 1 cm long, the color is purple-blue, slightly hairy [Wang et al. 2020]. The name *Scutellaria* comes from the Latin word ”scutella” meaning a cup, or a shield, this is the shape of the products of the calyx cover of *Scutellaria* flowers. It blooms from May to July. It occurs in wet meadows, near ponds and streams [Wang et al. 2012]. In recent years, studies have been carried out on the chemical composition of *S. barbata*, which have proven the presence of numerous compounds that are

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used in the treatment of human diseases and ailments. *S. barbata* contains alkaloids, steroids, flavonoids, diterpenoids, volatile oils, polysaccharides, and aromatic components. The great importance has been attributed to neo-clerodane diterpenoids from *S. barbata* due to their anti-inflammatory, antiviral, and antitumor effects [Feng et al. 2021, Li et al. 2023]. Single chemical compounds or extracts from *S. barbata* have anticancer activity against gynecological cancer cells, ovarian cancer, breast cancer, prostate cancer, liver cancer, lung cancer, skin and blood cancer [Perez et al. 2010, Wang et al. 2012, Brearley et al. 2014, Sun et al. 2024]. Flavonoids like scutellarin, carthamidin, apigenin, and luteolin are mainly responsible for the anticancer properties of the plant [Chen et al. 2012, Gao et al. 2019, Lema-Rumińska et al. 2023]. According to numerous studies, *S. barbata* also has strong cardiovascular, antibacterial, antiviral, anti-inflammatory, and antioxidant activity, can alleviate memory deficits and neuronal damage, and possesses insecticidal activity [Chen et al. 2020].

Due to the great interest in herbal medicines, the demand for herbal raw materials is growing. Intensive exploitation of herbs in their natural environment, together with human impact on nature, may lead to a reduction in the population of these valuable species. Tissue and cell culture enable the preservation of biological diversity and may be helpful in the rational management of natural resources. By properly selecting micropropagation methods, we can obtain plants with preserved genetic integrity with the mother plant, which ensures high quality of the medicinal raw material, also allows for controlled breeding conditions and obtaining a larger number of healthy plants. Moreover, thanks to this technique, it is also possible to eliminate diseases and pathogens that appear in traditional cultivation methods, which contributes to increasing the safety and quality of plant raw materials [Pant 2014, Brearley et al. 2014].

As demonstrated in the study by Pasternak and Steinmacher [2024] for the optimization of *in vitro* culture, the balance of nutrients and the presence of competent cells that can differentiate into stem cells, and the regulation of endogenous synthesis of hormones such as auxins and their distribution in the plant are also important. Auxin is a hormone responsible for the processes of shoot and root morphogenesis *in vitro*. It can be transported polarly, create gradients, and determine cell functions involved in the formation of all plant organs, including primary and lateral roots [Roychoudhry and Kepinski 2022]. Auxins and cytokinins are the main regulators of plant growth and development. Acting at low concentrations, which exclude their nutritional effect, they influence plant growth and development. In addition, cytokinin and auxin regulate the synthesis of each other, often act as an antagonistic hormone pair, showing a mutual feedback mechanism, which is important for many developmental processes in plants [Kurepa and Smalle 2022]. The main effect of cytokinin in *in vitro* tissue culture is the induction of shoots. It should be noted that cytokinins induce axillary as well as adventitious shoot formation from meristematic explants. The physiological function of cytokinins is, among others, the stimulation of cell division. In addition, they activate RNA synthesis and stimulate protein synthesis and affect enzyme activity. Application of cytokinins effectively limits the length of shoots while increasing their number, limits the surface area of leaves, and stimulates the formation of meristematic centers [Mishra et al. 2019, Hnatuszko-Konka et al. 2021, Pasternak and Steinmacher 2024, Figas et al. 2025].

Among the factors that have the greatest influence on the processes of growth and multiplication in *in vitro* cultures is the type and concentration of the carbon source applied to the medium. This factor influences the number of plants obtained in the process of micropropagation [Brearley et al. 2014]. In *in vitro* culture conditions, carbohydrates are a source of carbon that is used as energy, and regulate the osmotic potential for various physiological processes requiring energy [Yaseen et al. 2013, Naidu Mahadev et al. 2014, Van den Ende 2014]. Carbohydrates can protect against stress factors, and they participate in the regulation of defense reactions of plants exposed to stress. They have an osmoprotective effect and participate in signal transmission [Ciereszko 2018].

Previous studies on *S. barbata* conducted by Lema-Rumińska et al. [2023] included seven new genotypes with significant differences in morphology and metabolite content. However, these studies did not examine the effect of growth regulators on micropropagation and rooting rates. Therefore, our study complements earlier studies and focuses on the L7 line, which

is the most efficient in terms of the valuable metabolite (scutellarin) content.

In the described experiment, the influence of different plant growth regulators (PGRs) and carbon source was investigated to determine the most efficient and effective micropropagation protocol for the new L7 line of *S. barbata,* which will help in rapid reproduction of desired lines of plant with valuable medicinal properties and increase the herbal biomass production for pharmaceutical and medical industries.

MATERIAL AND METHODS

The research was conducted at the Department of Biotechnology of the Bydgoszcz University of Science and Technology (Poland). The plant material (ten homogeneous plants from the L7 line) came from previous studies published by Lema-Rumińska et al. [2023]. The explants used for the research were single-node shoot fragments (explants) of *S. barbata* isolated from mother plants growing in greenhouse conditions.

Preliminary test

A preliminary test was performed to determine the most effective and consistent sterilization procedure for the plant material used in the *in vitro* culture of *S. barbata*. In the first stage of disinfection, the explants were rinsed in running water with detergent, then they were placed in 70% C_2H_5OH (Chempur, Piekary Śląskie, Poland) for 1 minute. In the next stage, the explants were treated with sodium hypochlorite – NaClO (Warchem, Zakręt, Poland) solutions at concentrations of 2.0%, 3.0%, and 5.0% with the addition of Tween 20 – 300 µL/100 mL (Sigma-Aldrich, Burlington, MA, USA) for 12 minutes. The process of disinfection of shoot explants was completed by rinsing the disinfected shoot fragments three times in sterile double-distilled water. The control was explants sterilized with only 70% C_2H_5OH (EtOH). Sterile explants were inoculated into MS medium [Murashige and Skoog 1962] without PGR. The composition of the medium used was: 4.4 g·dm^{-3} MS basal medium (Sigma-Aldrich, Burlington, MA, USA), 30 g·dm^{-3} sucrose (Chempur, Piekary Śląskie, Poland), 7 g·dm^{-3} agar (Vitro LAB-AGAR, BioMaxima, Lublin, Poland). The pH of the medium was set at 5.8. In each of the sterilization variants 10 single-node shoot fragments were inoculated onto the medium. *In vitro* cultures were grown at a temperature of 24 ±1 °C, light intensity of 40 µmol·m^{-2}·s^{-1} (fluorescent lamps L36W/77, OSRAM, Munich, Germany) and photoperiod of 16 h light/8 h dark in test tubes, each containing 20 mL of solidified MS medium and one explant. The effectiveness of the disinfection process was determined 14 days after inoculation of the explants onto the culture medium.

Induction of axillary bud development

Stimulation of the development of axillary buds on nodal explants derived from mother plants, sterilized with NaClO at the concentration selected in the preliminary experiment, was carried out on the MS medium with 3 mg·dm^{-3} 6-benzylaminopurine (BAP), 1 mg·dm^{-3} BAP + 0.5 mg·dm^{-3} 1-naphthaleneacetic acid (NAA), or 0.5 mg·dm^{-3} BAP + 1.0 mg·dm^{-3} NAA. The control medium was MS without growth regulators; 10 explants were placed on each medium variant, and the culture was carried out for 12 weeks. This stage of the culture was carried out in test tubes, each containing 20 mL of solidified MS medium and one explant (Table 1).

Shoot multiplication

Shoots obtained by stimulating the development of axillary buds were divided into nodal fragments and transferred in the amount of 30 in each variant to propagation medium containing BAP, gibberellic acid (GA_3) and kinetin (KIN) in the amount of 1 mg·dm^{-3} and 1 mg·dm^{-3} BAP, KIN, thidiazuron (TDZ), zeatin (ZEA) supplemented with 0.1 mg·dm^{-3} NAA (Table 2) and 0.09 M sucrose or glucose as a carbon source. The explants were placed into Erlenmeyer flasks containing 25 mL of solidified MS medium, with three explants allocated per flask. After 12 weeks of culture the number of shoots obtained in the multiplication process was counted. Observations of the plant's shape and measurement of the length of shoots were also carried out. The morphological traits were assessed by manual measurements with the aid of millimeter-scale graph paper.

Induction of root formation

The shoots were transferred to rooting medium (MS) supplemented with 30 g·dm^{-3} sucrose, with

Table 1. Efficiency of stimulation of the development of axillary buds on the nodal explants of *S. barbata*

PGR (mg·dm^{-3})		Number of explants	Mean number of shoots/explant	Mean length of shoots (cm)
BAP	NAA			
0.0	0.0	10	4.6 ±2.63	1.52 ±0.82
3.0	0.0	10	16.4 ±6.01	0.53 ±0.32
1.0	0.5	10	9.0 ±3.74	2.48 ±1.14
0.5	1.0	10	6.8 ±4.26	1.14 ±0.55
$LSD_{0.05}$		–	2.167	0.295

Results are mean ± SD (standard deviation); LSD – the lowest significant difference (Tukey's confidence half-interval) at $p \leq 0.05$; PGR – plant growth regulators; BAP – 6-benzylaminopurine; NAA – 1-naphthaleneacetic acid

1 or 2 mg·dm^{-3} indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA), 30 shoots were used for each medium variant and cultured in a growth room for 12 weeks in Erlenmeyer flasks containing 25 mL of solidified MS medium, with three shoots per flask.

Acclimatization of rooted shoots

The rooted shoots were adapted to the greenhouse conditions. The plants were placed in containers filled with a substrate consisting of a mixture of horticultural substrate it contains high peat (0–6 mm), supplemented with neutralizing minerals, multicomponent fertilizer, specialized micronutrient fertilizer and Hydrofil (Hartmann, Poznań, Poland), sand, and perlite (Biovita, Tenczynek, Poland) in the proportions 2:1:1. The acclimatization process lasted 14 days and took place in multi-pots under a foil tent to limit transpiration, and then the plants were planted in pots with gardening substrate containing high peat (0–20 mm), supplemented with mineral neutralizers and multicomponent fertilizer (Hartmann, Poznań, Poland) and placed in greenhouse conditions.

Statistical analysis

The obtained analytical results were statistically processed in MS Excel and Statistica 13.3. In the case of results obtained from the micropropagation of *S. barbata*, analysis of variance was performed in a completely random design. At the stage of bud induction and rooting, one-way analysis of variance was used to determine the number of shoots or roots per explant and the length of shoots or roots. Two-way analysis of variance was performed to determine the number of shoots per explant and shoot length during the shoot multiplication phase. Using Tukey's single confidence intervals for the significance level at $p \leq 0.05$, the significance of differences (the lowest significant difference, LSD) was determined.

RESULTS AND DISCUSSION

Preliminary test

Sterilization efficiency of nodal shoot fragments was achieved at 80% using 5% NaClO for 12 min. In the case of a 2% NaClO solution, the disinfection efficiency was lower, it was 40%, slightly better results were achieved when a 3% NaClO solution was used, in this case, the disinfection efficiency was 60%. Of the tested explants, only some of them started growth, the highest number of explants able to start growth and development was recorded in the two highest NaClO concentrations, i.e. 3% and 5%. Sterile explants could not be obtained when only EtOH was used (Fig. 1). The results are consistent with the literature, which indicates that NaClO solutions are used for the elimination of contaminants, although their effect may be due to the concentration, action, and time of treatment [Mganga et al. 2025]. Effective sterilization of explants with active Cl was also achieved in *S. barbata* [Lema-Rumińska et al. 2023], *Leonurus sibiricus* L. [Figas et al 2025]. 10% NaClO has been successfully used to sterilize the stem node and shoot tip of lavender (*Lavandula* sp.) [Kara and Baydar 2012]. NaClO has a disinfecting effect also at lower concentrations. 4% NaClO for the sterilization of nodal segments was used by lavender (*Lavandula angustifolia* Mill.) [Kumari et al. 2024].

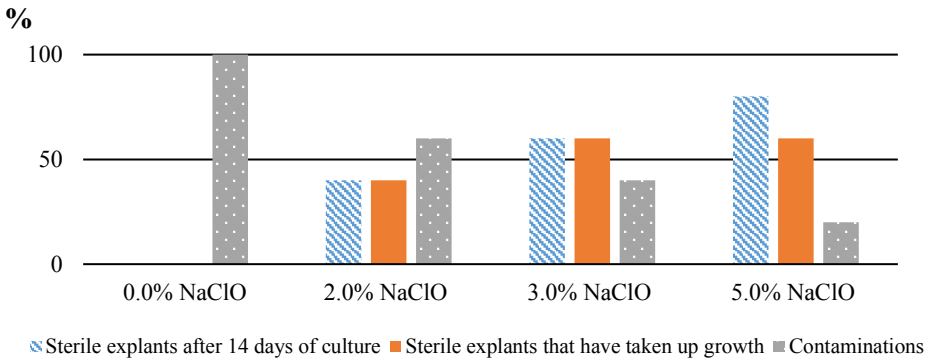

Fig. 1. Effectiveness of the methods used to sterilize *S. barbata* single-node explants

Induction of axillary bud development

The stimulation of the development of axillary buds was performed on MS medium with 3 mg·dm^{-3} BAP, 1 mg·dm^{-3} BAP + 0.5 mg·dm^{-3} NAA, or 0.5 mg·dm^{-3} BAP + 1.0 mg·dm^{-3} NAA. Significant differences were observed in the number and shape of the obtained shoots between the variants of the medium used. The highest effectiveness of inducing this process was found for 3 mg·dm^{-3} BAP. In this variant, it was recorded that one explant yielded on average over 16 shoots, which were characterized by short internodes and a small surface of leaf blades. Lower efficiency of shoot induction was observed on medium with 1 mg·dm^{-3} BAP + 0.5 mg·dm^{-3} NAA or 0.5 mg·dm^{-3} BAP + 1.0 mg·dm^{-3} NAA, where an average of 9 and 6.8 shoots per explant were obtained, respectively. The least useful for induction was the medium without growth regulators (average 4.6 shoots/explant). Measurements and observations were carried out after 12 weeks of culture. The longest shoots were observed on the medium containing 1 mg·dm□^{3} BAP + 0.5 mg·dm□^{3} NAA (2.48 cm) and on the control medium (1.52 cm), whereas the shortest shoots were recorded on the medium with 3 mg·dm□^{3} BAP (0.53 cm), see Table 1. Numerous literature data confirm this regarding the influence of the presence of cytokinins and cytokinins combined with auxins on the efficiency of shoot formation induction.

As other authors have shown, the presence of BAP in the medium is effective in shoot induction in various plant species. In the case of *Phytolacca dodecandra* L'Heit, the authors analyzed the effect of concentration ranging from 2.22 µM BAP to 22.2 µM BAP and achieved 80% shoot induction efficiency on nodal explants and 70% on shoot apical explants at the lowest doses [Daksa et al. 2015]. Good results were also obtained in studies on *Scutellaria alpina* L., where a high average number of axillary shoots was obtained [Grzegorczyk-Karolak 2015].

Analyzing the effect of different BAP concentration on micropropagation of *Scutellaria integrifolia* L., the maximum number of shoots from shoot apices (23 per explant) was obtained on MS medium supplemented with 2.2 µM BAP [Joshee et al. 2007]. In the case of *S. baicalensis* the highest average number of shoots (5.8) was, achieved for the MS + 1.0 mg·dm□^{3} BAP [Dyduch-Siemińska and Gawroński 2024]. Considering the commonly used cytokinins, the application of 5 µM BAP in *in vitro* culture of *S. barbata* and *Scutellaria racemosa* Pers. also resulted in obtaining numerous shoots with nodal explants [Brearley et al. 2014]. The stimulating effect of BAP increases with increasing concentration in Shine Muscat grapevine *in vitro* culture [Kim et al. 2023]. The rate of regeneration process in the presence of different BA doses ranged from 90.4% to 97.8%. The highest efficiency was observed for 2 µM BA, at higher concentration the rate of bud induction decreased. On the other hand, increasing BA concentration increased the number of shoots obtained from nodal explants from 1.05 at a concentration of 1.0 µM BA up to 1.38 at a concentration of 8 µM BA, however, the highest concentra-

tion (16 μM BA) turned out to be less useful in this case because the number of shoots per nodal explant was 1.29 [Kim et al. 2023].

Shoot multiplication

Shoot multiplication occurred most intensively on medium with sucrose under the influence of 1 mg·dm^{-3} KIN, where approximately 10 shoots per explant were obtained on average. Other results were presented by Grzegorczyk-Karolak et al. [2015] in the *in vitro* culture of *S. alpina* where the most effective cytokinin was BAP at a concentration of 2 μM, with the highest average number of axillary shoots (25.0 per explant). Kinetin supported the overall increase in shoot length, but did not have such a pronounced effect on the number of shoots as BAP [Grzegorczyk-Karolak 2015]. The combination of two cytokines, BAP and TDZ, effectively induced shoot organogenesis in *in vitro* cultures of *Scutellaria araxensis* Grossh. Optimal effects were obtained using BAP (0.5–2 mg·dm^{-3}) with TDZ (0.1–1.5 mg·dm^{-3}), which were more effective than the combinations of BAP with auxins (NAA and IBA) [Gharari et al. 2022].

In our research, the highest multiplication efficiency was obtained for KIN. BAP also has a positive effect on shoot proliferation, although its action was less effective compared to KIN. The use of BAP at a concentration of 1 mg·dm^{-3} contributed to the improvement of shoot growth, but this effect was less pronounced than in the case of KIN. The longest shoots of *S. barbata* were obtained on the medium with 0.09 M sucrose and 1 mg·dm^{-3} GA_3, where the average shoot length was 10 cm. GA_3 has also been successfully used for elongation *in vitro* cultures of

Table 2. Effect of different PGRs and carbohydrates on shoot number and shoot length of *S. barbata*

Carbohydrate	MS medium and PGR (mg·dm^{-3})	Mean number of shoot/ explant	Mean length of shoots (cm)
0.09 M sucrose	1.0 BAP	6.73 ±3.90	4.60 ±3.01
	1.0 GA_3	1.70 ±0.96	10.00 ±2.36
	1.0 KIN	9.80 ±3.68	5.00 ±2.77
	1.0 BAP +0.1NAA	6.10 ±3.93	4.20 ±3.21
	1.0 KIN +0.1NAA	6.50 ±3.86	6.00 ±3.40
	1.0 TDZ +0.1NAA	4.70 ±2.84	4.30 ±2.52
	1.0 ZEA +0.1NAA	5.00 ±3.38	4.20 ±2.89
Mean		5.78 ±2.12	5.47 ±1.81
0.09 M glucose	1.0 BAP	4.90 ±1.90	4.00 ±2.02
	1.0 GA_3	1.30 ±0.71	6.20 ±1.48
	1.0 KIN	6.00 ±2.78	4.20 ±1.22
	1.0 BAP +0.1NAA	4.90 ±2.56	3.60 ±2.05
	1.0 KIN +0.1NAA	4.70 ±2.02	4.70 ±2.10
	1.0 TDZ +0.1NAA	4.30 ±2.07	4.10 ±2.08
	1.0 ZEA +0.1NAA	4.20 ±2.01	3.80 ±2.45
Mean		4.33 ±1.26	4.37 ±0.76
LSD $_{0.05}$ for PGR (I)		0.462	0.456
LSD $_{0.05}$ for sugars (II)		1.278	1.291
Interaction I/II		0.435	0.460
Interaction II/I		1.746	1.848

Results are mean ± SD (standard deviation); LSD – the lowest significant difference (Tukey's confidence half-interval) at $p \leq 0.05$; MS – Murashige and Skoog medium; PGR – plant growth regulator; BAP – 6-benzylaminopurine; NAA – 1-naphthaleneacetic acid; GA_3 – gibberellic acid; KIN – kinetin; TDZ – thidiazuron; ZEA – zeatin

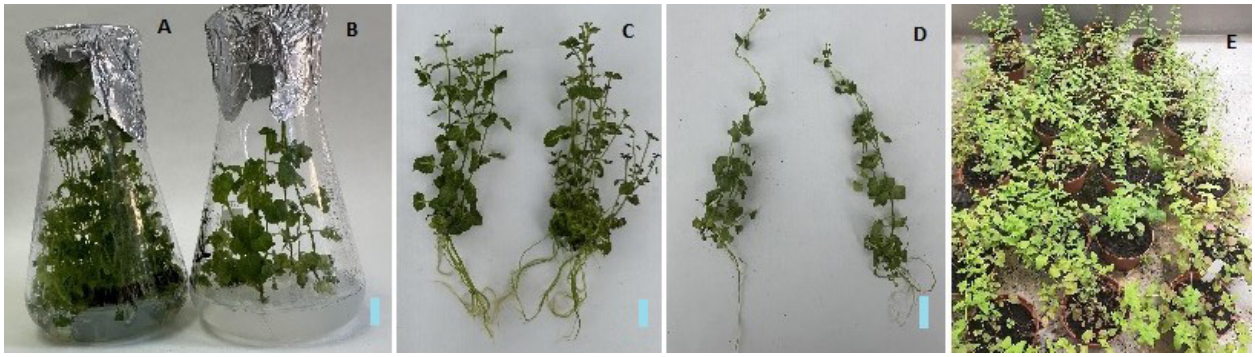

Fig. 2. Shoot multiplication of *S. barbata* on Murashige and Skoog medium [1962] with carbon source: sucrose (A), glucose (B), the rooted plants of *S. barbata* on MS medium with 2 mg·dm^{-3} IBA (C) and 2 mg·dm^{-3} IAA (D), acclimatized plants of *S. barbata* in greenhouse conditions (E), (bar = 1 cm)

S. araxensis [Gharari et al. 2022], *Dietes bicolor* Stend. [Silva 2020], and *Dalbergia latifolia* Roxb. [Boga et al. 2012]. GA_3 affects stem elongation when plants are exposed to different temperatures between day and night [Andini et al. 2020]. This growth phytohormone promotes cell expansion and, even at low concentration, enhances the activity of various cytokinins, thereby facilitating *in vitro* shoot and root elongation, multiple shoot induction, and cell differentiation [Ahmad et al. 2021]. The physiological function of gibberellins is cell elongation by changing the elasticity of the cell wall. They plays an important role in seed germination, stem elongation, leaf expansion and reproductive development [Shani et al. 2013, Teszlák et al. 2013]. In our study, the number of shoots obtained and their length on medium with sucrose were higher than with glucose (Table 2, Fig. 2A, B). In contrast to studies of Brearley et al. [2014], which prove that the addition of fructose or glucose to the medium results in a higher average shoot in *S. barbata* than the addition of sucrose. However, for *S. racemosa*, the results for sucrose after 21 days of culture were higher together with maltose compared to other carbon sources. It follows that the selection of the appropriate type of carbohydrate depends on the plant species and the type of explant. Sucrose is a commonly used carbon source in *in vitro* cultures. In most plant species, sucrose is the main form of assimilated carbon produced during photosynthesis. Long-distance sucrose distribution from the green source tissues to energy-demanding sink tissues is mediated by phloem [Naidu Mahadev et al. 2014, Bavnhøj et al. 2023]. The increase in seedling growth rate and size is related to the volumetric increase in the cell, which is influenced by the osmotic pressure regulated by sucrose [Sumaryono et al. 2012]. Sucrose was used in the effective propagation of *Mentha piperita* L. [Sujana and Naidu 2011], *Stevia rebaudiana* Bertoni [Preethi et al. 2011], *Solanum nigrum* L. [Sridhar and Naidu 2011], and *Solanum viarum* Dunal [Naidu Mahadev et al. 2014]. The effect of sucrose is difficult to determine because autoclaving contributes to the hydrolysis of sucrose into glucose and fructose and a large amount of it is decomposed during seedling growth [Rahman et al. 2010]. As a result of hydrolysis during *in vitro* culture, the concentration of sucrose also decreases due to the action of invertase, the source of which is explants. By examining the composition of the medium during culture, it is possible to determine the presence of both sucrose and glucose and fructose [Naidu Mahadev et al. 2014]. The evaluation of the effect of different carbohydrates on the regeneration process was carried out by Akyüz [2025]. Among the carbohydrates tested for hybrid chestnut cultivar Marsol (*C. sativa* × *C. crenata*) sucrose and then glucose added to the medium resulted in the best regeneration. A much weaker effect was noted for the other analyzed sugar – maltose. Such a diverse effect of carbohydrates used in *in vitro* cultures results from the different ability of plants to metabolize these compounds. Sucrose in *in vitro* plant cultures is usually applied at a concentration of 2–3%. In banana (*Musa banana* L.) cultures, shoot induction was more effective on sucrose medium, while shoot multiplication efficiency was highest in the presence of both 3% sucrose and 3% glucose

[Madhulatha et al. 2006]. As described by Tarinejad and Amiri [2019] in micropropagation of grapevine (*Vitis vinifera* L.), 3% sucrose proved to be the best carbon source, enabling the highest cutting height and the maximum number of shoots obtained from one explant. It was found, that 3% sucrose is also the most useful carbohydrate for effective micropropagation of *Nauclea diderrichii* (De Wild.) Merr. [Pitekelabou et al. 2015] and kohlrabi (*Brassica oleracea* var. *gongylodes* L.) [Ćosić 2020].

Rooting and acclimatization

Auxins such as IAA and IBA were used in the rooting process of *S. barbata* plants. The main factors enabling root formation in *in vitro* cultures are intensive auxin biosynthesis in developing shoots and efficient auxin transport through vessels from the site of synthesis [Pasternak and Steinmacher 2024]. An important role in the process of shoot and root formation is played by the concentration of auxins in tissues and organs, and their reactivity [Koike et al. 2020, Roychoudhry and Kepinski 2022]. In our experiment, the elongated shoots were transferred to MS medium with 1 or 2 mg·dm^{-3} IBA and IAA. The best rooting response was observed on a rooting medium supplemented with 2.0 mg·dm^{-3} IBA (Fig. 2C), with the highest number of rooted plants – 26 and the highest average number of roots – 8 (Table 3). In the plant, endogenous hormones play a fundamental role in morphogenesis. Exogenous growth regulators are applied to ensure balance with endogenous hormones, thereby influencing physiological responses as stimulators of cell division and elongation [Nurhanis et al. 2019]. Exogenous IBA proved to be most useful for stimulating the rhizogenesis process. Similar results were obtained by authors studying *in vitro* plants of the *Scutellaria* genus, namely *S. integrifolia* [Joshee et al. 2007], *S. barbata*, *S. racemosa* [Brearley et al. 2014], *S. havanensis* [Irvin et al. 2021], *S. araxensis* [Gharari et al. 2022], and other species such as *Centella asiatica* (L.) [Panathula et al. 2014]. The use of auxins in different concentrations clearly influenced the number of rooted shoots and root morphology, which is confirmed by the results of Joshee et al. [2007]. They found out that a lower concentration of IBA (4.9 M) was more beneficial for the rooting of *S. integrifolia* plants than 9.8 M IBA, because the higher concentration also caused callusing and swelling in addition to rooting. In our study, the use of IAA in the rhizogenesis process in *S. barbata* did not have a significant effect on the number of roots, but 2 mg·dm^{-3} IAA led to the development of the longest roots – 9.0 cm (Table 3, Fig. 2D). As a main auxin, IAA is a key factor in cell elongation, which explains the observed results. Exogenously supplied IAA affects protein synthesis in plant tissues, changes in the permeability of cell walls occur, and cell division and elongation are intensified, which may result in increased root length [Nurhanis et al. 2019].

After 12 weeks, the rooted plants were transferred to the multi-pots under a foil tent. In the final phase of acclimatization, the plants were placed in individual pots and placed in a greenhouse (Fig. 2E). The accli-

Table 3. Rooting efficiency of shoots on different medium variants after 12 weeks of *S. barbata* culture

MS medium and PGR (mg·dm^{-3})	Number of rooted plants	Mean root length (cm)	Mean number of roots/explant
1.0 IBA	18	5.0	6.0
1.0 IAA	20	8.0	3.0
2.0 IBA	26	5.5	8.0
2.0 IAA	22	9.0	6.0
$LSD_{0.05}$	–	1.533	1.252

Results are mean ± SD (standard deviation); LSD – the lowest significant difference (Tukey's confidence half-interval) at $p \leq 0.05$; MS – Murashige and Skoog medium; PGR – plant growth regulator; IBA – indole-3-butyric acid; IAA – indole-3-acetic acid

matization efficiency of *S. barbata* plants in the greenhouse was 60%.

CONCLUSIONS

An efficient plant regeneration system for the new L7 line of *S. barbata* (with the high scutellarin content) using nodal explants was developed. The conducted preliminary studies have shown the high effectiveness of 5% NaClO for the sterilization of nodal explants (80%). Among the growth regulators used, the highest effectiveness in the process of shoot development induction was demonstrated for 3 mg·dm^{-3} BAP. Shoot multiplication occurred most intensively on the medium with the combination of 0.09 M sucrose and 1 mg·dm^{-3} KIN, however, the highest degree of shoot elongation was observed after the application of 1 mg·dm^{-3} GA_3. Root formation was most intensive when the medium was supplemented with 2 mg·dm^{-3} IBA, although the longest roots were noted in the presence of 2 mg·dm^{-3} IAA. According to the presented results, the described micropropagation procedure for the L7 line of *S. barbata* may help in the rapid propagation of desired lines of plant with valuable medicinal properties and can be successfully used in commercial micropropagation.

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EFFECTS OF BACTERIAL AND FUNGAL BIO-FERTILIZERS ON YIELD AND QUALITY OF SOILLESS-GROWN CLUSTER TOMATO

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ABSTRACT

This study evaluated the effects of bacterial (*Arthrobacter globiformis*, *Streptomyces griseus*) and fungal (*Aspergillus oryzae*) preparations on the growth, yield, and fruit quality of cluster tomato (*Solanum lycopersicum* L. cv. Cletego F1) grown in cocopeat substrate under greenhouse conditions. Treatments significantly improved plant growth, cluster number, and yield compared with the control, with *Streptomyces griseus* producing the highest yield (58.6 t da^{-1}) and superior fruit quality (SSC = 4.85%, acidity = 0.28 g citric acid 100 mL^{-1}). The control recorded the highest vitamin C content. The study concludes that microbial inoculation enhances yield and quality in soilless tomato cultivation, supporting eco-friendly and sustainable production systems.

Keywords: *Arthrobacter globiformis*, *Aspergillus oryzae*, cocopeat, soilless agriculture, *Streptomyces griseus*

INTRODUCTION

Tomatoes (*Solanum lycopersicum* L.) have been shown to offer significant benefits to human health by strengthening the immune system. This is due to the phenolic compounds, especially lycopene, vitamins, and minerals they contain [Rao and Agarwal 2000, Barber and Barber 2002, Imran et al. 2023]. Tomatoes are a very important vegetable (13 million tons) that is widely cultivated worldwide, including in Turkey. A substantial proportion of tomatoes, which are the most widely cultivated vegetable in both global and Turkish production, are produced soilless greenhouse [TÜİK 2024, FAO 2024]. Soilless agriculture, which exhibits numerous advantages in comparison to traditional cultivation techniques, is categorized into two distinct methods: hydroponic culture and solid media culture. In the context of commercial tomato cultivation, solid media culture is a prevalent practice, owing to its cost-effectiveness and its role in creating a protective barrier around the plant's root zone [Toprak and Gül 2013]. Solid medium culture supports the plant grown on the growing medium, thereby increasing success. In soilless farming, many organic and inorganic media are used in solid medium culture. Cocopeat has become the preferred choice due to its high capacity to retain water and nutrients, its light weight, high air permeability, and

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ability to provide healthy cultivation [Frolking et al. 2001].

It has been established that microorganisms residing in the root zone of growing media employed in solid media culture exert an impact on crop health through antagonism against pathogens. Future efforts to enhance soilless yield should prioritize the development of intelligent environmental control methodologies and the advancement of microbiome science [Masquelier et al. 2022, Tuxun et al. 2025]. The emergence of pathogens over time within the growing environment poses a significant challenge. Consequently, the utilization of beneficial microorganisms in soilless agriculture is of paramount importance, as they ensure the effective use of nutrient solutions and promote the formation of beneficial microflora by suppressing fungal pathogens [Grover et al. 2021, Masquelier et al. 2022, Aktaş and Hor 2024, Tuxun et al. 2025]. The utilization of fungal and bacterial microorganisms as biocontrol agents within biological control approaches represents a novel methodology for combating issues pertaining to fungicide resistance. A variety of *Bacillus* and *Trichoderma* species, which possess strong biocontrol potential are employed extensively in the management of plant diseases, including early blight in tomatoes [Mazrou et al. 2020, Stracquadanio et al., 2020, Castro-Restrepo et al. 2022, Narware et al. 2023, Imran et al. 2023]. Similarly, it reveals that naturally occurring bacterial microbes can control many diseases in vegetable cultivation and increase pathogen resistance in plants [Imran et al. 2022, 2023, Abo-Elyousr et al. 2022, 2024]. Bio-fertilizer is defined as a material consisting of living microorganisms which, when applied to seed, plant surface, soil or substrate are capable of fixing atmospheric nitrogen, increasing the uptake of mineral elements from both organic and inorganic sources, or promoting plant growth through the production of secondary metabolites [Grover et al. 2021, Tuxun et al. 2025]. The composition of the material to be used in the growing medium is of paramount importance. It is a commonly held view amongst researchers that it is correct to develop the growing medium to be used in conjunction with the microorganisms to be used. In the context of sustainable tomato production, the growth, flowering and fruit formation of tomatoes are contingent on the relationship between the mineral enrichment of the growing medium, primarily calcium uptake, and microorganisms [Orta-Guzmán 2021, Mahapatra et al. 2022, Masquelier et al. 2022, Gulia et al. 2022].

Soilless tomato cultivation is a profitable production method due to its high yield. However, in comparison with soil-based cultivation, the soilless growing media is characterized by a deficiency in microorganisms. The agricultural practice of cultivation is undertaken with the application of intensive fertilization in commonly utilized growing media, such as cocopeat substrate. The number of studies investigating the effects of changes in the quantity and diversity of microorganisms in growing media on tomato yield and quality is limited. Therefore, this study was conducted with the objective of ascertaining the effect of bacterial (*Arthrobacter globiformis* and *Streptomyces griseus*) and fungal (*Aspergillus oryzae*) preparations applied to cocopeat used in substrate culture on the plant growth, yield, and quality of tomatoes cultivated in a soilless culture.

MATERIAL AND METHODS

Experimental time and location. The study was carried out between 15 March – 20 July 2022 in the greenhouse of Tutku Agriculture greenhouse enterprises. The greenhouse where the cultivation part was carried out is a plastic greenhouse located at '39.004814, 33.943019' on 40 acres of land in Sarıyahşi district of Aksaray province. Fruit quality analyses were carried out in the laboratories of Ordu University, Faculty of Agriculture, Department of Horticulture.

Plant material. In the study, seedlings of the cultivated tomato variety Cletego F1 (*Solanum lycopersicum* L.) (Syngenta, İzmir, Türkiye), which is particularly suitable for soilless agriculture, were used.

Experiment description. The prepared growing bags (Cocopeat; 100 × 20 × 5 cm) were placed in 60 m long, 25 cm wide and 1.5% slope channels in the greenhouse. The seedlings of the Cletego F1 tomato cultivar were then planted in the growing bags at equal intervals (60 cm between rows and 30 cm above rows) with 4 plants per slab. Following this, the macro and micronutrients utilised in the fertilization process were meticulously prepared as stock solutions in two tanks (A and B), each with a capacity of 2000 L. The nutrient solutions to be used in the experiment [Hoagland and Arnon 1938] are given in Table 1.

The stock solutions presented in Table 2 were administered at varying doses, contingent upon the growth and developmental phases of tomato plants. Temperature values recorded with data loggers in the greenhouse ranged from 17 °C to 28 °C, while relative humidity values varied between 43 and 86%. When relative humidity values exceeded 70%, the ventilation system was used to control them.

The fertilization process was initiated at 1.5 hours after sunrise and concluded at 2 hours before sunset during the growing period. During the growing period, the tanks containing stock solutions were re-prepared according to necessity. Conductivity (EC) and pH were measured continuously from the water extracted from the growing media from seedling planting to harvest. When elevated levels of salinity were recorded, the media was subjected to a 10-minute cycle of water alone. This process was repeated until the desired level of salinity reduction was achieved.

The study utilised a range of bio-fertilizers, namely, *Aspergillus oryzae* (Mılıcard, MCC075 pure culture), *Arthrobacter globiformis* (Mılıcard, MCC296 pure culture), and *Streptomyces griseus* (Mılıcard, MCC1973 pure culture) were used. One of the bio-fertilizers is of fungal origin (*Aspergillus oryzae* 1 × 1010 cfu/gr) and two are of bacterial origin (*Arthrobacter globiformis* 1 × 1010 cfu/gr, *Streptomyces griseus* 1 × 1010 cfu/gr).

Table 1. Macro and micronutrient solutions and ratios to be applied in soilless culture [Hoagland and Arnon 1938]

Stock solutions	Chemical substances	The amounts
A (2000 L)	potassium nitrate	50 kg
	calcium nitrate	260 kg
	nitric acid	1 L
	iron chelate	3 L
	potassium chloride	10 kg
	previcur energy	600 mL
B (2000 L)	potassium nitrate	52 kg
	mono potassium phosphate	54 kg
	potassium sulfate	84 kg
	magnesium sulfate	84 kg
	zinc sulfate	430 g
	manganese sulfate	1000 g
	sodium molybdate	24 g
	copper sulfate	60 g
	borax	570 g

Table 2. Fertilizer doses to be applied to tomatoes according to growth and development periods

Developmental periods	N	P	K	Ca	Mg
	mg/L				
Until flowering	200	50	275	250	70
4–5. after the inflorescence appears	230	55	360	230	70
7–8. after the inflorescence appears	220	55	470	230	60

The fungal and bacterial applications were made during the seedling period (1 month). Mixtures (10 g L^{-1}) were prepared, comprising 1 litre of each organism. Each plant was treated with 100 mL of the mixture, and 6 plants were used for each experiment. A total of 24 plants were incorporated into the experimental design, with 2 applications of the 10 g L^{-1} solution administered at 2-week intervals during the vegetation period, subsequent to the seedling stage. It should be noted that no microorganisms were used as a control in the study. In order to ensure that the control applications and all microorganism applications are isolated from each other, the growing bags used for the applications are placed in different slope channels.

Planting preparation, planting and cultivation

Prior to the planting of the seedlings, the distances were determined, and the planting sites were opened in the growing bags. Subsequent to the creation of the planting sites, drainage holes were made in the growing bags at a height of 2–3 cm above the bag. The tomato seedlings were planted in the second week of April (25.04.2022). Post-planting, drip irrigation pipes were affixed to the surface of the bags, with 200 ml of living water allocated to each plant. On the day of planting, only living water was administered to the plants, with the nutrient solution commencing from the subsequent day.

Pruning, a widely employed practice in tomato cultivation, was meticulously executed on the shoots emerging from the leaf axils and the lower old leaves, with the systematic removal of three leaves per week. Old leaves located beneath harvested clusters were systematically removed, ensuring the retention of five fruits per cluster. Subsequent green fruit clusters were pruned, with the removal of three leaves, leaving a total of 12 leaves on the plant. Throughout the cultivation period, a range of other maintenance, spraying and cultural procedures were applied as required. Armpit removal and pruning were executed on a weekly basis. According to the EDT (economic damage threshold) method, cultural precautions such as collecting pest leaves, placing pheromone water traps and sticky traps were carried out.

Measurements and observations on growing plants

In the present study, bio-fertilizer applications were initiated at the 6 cluster stage. However, given that the research was conducted within a commercial enterprise, measurements and observations were determined by following up until the end of cultivation. The fruit quality characteristics were evaluated on a scale of 1 to 5. The fruit samples were obtained from clusters 3–5.

The height of the plants was measured in centimetres from the root collar to the tip of the growth, with the aid of a tape measure. The stem diameter was measured in millimetres from the root collar using a digital calliper. The number of leaves was counted manually from the time of planting and recorded accordingly. The root dry weight was measured by washing and separating the roots so that there would be no root loss during the uprooting process. The separated roots were then placed in paper bags and placed in an oven at 80 °C. The drying process was carried out for a minimum of 48 hours. During this period, the weight change method was employed for the samples that had not yet completed drying, and the determination was made as to whether the drying process had been completed or not. Once it was established that the samples had undergone complete desiccation, their dry weights were determined by means of a balance with a sensitivity of 0.01 g. The number of cluster was determined by manual enumeration. The number of fruits per cluster was determined as the mean value by means of manual enumeration of the fruits in the cluster since planting. Measurements were taken at regular intervals, with each interval spanning a duration of 20 days. The final measurements were taken 9 months after planting the seedlings.

Throughout the growing season, all fruits that reached the pink stage were harvested and measured. The yield, weight and fruit diameter of each fruit were determined. The fruit weights were measured by means of a scale that was sensitive to 0.1 g. The fruit weight was determined in grams by averaging the obtained fruit weights. Finally, the yield per plant was calculated in kilograms by summing the weights of the harvested fruits (marketable product amount).

The colour of the fruit skin was determined in accordance with the CIE Chroma and Hue colour models. The colour of the fruit was measured using a colourimeter (Minolta, model CR-400, Tokyo, Japan) by taking a measurement from two opposite sides of the equatorial side of 10 fruits obtained from each repli-

cate of each treatment. Flesh firmness was measured by lifting the peel from two different sides of the equatorial part of 10 fruits in each treatment. A hand penetrometer (4301, Instron, USA) with a 7.9 mm tip was then used, and the force required to pierce the fruit was expressed in Newton (N) [Kılıç et al. 1991]. The water soluble solids content (SSC) was determined by shredding 10 fruit slices from each treatment in each replicate with an electric mixer, and the juice obtained was passed through cheesecloth. A sufficient volume of juice sample was collected, and the refractive index was measured using a digital refractometer (PAL-1, McCormick Fruit Tech, Yakima, USA). The results were expressed as a percentage. Titratable acidity was determined by diluting 10 mL of the juice sample with 10 mL of distilled water and titrating with 0.1 N sodium hydroxide (NaOH) until the pH reached 8.1. The results were expressed as citric acid (g citric acid 100 mL^{-1}) based on the amount of NaOH consumed in the titration. The extraction of vitamin C from 25 g of tomato fruits was achieved through a blending process involving 25 mL of oxalic acid (0.4%) with a blender, followed by filtration through filter paper. The amount of vitamin C (L-ascorbic acid) in the samples taken from this filtrate was measured with 2,6-dichloroindophenol using the titrimetric method AOAC [1995] at a wavelength of 518 nm in a spectrophotometer. The results were given as milligrams of vitamin C per 100 g of wet weight. The quality characteristics of the fruit were evaluated in samples obtained from the fourth to sixth cluster, three months after the seedlings were planted.

Statistical analysis

The study comprised four treatments: three biological fertilizers (*Aspergillus oryzae*, *Arthrobacter globiformis*, *Streptomyces griseus*) and one control. Experiments were conducted in a randomized plot design with three plot and with 18 plants in each replication. In total, 54 plants were measured for each treatment. The normality assumption of the data was examined using the Shapiro-Wilk test, and it was determined that the data met the normality assumption ($p > 0.05$). Furthermore, the Levene's test for variance homogeneity revealed that the variances were homogeneous ($p > 0.05$). According to these conditions, it was determined that the data were suitable for variance analysis, and statistical analyses were performed according to one-way analysis of variance (One Way ANOVA). Differences between groups were determined using the Duncan multiple comparison test. The OMU licensed SPSS 21 package programme was used in the analysis of the data.

RESULTS

The present study investigates the effects of different bio-fertilizers (Fungus: *Aspergillus oryzae* and Bacteria: *Arthrobacter globiformis*, *Streptomyces griseus*) on plant height, stem diameter, number of leaves and root dry weight in soilless tomato cultivation (Table 3). According to the results obtained, the effects of treatments on number of leaves and root dry weight were found to be significant ($p < 0.05$). While the highest root dry weight (20.3 g was obtained from

Table 3. The effect of biological fertilization with pure cultures of *Aspergillus oryzae, Arthrobacter globiformis* and *Streptomyces griseus* on tomato plant height, stem diameter, number of leaves and root dry weight

Applications	Plant height (cm)	Stem diameter (cm)	Number of leaves	Root dry weight (g)
Control	734.7	1.47	87.7 b	16.4 ab
Aspergillus oryzae	746.7	1.50	93.3 ab	11.8 b
Arthrobacter globiformis	753.5	1.53	97.5 a*	20.3 a*
Streptomyces griseus	758.0	1.53	94.3 ab	13.6 ab
SEM	*5.03*	*0.20*	*1.87*	*1.08*

Notes: Means in the same column followed by different letters were significantly different (* $p < 0.05$); SEM: standard error of the means

the *A. globiformis* bio-fertilizer application, it was determined that the control application with *S. griseus* was also in the same group. The application of *A. globiformis* resulted in the highest number of leaves (97.5). The lowest number of leaves was recorded in the control application, with a measurement of 87.7. While no statistical difference was detected, bio-fertilizers performed better than the control group on plant height and stem diameter, and bacterial treatment was more effective than fungal treatment. However, root dry weight values were similar between the bacterial and control groups. The study concluded that the effect of biological fungal fertilizers on root dry weight was limited.

It was determined that different bio-fertilizer (fungi and bacteria) applications had significant ($p < 0.05$) effects on the number of cluster, number of fruits in cluster and yield values in soilless tomato cultivation (Table 4). The *Streptomyces griseus* application demonstrated the highest number of cluster (28.3) and number of fruits per cluster (6.63). However, no significant differences were observed among the other bio-fertilizer applications. The lowest values were obtained from the control application. The *Streptomyces griseus* application yielded the highest yield of 58.6 t da^{-1}, while the *Aspergillus oryzae* application achieved similar values of 53 t da^{-1}. The lowest recorded yield values were measured at 47.6 t da^{-1} in the control application. No statistical differences were determined for the average fruit diameter and average fruit weight values.

The impact of diverse bio-fertilizers on fruit color (Chroma and Hue°), soluble solids content (SSC), titratable acidity and ascorbic acid was found to be sig-

Table 4. The effect of biological fertilization with pure cultures of *Aspergillus oryzae, Arthrobacter globiformis* and *Streptomyces griseus* on tomato cluster number, number of fruits per cluster, average fruit diameter, average fruit weight and yield in soilless tomato cultivation

Applications	Cluster number	Number of fruits per cluster	Average fruit diameter (cm)	Average fruit weight (g)	Yield (t da^{-1})
Control	26.3 b	3.38 b	6.63	116.8	47.6 b
Aspergillus oryzae	28.0 a	4.13 a	5.72	117.6	53.0 ab
Arthrobacter globiformis	27.3 ab	4.29 a	5.48	98.6	40.4 b
Streptomyces griseus	28.3 a*	4.63 a*	6.42	125.4	58.6 a*
SEM	*0.58*	*3.54*	*1.87*	*5.94*	*928.4*

Notes: as in Table 3

Table 5. The effect of biological fertilization with pure cultures of *Aspergillus oryzae, Arthrobacter globiformis* and *Streptomyces griseus* on tomato firmness, titratable acidity, SSC and vitamin C in soilless tomato cultivation

Applications	Chroma	Hue°	Firmness (N)	Titratable acidity (g citric acid 100 mL^{-1})	SSC (%)	Vitamin C (mg 100 g^{-1})
Control	25.6	55.7 a*	17.74	0.21 b	4.25 c	23.1 a*
Aspergillus oryzae	23.1	51.9 b	18.15	0.24 b	4.48 bc	19.3 b
Arthrobacter globiformis	26.7	54.2 ab	18.93	0.26 a	4.65 b	15.5 c
Streptomyces griseus	26.9	54.1 ab	19.82	0.28 a*	4.85 a*	11.8 d
SEM	*4.72*	*2.31*	*0.78*	*0.012*	*0.05*	*0.01*

Notes: as in Table 3; SSC: soluble solids content

nificant ($p < 0.05$). The most effective coloration was observed in the bio-fertilizer treatments, while *Aspergillus oryzae* bio-fertilizer application was notable for its impact on SCC (4.85%) and titratable acidity (0.28 g citric acid 100 mL^{-1}). Similar values were obtained in titratable acid values with *Arthrobacter globiformis* bio-fertilizer application of 0.26 g citric acid 100 mL^{-1}. The highest vitamin C content (23.07 mg 100 g^{-1}) was determined in the control treatment (Table 5).

DISCUSSION

The microbial content and diversity of the plant root zone (rhizosphere) plays a pivotal role in the suppression of soil-borne plant pathogens, thereby enhancing the natural suppressive capacity of the soil. Indeed, the rhizosphere is home to a plethora of species with beneficial properties that influence plant growth. Consequently, the rhizosphere provides plant health and protection against harmful soil-borne plant pathogens [Mendes et al. 2011, 2013, Anzalone et al. 2022]. The use of fungal and bacterial microorganisms in the root zone for biocontrol purposes is emerging as a new approach to combating diseases. Various *Bacillus* and *Trichoderma* species with strong biocontrol potential are widely used to control many plant diseases in tomatoes [Mazrou et al. 2020, Stracquadanio et al., 2020, Castro-Restrepo et al. 2022, Narware et al. 2023, Abo-Elyousr et al. 2022, Imran et al. 2023].

In a study investigating the effects of root microbiome in soilless tomato cultivation in greenhouses, microorganisms (bacteria and fungi) taken from soil were applied to cocopeat media. It has been determined that fungi form a more effective growth and network in the root zone, while the amount and diversity of bacteria decrease over time. Nevertheless, a multitude of bacterial species have been identified as exerting a favourable influence on biological control and root development of the plant [Jacobsen et al. 2004, Anzalone et al. 2022]. In the present study, it was established that the application of bacteria led to an augmentation in root dry weight. In many studies carried out on soilless tomato cultivation, it has been determined that similar results were obtained in growth and yield values when the same growing periods were considered [Öztekin et al., 2017, Orta-Guzmán 2021, Cela et al. 2024, Erdal et al. 2024]. In a separate study, it was reported that bacterial applications yielded higher values than mineral fertilizers and fungal biological fertilizers [Dasgan et al. 2023]. The present study investigated the effects of two different microbial fertilizers on the cultivation of soilless lettuce (*Lactuca sativa* L.) and celery (*Apium graveolens* L.). The findings demonstrated that microbial fertilizer applications led to substantial enhancements in several key parameters, including aboveground fresh weight, belowground fresh weight, root length, leaf length, and leaf number, in both lettuce and celery. Furthermore, Wang et al. [2023] reported significant enhancements in root activity, net photosynthesis rate, stomatal conductance and total chlorophyll content in both lettuce and celery. The study demonstrated that bio-fertilizers promote leaf photosynthesis by enhancing root development and root nutrient uptake [Wang et al. 2023].

The present study set out to investigate the effects of bio-fertilizer application on tomato yield in hydroponic systems. The study established that the application of bio-fertilizer (0%, 25%, 50%, 75% and 100%) in conjunction with the control (100% inorganic fertilizer) led to an augmentation in the population of endophytic bacteria, *Azotobacter* sp., *Azospirillum* sp., phosphate solubilizing bacteria and nitrogen content. It was determined that different bio-fertilizer combinations did not change the phosphorus and potassium content compared to the control, but increased fruit quality. In particular, the combination of inorganic fertilizer and bio-fertilizer resulted in a significant increase in fruit weight compared to the control group [Setiawati et al. 2023]. Soilless systems represent a popular cultivation technique that aims to maximize plant productivity whilst minimizing resource use. Nevertheless, the absence of a soil matrix gives rise to challenges that necessitate precise management of nutrients, effective control of salinity stress and proactive strategies to specialize in disease management. The utilization of plant growth-promoting microorganisms has emerged as a promising solution to address these challenges. The utilization of microbial inoculation in soilless growing systems has been demonstrated to enhance nutrient management, disease control and the mitigation of salinity issues [Mourouzidou et al. 2023]. A study was conducted in which four distinct treat-

ments were investigated: 1) poultry manure, 2) poultry manure + effective microorganisms, 3) leaf compost, 4) leaf compost + effective microorganisms. These treatments were applied in soilless tomato cultivation using an organic substrate and effective microorganisms. It is stated that the application of microorganisms in combination with different animal manures has a significant effect on yield and quality [Sajid et al. 2023].

In a study comparing organic and chemical fertilizers in soilless tomato cultivation, organic and chemical fertilization were used to determine the values of SCC (organic: 5.9% and chemical: 5.9%), titratable acidity (organic: 1.5 mg/100 g and chemical: 1.3 mg/100 g) and vitamin C (organic: 15.3 mg/100 mL and chemical: 23.1 mg/100 mL) [Bozköylü and Daşgan 2010]. The study revealed significant variations in vitamin C levels in organic and chemical fertilizations, with comparable outcomes observed in our study. In a separate study, the effects of various organic and inorganic growing media on soilless tomato cultivation were investigated, and fruit flesh firmness was measured as 11.96–12.46 N, SCC 3.75–4.01%, titratable acidity 2.92–3.85%, and vitamin C 12–20 mg/100 g [Tzortzakis and Economakis 2008]. In a study investigating the effects of growing media as an alternative to coconut fiber in soilless tomato cultivation, the values of 5.2–5.9% titratable acidity and 0.41–0.52 mg/100 g vitamin C were determined [Kartal and Geboloğlu 2023]. In a separate study investigating the effects of different growing media (perlite and cocopeat) on tomato yield and fruit quality in soilless tomato cultivation, chroma (32–39), hue° (61–62), titratable acidity (5.1–6.9 mval/100 mL), SCC (4.5–5.58%) and vitamin C (21–22.6 mg/100 mL) values were determined [Kartal and Geboloğlu 2023]. In the study, cocopeat was found to yield superior results in terms of quality [Toprak and Gül 2013]. In the study in which effective microorganisms were used in soilless tomato cultivation, the values of SSC ranged between 3.90% and 4.56%, with the highest value being determined with microorganism application [Sajid et al. 2023]. In the present study, titratable acidity values, which have been demonstrated to exert significant influence on fruit quality, taste and aroma in soilless tomato cultivation with the use of different bio-fertilizers, yielded analogous results with the exception of titratable acidity values.

CONCLUSION

In our study, it was determined that the use of commercial bio-fertilizers of different origin (fungi and bacteria) significantly affected the yield and quality in soilless tomato cultivation. The highest yield (58.6 t da^{-1}) and fruit quality (SSC = 4.85%, acidity = 0.28 g citric acid 100 mL^{-1}) values were obtained from the *Streptomyces griseus* application. It is a bacterial bio-fertilizer-derived from *Streptomyces griseus*. The study concludes that microbial inoculation enhances yield and quality in soilless tomato cultivation. This is supported by the fact that another bacterial biological fertilizer, *Arthrobacter globiformis* significantly increased (20.3 g) root dry weight. Bacteria-based biological fertilizers have been demonstrated to promote plant growth, with a particular emphasis on root development. Furthermore, it is acknowledged that these fertilizers assist plants in combating diseases by counteracting pathogens. Consequently, the utilization of microorganisms as fertilizers will emerge as a pivotal alternative for transitioning to a sustainable and environmentally friendly production model in soilless tomato cultivation.

The incorporation of plant growth-promoting microorganisms into the growing medium has emerged as a promising solution to address the existing challenges in soilless vegetable cultivation. Future studies should therefore concentrate on increasing the diversity of microorganisms in growing media and on the use of microorganisms with high adaptation to saline conditions. In addition, it is known that the use of organic growing media helps microorganisms to live and multiply. Therefore, the use of different plant residues that could be alternatives to cocopeat substrate could be an important step towards zero waste.

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ROLE OF MUSHROOM SUBSTRATE IN THE TRANSFER OF FUNGICIDE RESIDUES TO *Agaricus bisporus*

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ABSTRACT

White button mushroom (*Agaricus bisporus*) is a significant component of the human diet due to its nutritional value, which includes digestible proteins, dietary fiber, phenolic compounds, and B-group vitamins. In recent years, interest in organic agricultural products has increased, accompanied by growing demands for food quality and safety. Organically cultivated edible fungi, produced without the use of plant protection products, are generally expected to be safe and health-promoting. However, despite the application of organic production methods, the presence of pesticide residues in mushrooms remains possible, thus requiring comprehensive analysis.

This study aimed to evaluate the presence of plant protection products residues in mushroom fruiting bodies cultivated using substrates from conventional and organic sources. A total of 158 mushroom samples and 128 substrate samples were analysed using QuEChERS-based methods to assess whether the growing substrate facilitates the pesticide transfer to mushrooms. The results revealed significant differences between organic and conventional mushrooms. However, residues in mushrooms were detected even without the direct application of pesticides during cultivation, indicating that chemical substances can be transferred from the substrate to fruiting bodies. The presence of residues in mushrooms labeled as organic suggests that substrate contamination is an underestimated pathway for pesticide transfer. The findings emphasize the need for more precise regulation of substrate materials employed in mushroom cultivation, particularly within organic systems, to ensure food safety and compliance with residue limits. Monitoring mushroom substrates is crucial for developing sustainable and safe agricultural practices.

Keywords: edible fungi, food safety, organic cultivation, plant protection products, pesticide transfer, QuEChERS-based methods

INTRODUCTION

The white button mushroom (*Agaricus bisporus*) is one of the most commonly consumed mushrooms in the world. Poland is the leader in mushroom production in Europe, with annual production exceeding 330,000 tons, 70% of which is exported [Siwulski et al. 2022, Smoleński 2022]. The production of organic mushrooms, like other vegetable crops, constitutes a relatively small percentage of the total output. However, this market demonstrates a consistent growth [Weber and Skorbiansky 2023]. These mushrooms

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are distinguished not only by their taste and availability, but also by their high nutritional value. They are a source of easily digestible protein, fibre, vitamins (mainly from the B group, but also D, E and K), microelements (Se, K, Cu) and bioactive compounds such as ergothioneine and polysaccharides with antioxidant, immunostimulating, antidiabetic and anticancer properties [Golak-Siwulska et al. 2018, Mattila et al. 2001, Muszyńska et al. 2017, Sidor 2019].

One of the most significant factors affecting human health and well-being is proper nutrition, including the quality of the food products consumed. The increasing demand for organic food has resulted in greater attention to food safety and the absence of chemical contaminants. However, the intensification of agricultural practices to achieve high yields of qualified plants has increased the need for chemical crop protection, thereby resulting in the growing importance of assessing food of plant origin for fungicide residues [Bursić et al. 2021, European Food Safety Authority 2019, Gomiero 2018].

Organic mushroom cultivation involves the exclusion of plant protection products from the production process. Nevertheless, the risk of their presence can be attributed to the use of raw materials in the production of mushroom substrate. There is a significant knowledge gap concerning the migration of pesticide residues from the substrate to the *A. bisporus* fruiting bodies, despite the recognised possibility of such penetration [European Food Safety Authority 2016, 2019, Goglio et al. 2024]. The substrate for mushroom cultivation is composed of high-quality cereal straw (mainly wheat and triticale), poultry manure, gypsum, and water [Tello Martin et al. 2022, Wang et al. 2023]. Straw is derived from crops, which are frequently cultivated using conventional methods. Despite the continuous use of fungicides in intensive wheat production, research on their potential impact on mushroom cultivation remains limited. Fungicide applications during wheat cultivation can result in residues in grain or straw. Current knowledge on how fungicides used in wheat cultivation may affect mushroom production is still incomplete. However, there is a risk that residues of growth regulators, fungicides, and other pesticides may end up in the substrate, even though the mushroom cultivation process itself is carried out without their use [Chalaux et al. 1993, Tello Martin et al. 2022, McGee 2018]. Consequently, the fruiting bodies may become contaminated via the substrate. In the context of organic mushroom production, raw materials must meet specific criteria and possess the necessary certificates that attest to their suitability for organic farming, as outlined in the general regulations for organic products standardized by Commission Regulation (EC) no 149/2008.

Accordingly, it is imperative that straw and chicken droppings must be sourced from certified farms, free from pesticide residues and cereal growth regulators. Gypsum used to reduce substrate pH, must be uncontaminated and produced without involving power plant processes for purifying exhaust gases from sulfur. An annual audit is conducted by the relevant certification body to verify compliance with the established standards for the production of organic substrate for mushroom cultivation. The responsibility for regulating the reliability of inspection bodies is held by the Inspection of Trade Quality of Agricultural and Food Products, operating under the authority of the Ministry of Agriculture and Rural Development [Regulation (EU) 2018/848].

In recent years, the presence of pesticide residue has attracted particular attention from food safety authorities. In this regard, the European Union has introduced a series of regulations and legislation, including Commission Regulation (EC) no 149/2008 and the Food and Nutrition Safety Act, which obliges all Member States to adhere to comprehensive food control measures. These measures focus on the presence of contaminants, including pesticides, in food products. The European Union has set maximum permissible levels for these compounds, also known as MRLs (maximum residue levels), which are specific to food products of plant origin, including mushrooms [Commission Regulation (EC) 149/2008, Stachniuk and Fornal 2016].

The objective of the present study was to assess the presence of pesticide residues in both the substrate and the fruiting bodies of *A. bisporus* from organic and conventional crops. The study particularly emphasized the presence of cereal growth regulators, such as chlormequat chloride and mepiquat chloride, as well as fungicides used in cereal cultivation. Additionally, the investigation involved the analysis of pesticides applied in mushroom cultivation.

MATERIAL AND METHODS

Analyzed samples

Samples of mushroom substrate and *A. bisporus* fruiting bodies were obtained from production facilities located in various regions of the country during the period 2021–2024. The samples analysed in this study were obtained from crops cultivated using conventional and organic methods (Table 1). The weight of each sample was 1 kg.

The samples were submitted to the Food Safety Laboratory (FSL) within 24 hours of collection and were assessed as undamaged and suitable for pesticide residue testing. The samples were immediately cooled to –18 °C and ground using dry ice. All procedures related to sample preparation and analysis were consistent with generally accepted procedures and guidelines for official laboratories in the EU [Document Nº SANTE/11312/2021v2 2021].

Analytical methods

To assess pesticide residues in samples tested, the following research methods were employed:

QuEChERS multiresidue method. This method is based on the EN 15662:2018 standard [European Committee for Standardization 2018]. In general, the method consists of using QuEChERS extraction with acetonitrile, followed by purification of the extracts using dispersive solid-phase extraction (d-SPE), and then subjecting the extracts to analysis using gas and liquid chromatography coupled with tandem mass spectrometry. The detailed procedure is as follows: 10 g of a soil sample was weighed into a 50 mL Falcon tube, and 10 mL of acetonitrile was added. The mixture was then vortexed for approximately 3 min. Then, a mixture of salt and extraction buffer (4 g of anhydrous magnesium sulfate, 1 g of sodium chloride, 1 g of sodium citrate dihydrate, and 0.5 g of sodium hydrogen citrate) was added and shaken for 3 min. The sample was centrifuged using a laboratory centrifuge for 5 min (7200 rpm, room temperature), and 1 mL of the supernatant was transferred to an Eppendorf centrifuge tube containing the purification mixture (150 mg of magnesium sulfate and 25 mg of PSA) and shaken for approximately 1 min. The sample was centrifuged using an Eppendorf MiniSpin centrifuge for 1 min. (8500 rpm, room temperature). The extracts prepared in this way were subjected to further analysis using two chromatographic techniques: gas and liquid chromatography.

For gas chromatography, 1 mL of the extract was transferred to an autosampler cup. Then, 50 µL of internal standard, 100 µL of acetonitrile, and 30 µL of

Table 1. Origin of mushroom substrates and fruiting body samples

Sample	Production category	Number of samples	Sampling voivodeship
Mushroom substrates	organic production	41	łódzkie (substrate producer)
		19	łódzkie (mushroom producer)
		2	wielkopolskie (mushroom producer)
	conventional production	23	łódzkie (substrate producer)
		40	łódzkie (mushroom producer)
		3	mazowieckie (mushroom producer)
Mushroom fruiting bodies	organic production	41	łódzkie (mushrooms producer)
		2	wielkopolskie (mushroom producer)
		15	wielkopolskie (market)
	conventional production	68	łódzkie (mushroom producer)
		2	wielkopolskie (mushroom producer)
		6	mazowieckie (mushroom producer)
		14	łódzkie (market)
		10	mazowieckie (market)

Table 2. Scope of substances analyzed in analytical methods

Method	Substances analyzed
QuEChERS Multiresidue (499 substances)	2-phenylphenol, abamectin, acephate, acetamiprid, acetochlor, aclonifen, acrinathrin, alachlor, aldicarb, aldicarb sulfone, aldicarb sulfoxide, aldrin, allethrin, ametoctradin, ametryn, amidosulfuron, aminocarb, amisulbrom, anthraquinone, atrazine, azaconazole, azadirachtin, azinphos-ethyl, azinphos-methyl, aziprotryne, azoxystrobin, beflubutamid, benalaxyl, bendiocarb, benfluralin, benfuracarb, benthiavalicarb isopropyl, benzovindiflupyr, bifenazate, bifenazate diazene, bifenox, bifenthrin, biphenyl, bitertanol, bixafen, boscalid, bromacil, bromfenvinphos, bromocyclen, bromophos, bromophos-ethyl, bromopropylate, bromuconazole, BTS 44595, BTS 44596, bupirimate, buprofezin, butachlor, butafenacil, butylate, cadusafos, captafol, captan, carbaryl, carbendazim, carbetamide, carbofuran, carbofuran-3-hydroxy, carbofuran-3-keto, carboxin, carfentrazone-ethyl, chinomethionat, chlorantraniliprole, chlorbenside, chlorbufam, chlordane,-cis, chlordane,-oxy, chlordane,-trans, chlorfenapyr, chlorfenson, chlorfenvinphos, chloridazon, chlormephos, chlorobenzilate, chloropropylate, chlorothalonil, chlorotoluron, chlorpropham, chlorpyrifos, chlorpyrifos-methyl, chlorsulfuron, chlorthal-dimethyl, chlorthiophos, chlothion, chromafenozide, clodinafop-propargyl, clofentezine, clomazone, clothianidin, coumaphos, crimidine, cyanazine, cyanophenphos, cyanophos, cyantraniliprole, cyazofamid, cycloate, cycloxydim, cyflufenamid, cyflumetofen, cyfluthrin, cymiazol, cymoxanil, cypermethrin, cyprazine, cyproconazole, cyprodinil, DDD-o,p', DDD-p,p', DDE-o,p', DDE-p,p', DDM, DDT-o,p', DDT-p,p', DEET, deltamethrin, demeton-S, demeton-S-methyl, demeton-S-methyl sulphone, demeton-S-methyl sulfoxide, desmedipham, desmetryn, dialifos, diazinon, dichlobenil, dichlofenthion, dichlofluanid, dichloroaniline 3,5-, dichlorobenzamide 2,6-, dichlorobenzophenone-p,p, dichlorvos, diclobutrazol, dicloran, dicofol, dicrotophos, dieldrin, diethofencarb, difenoconazole, diflubenzuron, diflufenican, dimethachlor, dimethenamid, dimethoate, dimethomorph, dimoxystrobin, diniconazole, dinitramine, dinobuton, dinoseb, dinotefuran, dioxabenzofos, dioxacarb, dioxathion, diphenylamine, disulfoton, disulfoton sulfon, disulfoton sulfoxide, ditalimfos, diuron, DMF, DMPF, DMST, dodemorph, edifenphos, emamectin B1a, emamectin B1b, endosulfan alpha, endosulfan beta, endosulfan sulphate, endrin, endrin keton, EPN, epoxiconazole, esfenvalerate, etaconazole, ethalfluralin, ethametsulfuron-methyl, ethiofencarb, ethion, ethirimol, ethofumesate, ethofumesate-2-keto, ethoprophos, ethoxyquin, ethylan, etofenprox, etoxazole, etrimfos, famoxadone, fenamidone, fenamiphos, fenamiphos sulfoxide, Fenamiphos sulphone, fenarimol, fenazaquin, fenbuconazole, fenchlorphos, fenfuram, fenhexamid, fenitrothion, fenobucarb, fenoxaprop-P-ethyl, fenoxycarb, fenpropathrin, fenpropidin, fenpropimorph, fenpyrazamine, fenpyroximate, fensulfothion, fensulfothion oxon, fensulfothion oxon sulphone, fensulfothion sulphone, fenthion, fenthion oxon, fenthion oxon sulphone, fenthion sulfoxide, fenthion sulphone, fenvalerate, fipronil, fipronil desulfinyl, fipronil sulfon, flazasulfuron, flonicamid, florasulam, fluchloralin, flucythrinate, fludioxonil, fluensulfone, flufenacet, flufenoxuron, flumetralin, flumioxazin, fluopicolide, fluopyram, fluorodifen, fluotrimazole, fluoxastrobin, flupyradifurone, fluquinconazole, flurochloridone, flurprimidol, flurtamone, flusilazole, flutianil, flutolanil, flutriafol, fluxapyroxad, folpet, fonofos, foramsulfuron, formetanate, formothion, fosthiazate, fuberidazole, furalaxyl, furathiocarb, gamma-cyhalothrin, halfenprox, halofenozide, heptachlor, heptachlor cis-epoxid isomer B, heptachlor trans-epoxid isomer A, heptenophos, hexachlorobenzene, hexachlorocyclohexane HCH alpha, hexachlorocyclohexane HCH beta, hexaconazole, hexaflumuron, hexythiazox, imazalil, imazapic, imidacloprid, indoxacarb, iodofenphos, iodosulfuron methyl, ipconazole, iprobenfos, iprodione, iprovalicarb, isocarbophos, isofenphos, isofenphos-methyl, isofetamid, isoprocarb, isoprothiolane, isoproturon, isopyrazam, isoxaben, isoxaflutole, isoxathion, kresoxim-methyl, lambda-cyhalothrin, lenacil, lindane, linuron, lufenuron, malaoxon, malathion, mandestrobin, mandipropamid, mecarbam, mepanipyrim, mepronil, metaflumizone, metalaxyl, metamitron, metazachlor, metconazole, methacrifos, methamidophos, methidathion, methiocarb, methiocarb sulphone, methiocarb sulphoxide, methomyl, methoprotryne, methoxychlor, methoxyfenozide, metobromuron, metolachlor, metolachlor-S, metosulam, metoxuron, metrafenone, metribuzin, metsulfuron-methyl, mevinphos, molinate, monocrotophos, monuron, myclobutanil, napropamide, nicosulfuron, nitenpyram, nitralin, nitrapyrin, nitrofen, nitrothal isopropyl, novaluron, nuarimol, omethoate, oxadiazon, oxadixyl, oxamyl, oxycarboxin, oxyfluorfen, paclobutrazol, paraoxon-methyl, parathion, parathion-methyl, penconazole, pencycuron, pendimethalin, penflufen, pentachloroaniline, penthiopyrad, permethrin, pethoxamid, phenmedipham, phenthoate, phorate, phorate sulfone, phorate sulfoxide, phosalone, phosmet, phosmet oxon, phosphamidon, phoxim, phthalimide, picolinafen, picoxystrobin, pinoxaden, piperonyl butoxide, piperophos, pirimicarb, pirimicarb desmethyl, pirimiphos-ethyl, pirimiphos-methyl, prochloraz, procymidone, profenofos, profluralin, prometon, prometryn, propachlor, propamocarb, propaquizafop, propargite, propazine, propetamphos, propham, propiconazole, propoxur, propoxycarbazone, propyzamide, proquinazid, prosulfocarb, prosulfuron, prothioconazole destio, prothiofos, pymetrozine, pyraclostrobin, pyrazophos, pyrethrins, pyridaben, pyridafol, pyridalyl, pyridaphenthion, pyrifenox, pyrimethanil, pyriofenone, pyriproxyfen, pyroquilon, pyroxsulam, quinalphos, quinclorac, quinoclamine, quinoxyfen, quintozene, quizalofop-ethyl, resmethrin, rimsulfuron, rotenone, saflufenacil, silafluofen, silthiofam, simazine, spinetoram c42, spinetoram c43, spinosyn a, spinosyn d, spirodiclofen, spiromesifen, spirotetramat, spirotetramat enol, spirotetramat enol-glucoside, spirotetramat ketohydroxy, spirotetramat monohydroxy, spiroxamine, sulfometuron methyl, sulfosulfuron, sulfotep, sulfoxaflor, tau-fluvalinate, tebuconazole, tebufenozide, tebufenpyrad, tecnazene, teflubenzuron, tefluthrin, tepraloxydim, terbacil, terbufos, terbufos oxon, terbufos sulphone, terbufos sulphoxide, terbuthylazine, terbutryn, tetrachlorvinphos, tetraconazole, tetradifon, tetrahydrophthalimide, tetramethrin, tetrasul, thiabendazole, thiacloprid, thiamethoxam, thiencarbazone-methyl, thifensulfuron-methyl, thiobencarb, thiodicarb, thiometon, thiophanate-methyl, tolclofos-methyl, tolfenpyrad, tolylfluanid, topramezone, tralkoxydim, triadimefon, triadimenol, tri-allate, triazophos, trichlorfon, tricyclazole, tridemorph, trifloxystrobin, triflumizole, triflumuron, trifluralin, triflusulfuron, triticonazole, tritosulfuron, vinclozolin, zoxamide
QuPPe-PO method	chlormequat, chlorates, cyromazine, fosetyl-al, mepiquat, perchlorates, phosphonic acid

the protector working solution (AP-MIX) were added. Extracts were analyzed using highly selective gas chromatography coupled with tandem mass spectrometry (GC-MS/MS). An Agilent Technologies 7890A gas chromatograph equipped with a 7000 Triple Quad GC/MS mass detector was used for the analyses. Compound separation was performed on a DB-5MS capillary column (30.0 m × 250 µm × 0.25 µm). Identification and quantification were performed using the Multiple Reaction Monitoring technique.

For liquid chromatography, 200 µL of the extract was transferred to an Eppendorf tube containing 700 µL of solvent A. Then, 50 µL of internal standard and 50 µL of acetonitrile were added. The whole mixture was filtered into an autosampler cup. The analysis was performed using highly selective liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). An Agilent Technologies 1200 Series liquid chromatograph equipped with a 6410 Triple Quad LC/MS mass detector was used. Compound separation was performed on an Agilent Eclipse Plus, C18, 2.1 × 100 mm, 1.8 µm column. Two solvents: A – water and 5 mM ammonium formate and 0.01% v/v formic acid, and B – water: acetonitrile (5:95) and 5 mM ammonium formate and 0.01% v/v formic acid were used. Measurements were made using an ESI ion source in positive polarity using the Dynamic Multi Reaction Monitoring ion scanning mode. The scope of the analyzed substances within the multi-residue method for the GC-MS/MS and LC-MS/MS techniques is described in Table 2. All the substances analyzed by the multi-residue method were detected at the 0.01 or 0.005 mg kg^{-1} limit of quantification (LOQ).

QuPPe-PO single residue method. The method consists of several variants, as described in guidelines published by EU Reference Laboratories [Anastassiades et al. 2023]. A method is described for the residue analysis of highly polar, non-QuEChERS-amenable pesticides. Residues were extracted from the test portion after adjusting the water content and acidifying with 1% formic acid in methanol. The mixture was centrifuged, filtered, and directly analyzed using liquid chromatography coupled with tandem mass spectrometry:

– Agilent Technologies 1260 Series liquid chromatograph equipped with a 6460 Triple Quad LC/MS mass detector. Compound separation was performed on a Zorbax Hilic Plus, 2.1 × 100 mm, 1.8 µm column. Two solvents: A – 20 mM ammonium formate with 0.36% (v/v) formic acid in water, and B – acetonitrile were used. Measurements were performed using an ESI ion source in negative polarization using the Multiple Reaction Monitoring ion scanning mode. The method was used for the determination of chlormequat chloride, mepiquat chloride, and cyromazine;

– Agilent Technologies 1290 Infinity II Series liquid chromatograph equipped with a 6470B Triple Quad LC/MS mass detector. Compound separation was performed on a Thermo Scientific Hypercarb, 2.1 × 100 mm, 5 µm column. Two solvents: A – 1% acetic acid in water, and B – 1% acetic acid in methanol and 10% water were used. Measurements were performed using an ESI ion source in negative polarization using the Multiple Reaction Monitoring ion scanning mode. The method was used for the determination of chlorates, fosetyl-Al, perchlorates, and phosphonic acid.

Quantification was performed employing isotope-labeled analogs of the target analytes as internal standards. These were added directly to the test portion at the beginning of the procedure to compensate for any factors influencing the recovery rates, such as volume deviations, analyte losses during sample preparation, and matrix effects during measurement.

Statistical methods

Statistical analysis was performed by using MS Excel 2019 with the Analysis ToolPak add-in. Pesticide residue data obtained from conventional and organic samples of substrates and mushrooms over a four-year period were analyzed. For each year, the percentage of samples containing particular active substances was calculated. Differences between residue levels in conventional and organic substrates and fruiting bodies were evaluated using Student's t-test with a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

Mushroom substrate from organic cultivations

The study involved the assessment of 62 samples of organic substrate, of which 21 samples (34.1%) were free from chemical residues (Fig. 1). Approximately 20% of the samples contained a single substance, 24% contained two substances, and 11% contained three

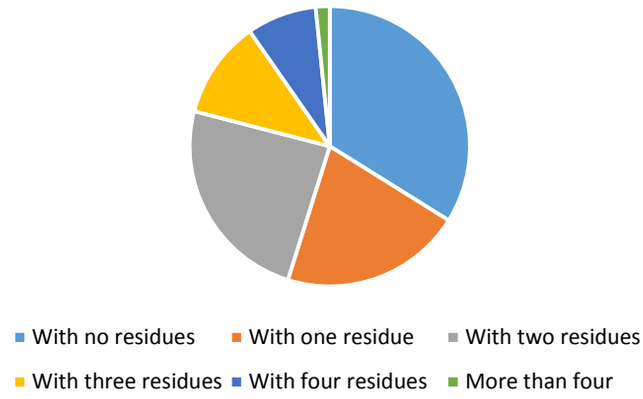

Fig. 1. Percentage of organic mushroom substrate samples with chemical residues

Table 3. Number of organic mushroom substrate samples containing chemical residues in the years 2021–2024

Year	2021	2022	2023	2024	Total
Number of samples	15	16	14	17	62
Chlormequat choride	2	4	–	1	7
Mepiquat chloride	–	–	4	–	4
Anthraquinone	–	1	2	–	3
Azoxystrobin	–	–	1	–	1
Benzovindyflupyr	–	–	1	–	1
Bromide ion	1	–	6	–	7
Chlorate	2	7	–	9	18
Epoxyconazole	–	1	–	–	1
Fluxapyroxad	2	–	–	–	2
Folpet	2	5	5	2	14
Melamine	–	–	–	7	7
Phthalimide	2	5	5	2	14
Tebuconazole	1	3	1	–	5
Tetraconazole	1	–	–	–	1
Samples without residues	9	5	3	4	21

substances. In the remaining samples, four chemical substances were detected.

Table 3 presents the chemicals detected in organic substrate samples from each year. In 2021, a total of 15 samples of mushroom substrate were analysed, of which 9 samples were free from any plant protection product residues. Two samples of substrate contained chlormequat chloride at a level of 0.007–0.012 mg kg^{-1}, while 5 samples contained other fungicides. In 2022, a total of 16 samples of organic substrate were analysed, and 5 samples did not contain any detected residues. The remaining samples contained various chemical substances. Four samples contained chlormequat chloride at 0.006–0.011 mg kg^{-1}, and 10 samples contained fungicides such as folpet and phthalimide (33% of samples) and tebuconazole (20%). Seven samples revealed the presence of chlorate residues. In 2023, 14 samples of organic substrate were analysed, of which three were free from any residues. Four samples contained mepiquat chloride at levels of

Table 4. Number of conventional mushroom substrate samples containing chemical residues in the years 2021–2024

Year	2021	2022	2023	2024	Total
Number of samples	11	18	22	15	66
Chlormequat choride	4	8	2	3	17
Mepiquat chloride	10	14	21	12	57
Azoxystrobin	5	7	5	8	25
Bixafen	–	–	–	2	2
Benzovindyflupyr	–	6	2	3	11
Bromide ion	–	–	9	2	11
Chlorate	1	6	11	5	23
Cypermethrin	1	1	2	1	4
Epoxyconazole	2	1	–	–	3
Fluxapyroxad	2	5	6	5	18
Folpet	3	9	15	5	32
Melamine	–	–	–	5	5
Phthalimide	2	9	15	5	31
Propiconazole	1	1	–	–	2
Pyraclostrobin	–	1	2	–	3
Tebuconazole	10	15	13	6	44
Tetraconazole	1	2	3	1	7
Triadimenol	3	–	–	–	3

0.005–0.052 mg kg^{-1}, as well as chemical products used in cereal protection (including bromide ion, phthalimide, and folpet). Seven samples contained residues of fungicides, mainly folpet, phthalimide, and bromide ion (54.5% of samples), as well as tebuconazole (27%). In 2024, 17 samples of organic substrate were analyzed, and of which 4 did not contain any residues. In one sample, residues of chlormequat chloride were detected at a level of 0.005 mg kg^{-1}, while in the remaining samples melamine and chlorate were identified.

The most frequently detected chemical substances in the tested organic mushroom substrate samples are presented in Figure 2. The samples were mainly contaminated with chlorates (29%), folpet and phthalimide (23%), and more than 11% contained bromide ion, melamine, and chlormequat chloride.

The frequency of detection of plant protection products residues and other substances in the substrate samples varied between years, indicating potential fluctuations in their utilisation or differences in persistence in raw materials. From 2021 to 2024, the percentage of residue-free substrate samples decreased, while the profiles of detected substances changed. A decrease in chlormequat chloride detection was observed in 2023, whilst detections of other pesticides and chlorates increased. Furthermore, over 20% of the samples were contaminated with chlorates, folpet, and phthalimide, while approximately 10% contained bromide ion and melamine.

Previous research has demonstrated that melamine residues are a prevalent occurrence in plant-based foods. Reports suggest that these may be from the metabolism of the triazine insecticide cyromazine or the triazine fungicide anilazine, or may originate from cyanamide fertilizer. The detection of melamine and its degradation products appears to be associated with the use of fertilisers containing cyanamide [Lütjens et al. 2023]. Nevertheless, the application of pesticides is prohibited in organic farming. Residues of these compounds, as well as others such as mepiquat chloride or chlormequat chloride, have been detected in mushroom substrates as a result of the use of fungicides and growth regulators in the protection of cereals [Chalaux

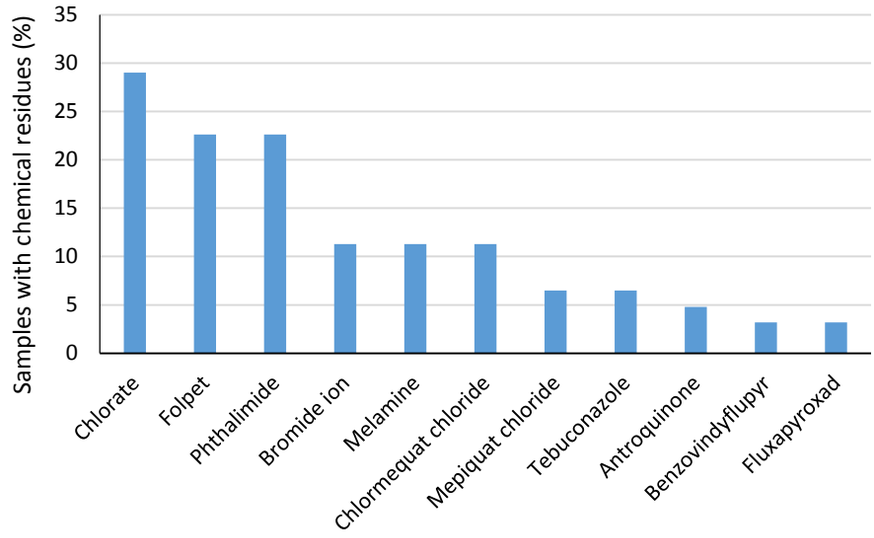

Fig. 2. Chemical residues detected in organic mushroom substrate samples

et al. 1993]. The detection of bromide ions in mushroom substrate derived from straw may be attributed to the natural bioaccumulation of bromine from the soil by wheat or other cereal crops, rather than to external contamination. Several studies have confirmed that plants can absorb bromide from the soil and water through natural physiological processes [Shtangeeva et al. 2019, Yamada 1968]. However, the application of bromine-containing pesticides in industry and agriculture resulted in an increase of bromide levels in soil. The process of bromine accumulation by plants is also influenced by the physicochemical properties of the soil [Shtangeeva 2017].

Mushroom substrate from conventional cultivations

A total of 66 samples of conventional mushroom substrate were examined. Only one sample was found to be free from chemical substances. The analysis revealed that in more than 45% of the samples, four or more of chemical substances were detected. Furthermore, 24% of the samples exhibited the presence of 4 substances, and 15% of samples contained 3 chemical substances. In the other substrate samples, 1 or 2 chemical substances were identified (Fig. 3).

Table 4 presents the number of conventional substrate samples collected each year, along with the chemicals detected. In 2021, residues of plant protection products were detected in all samples. Mepiquat chloride was detected in 10 samples, at concentrations from 0.006 to 0.029 mg/kg. Chlormequat chloride was identified in 3 samples. Furthermore, the presence of other fungicides in ten samples was detected, namely azoxystrobin, tebuconazole, folpet, phthalimide, fluxapyroxad, triadimenol, epoxiconazole, and propiconazole. In 2022, a total of 18 samples of conventional substrate were analyzed. Mepiquat chloride was detected in 14 samples, with concentrations ranging from 0.005 to 0.051 mg kg^{-1}, while chlormequat chloride was identified in eight samples. Furthermore, the following compounds were detected: tebuconazole, folpet, phthalimide, thiabendazole, azoxystrobin, benzovindiflupyr, cypermethrin, and fluxapyroxad. In 2023, mepiquat chloride was detected in 21 out of 22 samples of conventional mushroom substrate with concentrations ranging from 0.005 to 0.021 mg kg^{-1}. Two samples also contained chlormequat chloride. Folpet and phthalimide were the most frequently detected fungicides, followed by tebuconazole and chlorates. In 2024, analysis of 12 samples of conventional substrate revealed the presence of mepiquat chloride at levels of 0.008–0.014 mg kg^{-1} and chloride chlormequat at 0.006–0.009 mg kg^{-1} in 3 samples. Furthermore,

12 other chemical substances were identified in substrate samples with variable prevalence. The most frequently were: azoxystrobin, tebuconazole, folpet, phthalimide, fluxapyroxad and melamine.

Figure 4 shows the most frequently detected chemicals in the tested samples of conventional mushroom substrate. Almost 90% of the samples contained mepiquat chloride, over 60% contained tebuconazole, and more than 40% contained folpet and phthalimide. Azoxystrobin, chlorates, and chlormequat chloride were also detected in a significant proportion of samples.

Results of our study indicate that conventional mushroom substrates frequently exhibited the presence of multiple pesticide residues, with a higher probabili-

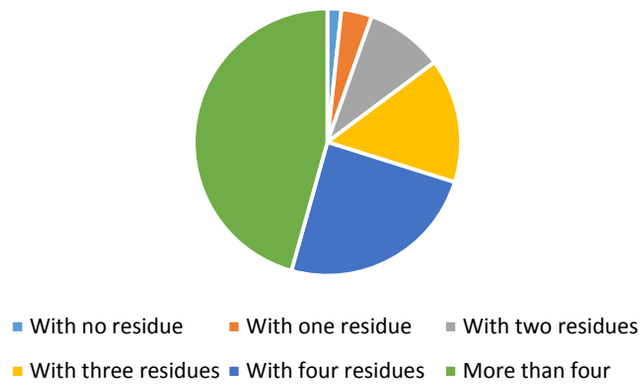

Fig. 3. Percentage of conventional mushroom substrate samples with chemical residues

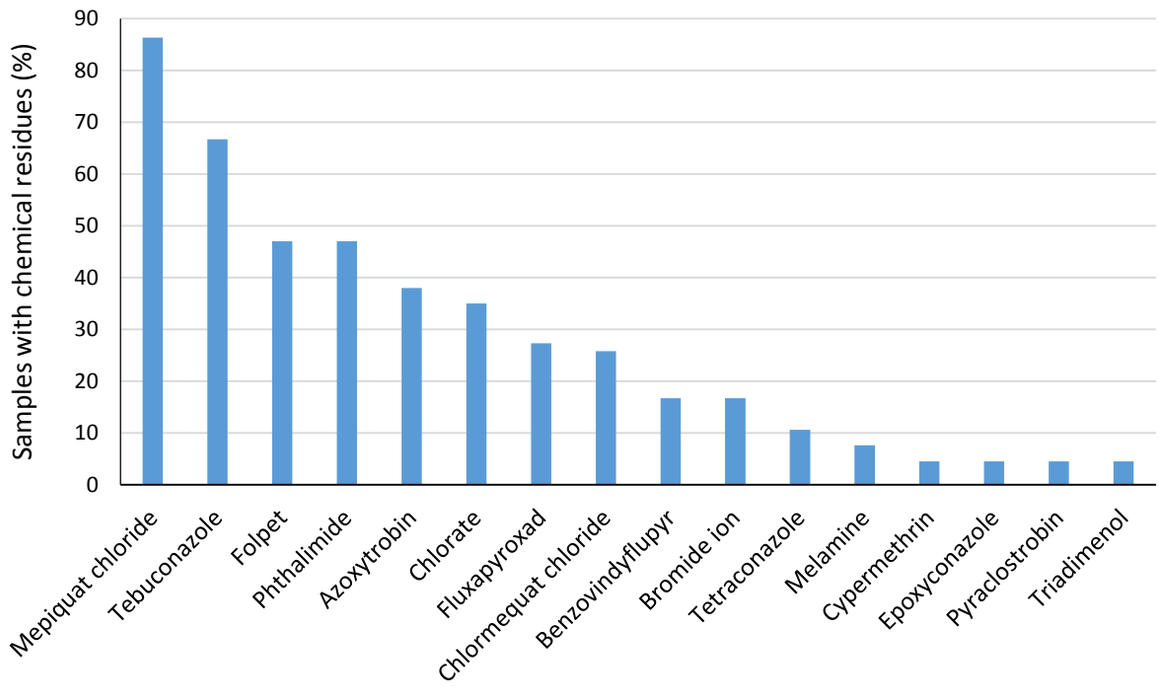

Fig. 4. Chemical residues detected in conventional mushroom substrate samples

ty of containing 4 or more residues in comparison to 3 or fewer. In some cases, analysis of individual samples revealed the presence of up to 14 different residues. Additionally, analysis of conventional substrate samples has revealed the presence of epoxiconazole and propiconazole, which have lost their EU permissions. These substances were derived from preparations previously applied for the control fungal diseases in cereal crops and were approved for use in the EU until 19/03/2020 [Commission Implementing Regulation 2018/1865; Commission Regulation 2020/749].

The occurrence of multiple pesticide residues in conventional and organic substrate samples was compared. It was stated highly significant differences between the two types of substrate. In conventional substrates, multiple residues were detected in 45%, 24%, and 15% of samples, corresponding to samples containing more than 4, 4, and 3 substances, respectively. In contrast, organic substrate samples contained multiple residues in only 2%, 8%, and 11% of samples at the same residue levels, respectively.

Statistical analysis confirmed significant differences in pesticide residues between organic and conventional substrates. Conventional samples exhibited a higher frequency of mepiquat chloride and fungicides (e.g., azoxystrobin, fluxapyroxad, tebuconazole, and tetraconazole). In contrast, chlorate, bromide ion, and melamine exhibited comparable levels across both analysed samples, while anthraquinone occurred exclusively in organic samples (5.1%). Residue-free samples were more common in the organic group (34.1%), whereas none were detected among conventional substrates. Conven-

Table 5. Number of samples with pesticide residues in organic and conventional mushroom substrates over a period of four years

Substances analyzed	Mean number of substrate samples with residues (%)	
	organic	conventional
Chlormequat chloride	11.1 ±10.8 A	27.5 ±15.9 A
Mepiquat chloride	7.1 ±14.3 B	86.0 ±8.5 A
Anthraquinone	5.1 ±5.8 A	0.0 ±0.0 A
Azoxystrobin	1.8 ±3.5 B	40.1 ±13.0 A
Bixafen	0.0 ±0.0 A	3.3 ±13.4 A
Benzovindyflupyr	3.3 ±7.1 A	3.6 ±6.7 A
Boskalid	0.0 ±0.0 A	1.4 ±2.8 A
Chlorate	27.5 ±25.0 A	31.4 ±16.8 A
Cyproconazole	0.0 ±0.0 A	1.4 ±2.8 A
Epoxynazole	1.6 ±3.1 A	5.9 ±8.6 A
Fluxapyroxad	3.3 ±6.1 B	26.6 ±6.2 A
Folpet	23.0 ±12.3 A	44.7 ±18.4 A
Fthalimide	23.0 ±12.3 A	42.4 ±21.5 A
Bromid ion	12.5 ±20.1 A	13.6 ±19.3 A
Melamine	10.3 ±20.4 A	8.3 ±17.3 A
Pyraclostrobin	0.0 ±0.0 A	3.6 ±4.7 A
Propiconazole	0.0 ±0.0 A	3.7 ±4.3 A
Tebuconazole	8.1 ±8.5 B	68.3 ±23.3 A
Tetraconazole	1.7 ±3.3 B	10.1 ±2.3 A
Triadimenol	0.0 ±0.0 A	6.8 ±13.6 A
Without residues	34.1 ±17.8 A	0.0 ± 0.0 B

Values are means of data ± standard deviation (SD); means in the same row with the same letter do not differ statistically ($P < 0.05$, Student's t-test)

tional production was found to be associated with a significantly higher prevalence of residue (Table 5).

It should be noted that the straw used in mushroom substrate production must be monitored for the chemical residues, due to the fact that fungicides are applied during the intensive wheat production in Europe and other regions of the world. Significant quantities of residues in grain or straw can result from these application, but slight information is available on possible effects of pesticides used during wheat cultivation on mushroom production.

Mushrooms from organic cultivation

An assessment of the residue of plant protection products was conducted on 58 samples of edible mushroom fruiting bodies from organic farms. The analysis revealed that approximately 40.1% of the samples were free from chemical contamination. The other samples contained mepiquat chloride (approximately 30% of samples), chlorate and melamine (15% and 12% of samples, respectively), and about 6% contained chlormequat chloride (Fig. 5).

Table 6 presents the number of fruiting body samples from organic farms from each year and the detected chemical substances. In 2021, no residues of plant protection products were detected in 61% of samples. In the remaining samples, the most common were mepiquat chloride, chlormequat chloride, and chlorates. In one sample, residues of fungicides and insecticides used in the protection of mushroom crops (metrafenone, prochloraz and cyromazine) were also found. All residue levels did not exceed the permissible limits. Table 7 presents the maximum residue limits (MRLs) for chlormequat chloride and mepiquat chloride in mushrooms, which are 0.9 mg kg^{-1} and 0.09 mg kg^{-1}, respectively, according to the European Food Safety Authority [European Food Safety Authority 2024].

In 2022, no residues were found in most fruiting body samples, while three samples contained chlorate residues at a level of 0.017–0.041 mg L^{-1}. These values were well below the permissible limit of 0.7 mg L^{-1} (Table 7). In 2023, 80% of organic samples contained mepiquat chloride residues, with an average level of 0.006 mg kg^{-1}. Moreover, no other plant protection products were detected in the mushrooms.

In 2024, two samples of organic fruiting bodies contained mepiquat chloride residues at a level of 0.006 mg kg^{-1}, and one sample contained chlormequat chloride 0.009 mg kg^{-1}. Furthermore, melamine residues were detected in all samples of fruiting bodies (0.022–0.12 mg kg^{-1}). In two samples, chlorate residues were also identified at a level of 0.015–0.35 mg kg^{-1}, not exceeding permissible levels.

The white button mushroom is cultivated on substrate derived from cereals straw. Given the extensive utilisation of chlormequat chloride in the cultivation of these crops, it is highly likely that the residues originate from mycelial uptake from the straw, rather than from direct application to mushroom cultivation. The mycelium responsible for mushroom development is characterised by extensive growth, coming into contact with a substantial mass of straw. The presence of chlormequat chloride residues in mushrooms is considered a reliable indicator of either high efficiency in the absorption of this compound or limited ability to metabolise it [Reynolds et al. 2004]. The presence

Table 6. Number of organic mushroom fruiting body samples containing chemical residues in the years 2021–2024

Year	2021	2022	2023	2024	Total
Number of samples	23	15	10	10	58
Chlormequat choride	3	–	–	1	4
Mepiquat chloride	7	–	8	2	17
Chlorate	3	3	–	2	8
Cyromazine	1	–	–	–	1
Melamine	–	–	–	10	10
Metrafenone	1	–	–	–	1
Prochloraz	1	1	–	–	1
Samples without residues	12	12	2	–	26

Table 7. Plant protection product residues detected in mushroom fruiting bodies from organic cultivation

Chemical substance	Concentration (mg/kg)	Mean	Standards range and assessment	
			MRL[1] (mg/kg)	result compliance[2]
Mepiquat chloride	0.005–0.012	0.008	0.09	compliant
Chlormequat chloride	0.006–0.012	0.008	0.9	compliant
Chlorate	0.015–0.35	0.075	0.7	compliant
Melamine	0.022–0.12	0.065	2.5	compliant
Metrafenone	0.022 ±0.011	0.022	0.5	compliant
Cyromazine	0.75 ±0.38	0.75	10.0	compliant
Prochloraz-Mn	0.038 ±0.019	0.038	3.0	compliant

[1] Maximum residues level, in accordance with Regulation (EC) No 396/2005 of the European Parliament and of the Council of 23 February 2005, as amended

[2] Decision rule for determining compliance/non-compliance according to Document Nº SANTE/12682/2019: Result is compliant, if x−U ≤ MRL; result is non-compliant, if $x-U$ > MRL

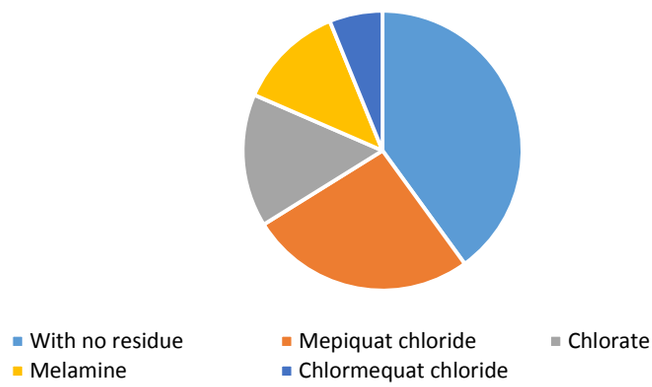

Fig. 5. Percentage of organic mushroom samples with chemical residues

of pesticides in mushrooms from organic farms have been also reported by Rembiałkowska and Badowski [2011]. Pesticides are widely applied in horticultural production worldwide, and organic farms are not isolated units within the agricultural environment. They are often located near conventional fields where pesticides are used. These chemicals can become airborne and disperse into areas where organic crops are cultivated. The efficacy of aerial pesticide applications is significantly diminished, with only approximately 25% of the chemical reaching the intended crop, while the remainder disperses into the surrounding environment. Another pathway contributing to the migration of agrochemicals is field irrigation. Water used for irrigation can transport residues via drainage canals. Water outflow from conventional crops often contains pesticide residues, which can be absorbed by plant cultivated in organic farms. Consequently, residues of chemical substances may be present in organic produce [Benbrook and Baker 2014].

Mushrooms from conventional cultivation

The study involved analyzing pesticide residues in a total of 100 mushroom samples from conventional crops. None of the samples contained any residues. The most frequently detected compound was mepiquat

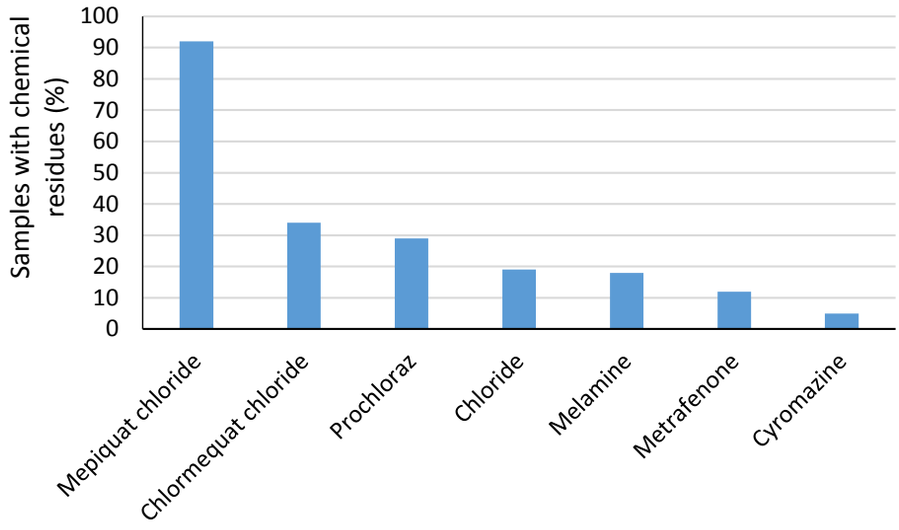

Fig. 6. Chemical residues detected in mushroom samples from conventional cultivation

chloride (92% of samples), followed by chlormequat chloride (34%) and prochloraz (29%), chlorates and melamine (20%), and metrafenone (12%), see Figure 6.

The analysis of mushroom samples collected from conventional cultivation each years is presented in Table 8. In 2021, only one mushroom sample did not contain any residues of plant protection products from straw, while the remaining samples contained chlormequat chloride and/or mepiquat chloride. Fungicide residues (prochloraz, metrafenone, or cyromazine) were detected in 13 samples, while chlorates were present in 3. All detected residues remained below the maximum residue limits (MRLs) for mushrooms (Table 9). In 2022, the residues of chlormequat chloride (0.005–0.010 mg kg^{-1}) and/or mepiquat chloride (0.005–0.050 mg kg^{-1}) were identified. Prochloraz residues were detected in eleven samples, while metrafenone and cyromazine were found in one sample. Chlorate residues were identified in three samples. In 2023, mepiquat chloride was the most prevalent detected compound. Chlorates, prochloraz-Mn and metrafenone were detected in 7, 8 and 3 samples, respectively. In 2024, mepiquat chloride was the dominant residue, while chlormequat chloride was detected in five samples. Metrafenone was present in four samples, prochloraz in one, and melamine was detected in all samples (at levels of 0.013–0.56 mg kg^{-1}).

The analysis of residues in mushrooms from conventional crops demonstrates the prevalence of chemicals in the majority of the samples. Moreover, conventional mushrooms exhibited higher prevalence of residues than those cultivated organically. A similar conclusion was drawn by Rembiałkowska and Badowski [2011], who stated that the probability of pesticide residues in organic products is reduced by a 3–4-fold compared to conventional products. The likelihood of organic raw materials containing multiple pesticides is up to 11-fold lower than for conventional materials. A large number of studies have further confirmed that the mean level of organic product contamination with pesticides is 3-fold or even 10-fold lower than the mean concentration of the same compound in conventional products. The most frequently detected pesticides in the conventional samples were tebuconazole, folpet, phthalimide and azoxystrobin, which were found in over 30% of the samples. In the organic samples, the most frequently observed compounds were folpet and phthalimide, although these were only present in 20% of the samples. Furthermore, the variation in the levels of detected fungicides was found to be significantly lower in organic samples than the conventional ones.

Table 8. Number of conventional mushroom fruiting body samples with chemical residues in the years 2021–2024

Year	2021	2022	2023	2024	Total
Number of samples	30	27	25	18	100
Chlormequat choride	10	13	6	5	34
Mepiquat chloride	26	25	24	17	92
Azoxystrobin	–	–	–	1	1
Benzovindyflupyr	–	–	–	1	1
Carbendazim	–	–	–	1	1
Chlorate	3	8	7	1	19
Cyromazine	4	1	–	–	5
Folpet	–	–	–	1	1
Melamine	–	–	–	18	18
Metrafenone	4	1	3	4	12
Phthalimide	–	–	–	1	1
Prochloraz	9	11	8	1	29
Tebuconazole	–	–	–	1	1
Tiametoxam	–	–	–	1	1
2-phenylphenol	–	–	–	1	1

Table 9. Levels of detected residues of chemical substances in conventional mushroom fruiting body samples

Chemical substance	Concentration (range, m/kg)	Mean concentration	Standards range and assessment	
			MRL[1] (mg/kg)	result compliance[2]
Chlormequat chloride	0.005–0.016	0.009	0.9	compliant
Mepiquat chloride	0.005–0.051	0.015	0.09	compliant
Chlorate	0.020–0.55	0.167	0.7	compliant
Cyromazine	0.12–1.47	0.51	10.0	compliant
Melamine	0.013–0.56	0.045	2.5	compliant
Metrafenone	0.007–0.087	0.022	0.5	compliant
Prochloraz-Mn	0.006–0.098	0.028	3.0	compliant

Explanations – see Table 7

The same results concerning analysis of occurrence of pesticides in organic and conventional food was preformed by Montiel-León et al. [2019].

Statistical analysis revealed significant differences in pesticide residues between organic and conventional fruiting bodies. Conventional mushrooms exhibited a significantly higher frequency of mepiquat chloride and chlormequat chloride. Other fungicide, such as azoxystrobin, benzovindiflupyr, folpet, fthalimide, 2-phenylphenol, tebuconazole, and thiamethoxam, were detected at relatively low levels (0–1.4%) in both groups of samples. Among organic mushrooms, 40.1% of samples were free from residues, in contrast to the lack of residue-free samples in the conventional ones (Table 10).

Residues of prochloraz and metrafenone, commonly applied in *A. bisporus* cultivation, were frequently detected in conventional mushrooms from 2021 to 2023.

Table 10. Number of samples with chemical residues in organic and conventional mushroom fruiting bodies over a period of four years

Substances analyzed	Mean number of mushroom samples with residues (%)	
	organic	conventional
Chlormequat chloride	5.7 ±6.8 B	33.5 ±10.6 A
Mepiquat chloride	32.6 ±30.3 B	92.4 ±4.1 A
Azoxystrobin	0.0 ±0.0 A	1.4 ±2.8 A
Benzovindyflupyr	0.0 ±0.0 A	1.4 ±2.8 A
Chlorate	13.3 ±9.4 A	18.4 ±12.3 A
Folpet	0.0 ±0.0 A	1.4 ±2.8 A
Fthalimide	0.0 ±0.0 A	1.4 ±2.8 A
2-phenylphenol	0.0 ±0.0 A	1.4 ±2.8 A
Tebuconazole	0.0 ±0.0 A	1.4 ±2.8 A
Tiametoxam	0.0 ±0.0 A	1.4 ±2.8 A
Without residues	40.1 ±27.8 A	0.0 ±0.0 B

Explanations – see Table 5

This finding is consistent with results from other studies conducted by Carrasco et al. [2017] and Schusterova et al. [2023]. Nevertheless, in 2024 only a single sample contained prochloraz–Mn. However, the authorisation for its use in mushroom cultivation was withdrawn on 1 October 2023. Furthermore, in 2023, one sample contained carbendazim residues, although this substance is no longer authorised for mushroom protection [Commission Implementing Regulation 542/2011]. Another sample showed a twofold exceedance of the permitted level of 2-phenylphenol. A study by Li et al [2022] revealed that the primary pesticides found in edible fungi samples were carbendazim, acephate, procymidone, prochloraz, and aldicarb sulfone. According to Commission Implementing Regulation No 542/2011, the authorisation of carbendazim as an active substance for use in plant protection expired on 30 November 2014. Consequently, products containing carbendazim were removed from the register in June 2016. In 2024, thiamethoxam was detected in one sample of mushroom fruiting bodies at a concentration of 0.03 mg kg^{-1}, which exceeds the maximum permitted level (0.01 mg kg^{-1}). Moreover, the use of thiamethoxam was authorised until 19 December 2018 [Commission Implementing Regulation 2018/785, Commission Implementing Regulation (EU) 2022/801]. Additionally, it was determined that in 2023 and 2024, a significantly lower number of samples (11 out of 43) contained chlormequat chloride residues compared to the years 2021 and 2022 (23 out of 57).

Chlorate residues were frequently detected in mushroom samples. The analysis revealed that 14% of organic samples and 19% of conventional samples contained these substances. This occurrence is likely associated with the use of disinfectants during the cleaning of mushroom growing chamber or water disinfection for irrigation purposes in mushroom cultivation [Kettlitz et al. 2016]. Kettlitz et al. [2016] obtained similar conclusions in their study on the chlorate residue in food. They revealed that 50.5% of the food samples contained chlorate above 0.01 mg kg^{-1}. However, this was not due to the use of chlorate as a pesticide. Instead, it was mainly due to the occurrence of chlorate as an unavoidable by-product of disinfection. Furthermore, Gómez-Ramos et al. [2020] indicated chlorate residues in a large part of the samples of tested organic food. According to Zhang et al. [2023], high levels of chlorate were also detected in *Agaricus blazei* mushrooms. The potential pathways through which mushrooms can be contaminated with chlorate are not yet finally elucidated. However, it

is reported that chlorate residues arise in many cases by using chlorinated water either for irrigation in the field or post-harvest for various food processing, i.e. washing of equipment and surface disinfection in mushroom growing chamber [Commission Regulation 2020/749, European Food Safety Authority CONTAM Panel 2015].

CONCLUSION

The results obtained provide awareness of the presence of pesticide residues in organic mushrooms, originating from the cultivation substrate. Furthermore, no residues were detected in 40.1% of the organic fruiting body samples, while 32.6% of the samples contained mepiquat chloride. Only 34.1% of the organic substrate samples were free from chemical residues. Chlormequat chloride was detected in 11.1% of the samples. The other samples were mainly contaminated with chlorates, folpet, and phthalimide, not exceeding the maximum residue limits (MRLs).

In contrast, all mushroom samples from conventional cultivation contained pesticide residues, generally below the established MRLs. The most frequently detected residues were mepiquat chloride and chlormequat chloride, followed by prochloraz, chlorate, and melamine. However, one mushroom sample exceeded the permissible concentration of 2-phenylphenol, and two other samples contained residues of unapproved substances, such as carbendazim and thiamethoxam. Moreover, the level of thiamethoxam residue exceeded the maximum acceptable limit. The present study confirmed that conventionally cultivated mushrooms were associated with a higher occurrence of residue, while organic production resulted in a greater proportion of residue-free samples.

The results of this study indicate the need for continuous monitoring of white button mushrooms from both organic and conventional cultivation with regard to the presence of plant protection product residues. Moreover, the presence of residues in organic mushrooms does not preclude them from being classified as organically produced. Although the number of analyzed samples was limited, such monitoring is essential to ensure the safety and quality of agricultural products.

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RESEARCH PAPER

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THE COMPARISON OF ANTIOXIDANT CAPACITY AND HPTLC POLYPHENOLIC PROFILE OF GREEN FRUITS AND LEAVES OF WALNUT (*Juglans regia* L.) CULTIVARS GROWN IN POLAND

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ABSTRACT

Walnut (*Juglans regia* L.) has a wide range of applications in food, pharmaceutical and cosmetics industries, however both unripe fruits and leaves have not been widely evaluated for varietal variation so far. Due to, leaves and immature fruits of seven walnut cultivars (Lake, Mars, Franquette, U02, SK04/10, Broadview, and Resovia) grown in Poland were compared for the first time in terms of antioxidant capacity (DPPH and FRAP), total phenolic content and qualitative polyphenol profile (HPTLC). The highest phenolic content was found for the Franquette ($p < 0.05$), both for leaves and nuts. In general, lower variability was observed for leaves, in the case of green nuts the results were more diversified by cultivar. The rapid and cost-saving HPTLC method was used to compare the polyphenolic profiles of the extracts. Cultivar-dependent differences in phenolic acids (mainly syringic) and flavonoids (mainly derivatives of quercetin) presence were found. Despite of observed differences between cultivars, it was found that both part of walnut are abundant in polyphenols. However it has been confirmed that the selection of a phytochemically appropriate cultivar may be important in providing a particularly valuable raw material for the food, pharmaceutical and cosmetic applications, thus the further quantitative studies are required.

Keywords: phenolic acids, flavonoids, DPPH, FRAP, raw material

INTRODUCTION

Walnut (*Juglans regia* L.) is a popular tree, widely cultivated in Europe, including Poland. It is used because of its tasty nuts, which have a lot of culinary uses. Moreover, the leaves, flowers, bark, and unripe fruit are also valuable herbal raw materials [Britton et al. 2008]. Walnut leaves and green nuts are the abundant source of antioxidants, mainly flavonoids and phenolic acids, as well as compounds from the group of naphthoquinones [Santos et al. 2013, Schwindl et al. 2017]. Antimicrobial, antiparasitic and hypoglycemic properties have been demonstrated for the leaves and green nuts, which also have an antiproliferative effect on selected cancerous cell lines [Oliveira et al. 2008, Carvalho et al. 2010, Santos et al. 2013, Sharma et al. 2013, Wang et al. 2016, Rabiei et al. 2018, Vieira et al. 2019].

There are many cultivars of walnut cultivated in the world, varied in terms of yield, morphology and

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growing conditions. Widely used commercial cultivars include: Franquette – an old French variety, Mars – native to the Czech Republic, Broadview – bred in Canada, and the American cultivar Lake. U02 is a Polish walnut cultivar, whose mother tree originated from the remains of historical orchards at the Łańcut Palace in the Podkarpacie region. This cultivar is characterized by high frost tolerance, and is highly fertile. In turn, SK04/10 is a cultivar with nuts of a pink colored skin on the kernel. It originates from Silesia. Although it exhibits slightly lower frost resistance, it is highly fertile and enters the fruiting phase early. The red kernels additionally enhance the decorative value of the fruits [http://szczepionyorzech.pl]. The Resovia cultivar also comes from Podkarpacie, and was named after Rzeszów, the capital of this region. It is an early cultivar, resistant to frost and diseases [Zdyb 2003].

The wide genotypic diversity of walnuts translates into a diverse phenotype. In many regions of the world, selection for genotypes with specific characteristics, including high kernel quality, fat, protein, and phenolic compound content, as well as resistance to climatic conditions has been conducted [Cosmulescu 2013, Keles et al. 2014, Sarikhani et al. 2021, Balapanov and Artykhova 2021, Hamidirad et al. 2025]. The most important biochemical properties include the content of bioactive substances in nuts, especially polyphenolic compounds, tocopherols and fatty acids [Sarikhani et al. 2021, Temizyürek et al. 2025]. The selection of superior genotypes, and their inclusion in walnut breeding program is possible thanks to modern biotechnological and bioinformatic tools [Vahdati et al. 2020, Temizyürek et al. 2025].

The aim of the study was to compare seven walnut cultivars grown in the same soil and climate condition in terms of antioxidant capacity and phenolic profile of unripe fruits and leaves. To obtain qualitative polyphenol profiles, a rarely used method of high-performance thin-layer chromatography (HPTLC) was used, which allows for quick, easy and cheap comparison (screening) of many samples simultaneously. The tested cultivars were evaluated for the usefulness of these parts of the plant as raw materials for the preparation of tinctures or some pharmaceutical preparations and also in terms of indicating cultivars valuable for walnut cultivation.

MATERIALS AND METHODS

Plant material and extracts preparation

Leaves and unripe walnuts were obtained in May–June 2020 from the walnut plantation in Urzejowice (Podkarpackie, Poland, 50°0'49" N, 22°27'23" E). Research material of seven walnut cultivars: Lake, Mars, Franquette, U02, SK04/10, Broadview and Resovia was collected and deposited in the collection of Department of Chemistry and Food Toxicology, University of Rzeszów. After harvesting, the leaves were dried at a temperature below 40 °C, while the nuts were cut into quarters and freeze-dried using the Alpha 1–2 LD plus freeze dryer (Martin Christ, Osterode am Harz, Germany). The dried material was ground into powder using a grinder (MK-06M, MPM, Milanówek, Poland). Two grams of each sample were extracted using 40 mL of 80% (v/v) ethanol, using an ultrasound-assisted method (700 W, 40 kHz; SONIC-10, Polsonic, Warszawa, Poland). The extracts were filtered through a paper filter and stored in a freezer until further analyses.

Total phenolic content and antioxidant capacity

The total phenolic content was measured using Folin-Ciocâlteu method, as described by Dżugan et al. [2021]. Extracts were diluted 100-fold and aliquotes of 0.02 mL were placed into a 96-well plate. Then, 0.1 mL of 10% Folin-Ciocâlteu reagent followed by 0.08 mL of 7.5% (w/v) of Na_2CO_3 solution were added. Samples were kept in the dark for 60 min, and then the absorbance was measured against blank at 760 nm using a microplate reader (EPOCH 2, BioTek, Vermont, USA). The results were expressed as mg of gallic acid (GAE) equivalents per gram of dry mass (mg GAE g^{-1}) based on calibration curve prepared for GAE standard solutions in the range 0–250 μg mL^{-1} ($y = 0.0555x$, $R^2 = 0.9976$).

The FRAP assay (Ferric Reducing Antioxidant Power) was carried out according to Dżugan et al. [2021], as follows: to 0.02 mL of diluted plant extract, 0.18 mL FRAP reagent – 2.5 mL of a 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ) solution in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3$ and 25 mL of 0.3 M acetate buffer (pH 3.6) – was added and after 10 min incubation at 37 °C the absorbance of the reaction mixture was measured using a microplate reader (EPOCH 2)

against blank at 593 nm. The results were expressed as μmol of Trolox (TE) equivalents per gram of dry mass (μmol TE g^{-1}) based on calibration curve (y = 0.026x, R^2 = 0.9989).

The DPPH test was performed also according to Dżugan et al. [2021], as follows: to 0.02 mL of diluted plant extract 0.18 mL of DPPH radical methanolic solution (0.1 mM) was added and kept in the dark for 30 min. Then, the absorbance of tested (As) and control (Ao) samples was measured at 517 nm against methanol using microplate reader (EPOCH 2). The reduction of DPPH radical was calculated according to equation: DPPH% = [(Ao − As) / Ao] × 100. The results were calculated for Trolox equivalents using a calibration curve in the range 0.5–6 μmol of Trolox per sample (y = 15.553x, R^2 = 0.9970).

HPTLC polyphenols detection

The analysis of polyphenol profile was performed using a HPTLC set (Camag, Muttenz, Switzerland) consisted of the semi-automated application device (Linomat 5, CAMAG), automatic developing chamber (ADC2, CAMAG), the imaging device (TLC Visualizer, CAMAG) and the automated derivatizer of TLC plates (CAMAG Derivatizer). Extracts were applied to plates (HPTLC Silica Gel 60 F254, 20 × 10 cm, Merck Darmstadt, Germany) in a volume of 3 μL and the plates were developed with the mobile phase composed of chloroform, ethyl acetate and formic acid (5:4:1, v/v/v) to a distance 70 mm. Obtained results were documented using UV light (366 nm). Additionally, plates were derivatized with p-anisaldehyde sulfuric acid reagent. After derivatization, plates were heated at 110 °C for 10 min and imaged under UV 366 nm. Obtained chromatographic images were analyzed using the HPTLC software (Vision CATS 3.2, CAMAG).

Statistical analysis

All numerical data were obtained in triplicates. The mean values and standard deviations were calculated. Significance of differences was checked using ANOVA one-way analysis of variance followed by a Tukey's (HSD) test (p = 0.05). In order to demonstrate the similarity of the tested varieties, a cluster analysis was performed based on the results obtained. All calculations and analyses were performed using Statistica 13.1 software (StatSoft, Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

Interest in unripe walnuts as a culinary raw material with valuable nutritional and therapeutic properties is constantly growing [Mukarram et al. 2024]. On the other hand, walnut leaves are less known and less frequently used. However, both raw materials can be a source of bioactive substances for food fortification and the development of new therapeutic and cosmetic preparations.

Samples of leaves and unripe fruit of the tested walnut cultivars were assessed for the content of phenolic compounds and antioxidant activity. Data on total phenolic content are presented in Figure 1.

The obtained results showed considerable differentiation. Both raw materials are a valuable source of polyphenolic compounds, and the difference between leaves and nuts in each case reaches max. 20%. In some cultivars (Mars, U02, SK04/10), higher levels of phenols were recorded in the leaves, for others in green nuts. The Franquette containing over 90 mg GAE g^{-1} definitely stood out among the green fruits samples. Obtained results are in line with Jakopič et al. [2009] findings, who investigated the content of phenols in the unripe fruits of two *J. regia* cultivars, namely: Franquette and Elit. In cited study the amount of 135.27 mg GAE g^{-1} and 126.20 mg GAE g^{-1} were found, respectively. In turn, Pycia et al. [2019] determined lower polyphenol content (at the level of 7.15 mg GAE g^{-1} for Resovia and 21.49 mg GAE g^{-1} for Leopold) in nuts harvested in July, and further decrease was noted during maturation. Other authors give data for the green husk of walnut at the level of up to 74.08 mg g^{-1} [Oliveira et al. 2008]. Also in these studies Franquette turned out to be the richest in phenolic compounds among the cultivars tested by the authors, which is consistent with the results obtained in the present study.

In the case of leaves, the results were slightly less differentiated. The highest content of phenols (in the range of 65–70 mg GAE g^{-1}) was recorded for the Lake, Franquette and SK 04/10 cultivars. The obtained results are consistent with Einali et al. [2018] findings who provide the value of 52.48 mg GAE g^{-1}.

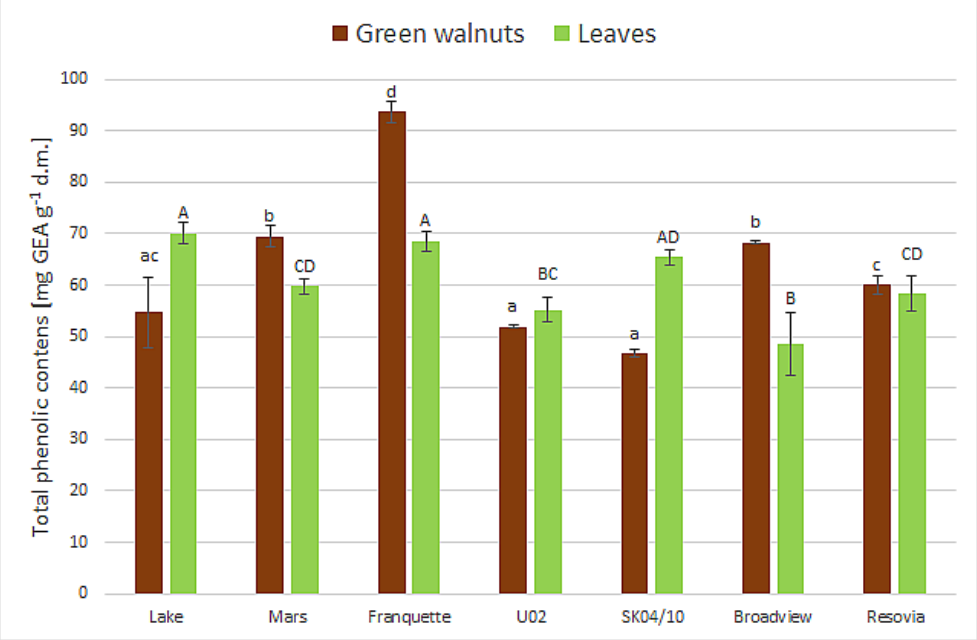

Fig. 1. The comparison of total phenolic content in extracts of green nuts and leaves extracts in terms of walnut cultivar. Means marked with different letters (uppercase for green walnuts and lowercase for leaves) are significantly different ($p < 0.05$)

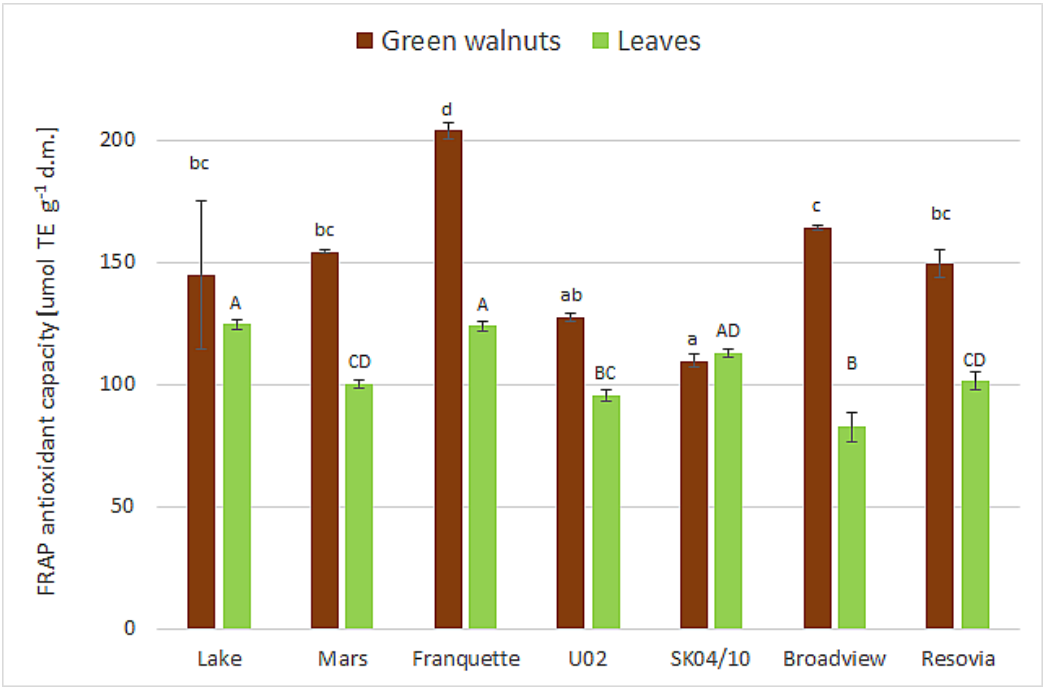

Fig. 2. The comparison of FRAP antioxidant capacity of green nuts and leaves extracts in terms of walnut cultivar. Means marked with different letters (uppercase for green walnuts and lowercase for leaves) are significantly different ($p < 0.05$)

However, the values reported in the literature show a large dispersion, from 25.3 mg GAE g^{-1} [Santos et al. 2013] to 410 mg GAE g^{-1} [Sharafati-Chalestori et al. 2011]. Such huge differentiation of the data may result from different sampling times or growing conditions as well as sample preparation and storage [Pakrah et al. 2020]. Cosmulescu and Trandafir [2011] observed seasonal variability of the phenol content in walnut leaves of several cultivars.

Literature data confirm strong correlation between total polyphenols content and antioxidant potential of both unripe fruits [Pycia et al. 2019, Cosmulescu et al. 2014] and leaves [Vieira et al. 2019, Pereira et al. 2008, Masek et al. 2019] of *J. regia*. Thus, the antioxidant capacity of the extracts was tested using two methods: FRAP (Fig. 2) and DPPH (Fig. 3).

According to both methods, the Franquette showed the highest activity among the samples of green nuts. Slightly different results were obtained by DPPH method, where Mars and Resovia did not differ significantly from the Franquette in terms of ability to scavenge free radicals. In the case of leaves, the differences were smaller The Lake, Franquette and SK 04/10 showed the strongest reducing abilities, and the Lake stood out in the free radical scavenging method.

There was a positive correlation coefficient between both methods (r = 0.773). The results for both assays were also strongly positively correlated with the total content of phenols: r = 0.761 and 0.832 for FRAP and DPPH, respectively. The correlation coefficients examined separately for leaves and green nuts are even higher (Table 1). High values of correlation coefficients indicate the dominant share of phenolic compounds determined by the Folin-Ciocâlteu method in shaping the overall antioxidant capacity of the tested extracts.

The obtained results were used to perform a cluster analysis, and construct the classification tree (Fig. 4). Based on the results, the closest relationships can be found between the Resovia and Mars, as well as

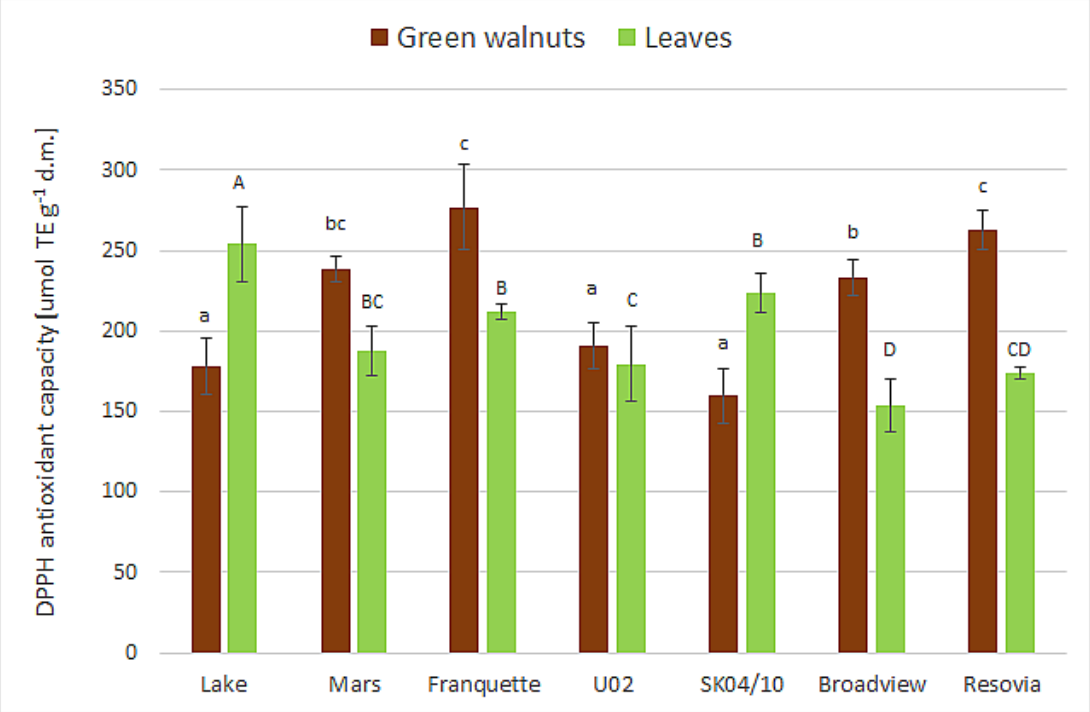

Fig. 3. The comparison of DPPH antioxidant capacity of green nuts and leaves extracts in terms of walnut cultivar. Means marked with different letters (uppercase for green walnuts and lowercase for leaves) are significantly different ($p < 0.05$)

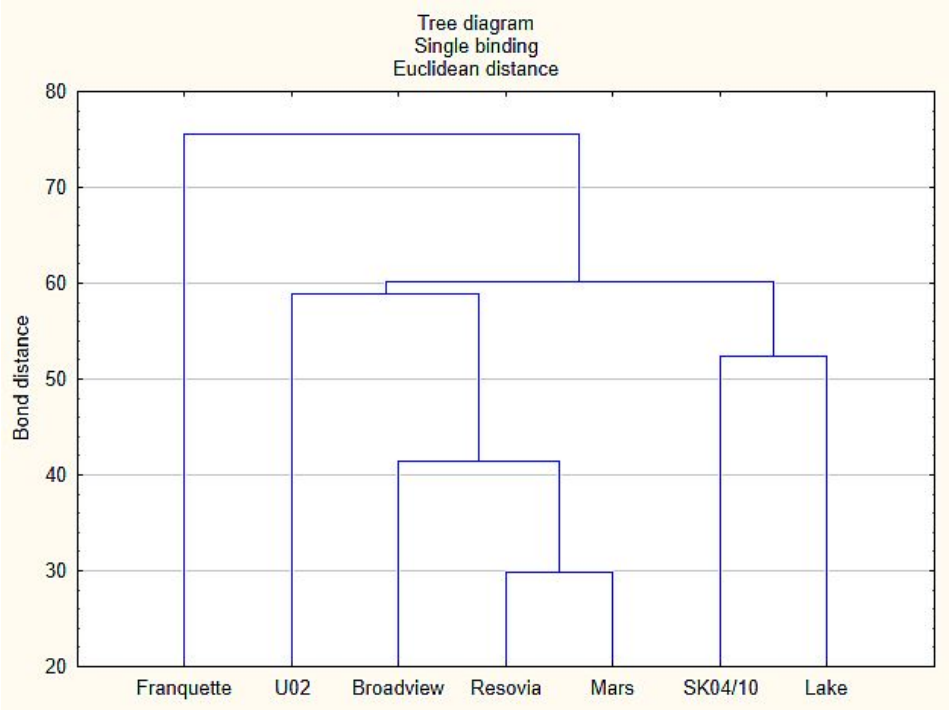

Fig. 4. Classification tree constructed on the basis of the results of the content of phenolic compounds and the antioxidant properties of leaves and green walnuts

Table 1. Pearson's correlation coefficients for tested parameters

Parameter	Leaves			Green walnuts			Overall		
	TPC	FRAP	DPPH	TPC	FRAP	DPPH	TPC	FRAP	DPPH
TPC	1	–	–	1	–	–	1	–	–
FRAP	0.969	1	–	0.990	1	–	0.761	1	–
DPPH	0.829	0.838	1	0.927	0.908	1	0.832	0.773	1

Broadview, which were characterized by similar antioxidant properties. The Franquette, for which the most favorable properties have been shown, differs significantly from the others.

The demonstrated differences between cultivars in the content of bioactive compounds and antioxidant properties may have a genetic basis, but the influence of environmental conditions cannot be excluded, although the tested raw materials came from trees grown in one location. It has been repeatedly shown that the genotypic variability of walnuts translates not only into phenotypic, but also phytochemical traits, including the content of important nutritional and bioactive components [Vahdati et al. 2020, Sarikhani et al. 2021].

In the present study, leaf and green nut extracts were analyzed for polyphenol profiles using the HPTLC method for the first time. This method is particularly useful for preliminary screening of multiple samples that are separated simultaneously, and the results obtained provide a direct comparison of component profiles under the same conditions [Choma et al. 2019]. Based on the set of obtained bands, a qualitative comparison of the fingerprints of each nut culti-

var can be made (number, color, intensity). Moreover, having Rf values for the expected standard substances determined under the same chromatographic separation conditions, it is possible to identify bands showing the same Rf value (Table 2). Images of HPTLC plates before and after derivatization with p-anisaldehyde are shown in Figure 5.

The compounds detected in HPTLC chromatograms include phenolic acids (blue bands in UV 366 nm) and flavonoids (yellow-green bands). Table 2 summarizes the presence of several reference substances, which were expected to occur based on the literature [Santos et al. 2013, Shi et al. 2018, Fernández-Agulló et al. 2020, Medic et al. 2021]. There was no confirmed presence of (–)-epicatechin, gallic acid, taxifolin, 1,4-naphtoquinone, and juglone in any of the tested samples using this method. Orange bands at the top of the plate indicate the presence of carotenoids and chlorophylls, especially in leaf extracts. The leaves showed more diversified phenolic profiles than unripe fruits which was manifested by a larger number of bands. Among the compounds distinguishing individual cultivars, (+)-catechin should be mentioned, present only in Lake leaves, the pattern of orange bands of photosynthetic pigments present in particular cultivars is also different. In the case of leaf extracts, the SK04/10, Franquette, Mars and Broadview have a clear blue band at Rf = 0.1, corresponding to an unidentified compound, most likely from the flavonoid group. The predicted presence of applied standard metabolites has not been confirmed in any case in green walnut extracts. The lack of detection of juglone, which is a characteristic metabolite, is probably related to the non-optimal extraction system used in the research. The 80% ethanol extraction used is more suitable for the recovery of polar phenolic compounds whereas non-polar solvents, such as petroleum ether, hexane, or chloroform, are optimal for juglone extraction [Strugstad and Despotovski, 2012]. There are also known examples of juglone extraction from green walnuts using pure ethanol or methanol [Jakopič et al. 2009, Cosmulescu et al. 2011], but not their mixtures with water. Juglone was also not detected in tinctures made of green nuts of the Mars with 40% ethanol [Miłek et al. 2022b], as well as using the analogous extraction system and HPTLC detection method in extracts of leaves and unripe walnuts extracts [Miłek et al. 2022a].

In order to precisely determine the polyphenolic profiles and estimate their content an accurate LC method coupled with MS should be used. Banding patterns on HPTLC plates may, however, be of value as an auxiliary classification and quality assessment method. Thanks to the ability to analyze up to 20 samples on a single chromatographic plate, relatively low analysis costs and time savings, the HPTLC method is particularly useful for the initial screening of samples and the qualitative comparison of their composition [Choma et al. 2019]. However, when using this technique, there are often problems with the identification

Table 2. Presence of polyphenolic compounds used as reference substances in fruit and leaf extracts

Compound	Rf	Color		Lake		SK 04/10		Franquette		Mars		Broadview		U02		Resovia	
		366 nm	366 nm after derivatization	GW	L	GW	L	GW	L	GW	L	GW	L	GW	L	GW	L
Quercetin 3-glucoside	0.01	yellow	greenish blue	–	+	–	+	–	+	–	+	–	+	–	+	–	+
Avicularin	0.02	black	blue	–	+	–	+	–	+	–	+	–	+	–	+	–	+
(–)-epicatechin	0.06	dark blue	black	–	–	–	–	–	–	–	–	–	–	–	–	–	–
(+)-catechin	0.08	blue	black	–	+	–	–	–	–	–	–	–	–	–	–	–	–
Gallic acid	0.11	dark blue	dark blue	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Taxifolin	0.22	blue	brown	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Syringic acid	0.50	blue	gray	–	+	–	+	–	+	–	+	–	+	–	+	–	+
1,4-naphtoquinone	0.58	gray blue	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Juglone	0.62	yellow	orange	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Rf – retardation factor, GW – green walnuts, L – leaves

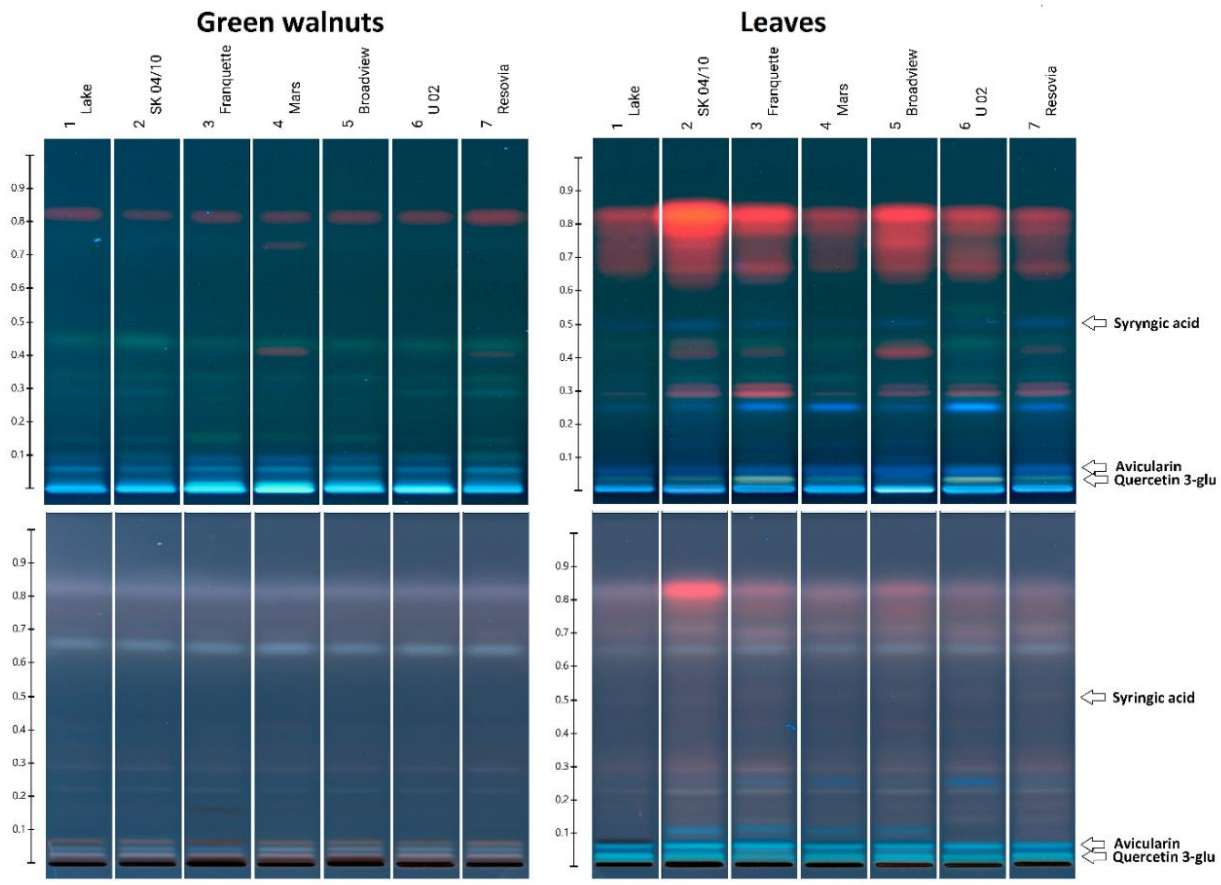

Fig. 5. HPTLC polyphenolic profiles for tested extracts with selected identified compounds marked, viewed in UV 366 nm, before (top) and after p-anisaldehyde reagent derivatization (bottom)

of compounds resulting from the need to use standards that are not always available and also from the frequent overlap of bands.

Previous studies of polyphenol profiles for various walnuts cultivars (using the UPLC-PDA-MS/MS method) showed that the qualitative composition is basically constant, however, thanks to the sensitive method, quantitative differences between cultivars were found, e.g. the Leopold was characterized by an early stage of ripeness nuts with a particularly high content of many compounds, e.g. procyanidins or quinic acid and its derivatives, so for this cultivar the total polyphenol content was significantly higher [Pycia et al. 2019]. In green walnut extracts, (+)-catechin and (–)-epicatechin as well as numerous phenolic acids were previously determined to be dominant [Cosmulescu et al. 2014]. Optimized extraction of green walnut shells with 50% ethanol allowed to identify polyphenolic compounds, as well as organic acids, lipids, terpenes and quinones using UHPLC–ESI–MS/MS [Savić and Savić Gajić, 2025]. Among the compounds identified in *J. regia* leaf extracts, caffeoylquinic and coumaroylquinic acids, as well as quercetin and kaempferol derivatives dominated [Amaral et al. 2004, Santos et al. 2013]. Hamdi et al. [2025] additionally identified numerous myricetin derivatives, juglone and its derivatives in ethanol extracts of leaves of Gran Jefe. Also for leaf extracts, differences between varieties were demonstrated by Nour et al. [2013] who concerned both compounds belonging to the class of

flavonoids, phenolic acids and naphthoquinones. The results of previous studies confirm that the composition of secondary metabolites depends on the plant genotype [Nour et al. 2013].

Illustrating differences in the qualitative composition of the tested extracts by HPTLC may help explain trends in their antioxidant activity. The similar total phenolic compound contents are reflected in similar band intensities in the chromatograms for leaves and green walnuts. The presence of pigments (chlorophylls and carotenoids), abundant in the leaf extracts, apparently does not affect the antioxidant activity. Therefore, it can be concluded that this activity is primarily due to the presence of polyphenolic compounds. The detected metabolites, e.g. flavonoid glycosides and especially syringic acid are known for their strong antioxidant properties [Srinivasulu et al. 2018] which confirms the observed correlation between the total phenols content and antioxidant capacity.

CONCLUSIONS

The preliminary analyzes carried out for selected seven cultivars of walnut grown in Poland confirm the high pro-health potential of both leaves and unripe fruits of these trees. The Franquette old cultivar, traditionally grown in Europe, turned out to be the best source of antioxidants. The HPTLC analysis of polyphenolic profiles allowed to create characteristic fingerprints for each cultivar and can be applied for appropriate and valuable walnut selection which can be introduced into wider cultivation. However, due to seasonal and climatic variability of the content of polyphenolic compounds in plant material, further long term research across growing season is needed to describe the effect of season on polyphenolic profiles of leaves and unripe fruits of *J. regia*. It is also necessary to conduct an in-depth phytochemical analysis of the tested raw materials using advanced extraction and sensitive analytical method as LC-MS/MS. Such detailed phytochemical analysis of raw materials is crucial for their use in various industries.

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The Authors declare no conflict of interest.

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SEASONAL AND PHENOLOGICAL DYNAMICS OF ESSENTIAL OIL CONSTITUENTS IN CULTIVATED *Satureja montana* L.

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ABSTRACT

Research on the qualitative and quantitative composition of essential oil was conducted in south-eastern Poland. The aim of the study was to evaluate the chemical composition of essential oil from *Satureja montana* L. depending on the plant's developmental stage. Seedlings of winter savory were planted in the field at a spacing of 30 × 30 cm. The herb was collected on the following dates: June (vegetative stage of the plant), mid-July (onset of flowering), mid-August (full flowering), and September (senescence). The highest essential oil content was found in plants at full flowering (1.88–2.20%) and at the beginning of flowering (1.63–2.20%). The oil from *S. montana* contained high levels of phenolic compounds, mainly carvacrol and thymol. The highest levels of carvacrol were found at the beginning of flowering (60.97–72.03%) and at full flowering (62.36–73.54%). The highest thymol content was found in 2018 – 19.47% – in the herb collected from plants that had finished flowering and 14.90% – in the herb from plants collected during the vegetative phase.

Keywords: winter savory, carvacrol, thymol, plant phenological stages

INTRODUCTION

Plants belonging to the Lamiaceae family represent a rich source of herbal raw materials containing essential oils (EO). Essential oils are the biologically active substances – secondary metabolites of many plants species whose properties are used in health prevention. These biologically active substances obtained from plants of the Lamiaceae family are of significant importance in phytotherapy, the pharmaceutical and cosmetics industries, as well as in food processing [Chorianopoulos et al. 2004, Jafari et al. 2016, Zawiślak and Nurzyńska-Wierdak 2017a, Vrancheva et al. 2022, Vilmosh et al. 2023, 2024, Jakupović et al. 2025, Kartal et al. 2025]. The genus *Satureja* includes approximately 200 species, native to the Middle East and Mediterranean Europe, as well as Western Asia, North Africa, and South America [Chorianopoulos et al. 2004]. One of the most interesting is *Satureja montana* L., whose essential oil demonstrates antiseptic, antioxidant, antifungal, carminative, and digestive properties [Momtaz and Abdollahi 2010, Maccelli et al. 2020, Abbad et al. 2025]. The herb of *S. montana* is commonly used in Mediterranean cuisine [Kartal et al. 2025], and its essential oil serves as a natural antibacterial agent in food packaging applications [Dima and Dima 2015]. Extracts from *S. montana* containing rosmarinic acid are effective against Gram-positive and Gram-negative bacteria [Gomes et al. 2020]. The biological activity of winter savory is mainly due to the presence of essential oil [Nurzyńska-Wierdak 2016, Rezende et al. 2022, Abbad et al. 2025]. According

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to literature data the main component of *S. montana* essential oil is carvacrol [Skočibušić and Bezić 2004, Bezić et al. 2009, Miladi et al. 2013, Pokajewicz et al. 2023] or thymol [Damjanović-Vratnica et al. 2011, de Oliveira 2011]. The potent antimicrobial activity of this essential oils has been attributed primarily to its high content of carvacrol and thymol, phenolic compound exhibiting synergistic antimicrobial effect [Maccelli et al. 2020, Šimunović et al. 2020].

Said-Al Ahl et al. [2024] pointed to the growing interest in essential oil from winter savory and its compounds, which have a beneficial effect on human health. However, *S. montana* does not occur naturally in Poland and is rarely cultivated in our country.

The yield and chemical composition of essential oils obtained from plants of the Lamiaceae family are variable and influenced by climatic conditions, cultivation practices, and harvest timing [Zawiślak 2013, Zawiślak and Nurzyńska-Wierdak 2017b, Drozd et al. 2024].

Moreover, many scientists have demonstrated that essential oil composition obtained from *S. montana* depends on the phenological stage of the plant and environmental conditions [Milos et al. 2001, Damjanović-Vratnica et al. 2011]. This relationship has also been confirmed for many other species of plants from the Lamiaceae family [Jordán et al. 2013, Moghaddam et al. 2015, Türkmen 2021]. However, studies by Miladi et al. [2013] showed that the carvacrol content in the essential oil of *S. montana* herb, collected in France at the peak of flowering, was 53.35%. A similar carvacrol content (52.4%) was found in essential oil from raw material harvested before flowering in Croatia [Mirjana and Nada 2004].

Therefore, this study aimed to evaluate the chemical composition of essential oil from *S. montana* cultivated in southeastern Poland, examining variations related to plant phenological stages at harvest during three years of the study.

MATERIALS AND METHODS

Plant material

The experiments were carried out at the Experimental Farm of the University of Life Sciences in Lublin, located in southeastern Poland (51.23° N; 22.56° E), in 2017–2019. The Lublin vicinity is a region with a long traditions of herbs cultivations. The average long-term temperatures in May are 13.2 °C, gradually increasing in the following months (June: 15.6 °C, July: 18.1 °C), slightly decreasing in August to 17.5 °C, and in September reaching 12.9 °C. The moderate summer temperature determines, among other the possibility of cultivating essential oil plants. Seeds of *S. montana* were sourced from the Botanical Garden collection of Maria Curie-Skłodowska University, Lublin, Poland. Seedlings were cultivated under greenhouse conditions, with sowing conducted on March 20 into trays containing peat-based substrate. Seed germination occurred within two weeks. Seedlings were subsequently transplanted into multi-cell trays, and then planted in the field in mid-May at a spacing of 30 × 30 cm. The cultivation plot was established following standard agronomic practices for *Satureja* sp. Soil analysis indicated grey-brown podzolic soil derived from loess deposits, containing approximately 1.6% organic matter. Based on this analysis, fertilization consisted of 60 kg N, 50 kg P_2O_5, and 100 kg K_2O per hectare. Weed control and soil aeration were performed manually. Chemical plant protection products were not applied, as pests and diseases were not observed throughout the experiment. The plants were harvested in the second year of cultivation at four developmental stages for three consecutive years.

1. June (vegetative stage),
2. Mid-July (onset of flowering),
3. Mid-August (full flowering),
4. September (senescence).

Plants were harvested by cutting approximately 8 cm above ground level. Collected material was dried in a thermal dryer at 30 °C. The mountain savory herb was placed in a Leśniczanka-type drying chamber. Not all parts of the raw material dried evenly, so the drying time was 8 days. Furthermore, the layer of fresh herb on a single sieve was approximately 15 cm thick, which also extended the drying time. In the case of the *S. montana* herb, the stems remained moist for a long time. When the stems broke with a characteristic cracking sound and the leaves rustled, the drying process was complete. The drying conditions in the conducted tests were consistent with the manufacturer's recommendations [Kołodziej 2018].

The dried herb was sieved (mesh size 4–5 mm) to obtain rubbed herb material, comprising leaves and

shoot tips, from which the essential oil was extracted. The water content in raw material intendent for testing depending on the harvest data from 6% to 14%.

Chemical analyses of plant material

Essential oil extraction. Essential oils were extracted by hydrodistillation using a Clevenger-type apparatus. Samples of 20 g dried herb were placed into a 1000 mL round-bottom flask, and 400 mL distilled water was added. The mixture was brought to boiling, and distillation was carried out for 3 hours, maintaining a consistent distillation rate throughout the extraction. After distillation, heating was stopped, and following a 15-minute cooling period, essential oil volumes were measured using a calibrated receiver according to standard protocols described in the Polish Pharmacopoeia IX [Farmakopea Polska IX 2011].

GC-MS analysis of essential oil. Chemical constituents of the essential oil were identified using a Varian 4000 ITMS GC-MS/MS system (Varian, USA), equipped with a CP-8410 auto-injector and a VF-5ms capillary column (30 × 0.25 mm i.d., 0.25 µm film thickness; Varian, USA). Helium was employed as the carrier gas at a flow rate of 0.5 mL/min. Injector and detector temperatures were maintained at 220 °C and 200 °C, respectively. A split ratio of 1:20 was used, and injection volume was 1 µL. The temperature program was as follows: initial temperature of 60 °C held for 0.5 min, increased by 3 °C/min to 246 °C, and maintained at 246 °C for 10 minutes. Ionization was performed at 70 eV, and mass spectra were recorded in the mass range of 40–1000 Da with a scan time of 0.80 s. Qualitative identification of compounds was performed by comparing obtained mass spectra to reference spectra in the NIST Mass Spectral Library, supplemented with confirmation by comparing calculated retention indices with literature values [Adams 2007]. The retention index was determined according to Van Den Dool and Kratz [1963].

Statistical analysis

The obtained results are presented as the means and were statistically analyzed by ANOVA, and the averages were compared using Tukey's HSD test at the probability level $\alpha = 0.05$. Statistical analyses were calculated with Statistica 13.3 PL software (StatSof Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

The essential oil content in *S. montana* herb collected at the beginning of flowering (1.63–2.20%) and at full flowering (1.88–2.20%) was indeed higher than in other term of extraction. This trend persisted throughout all years of the study (Table 1). According to Zawiślak and Nurzyńska [2017 a], the oil content in winter savory herb collected at the beginning of flowering ranged from 1.44 to 2.04%. In the studies by Skočibušić and Bezić [2004], the oil content in flowering plants was 1.7%. In the studies conducted, the herb collected from plants in the vegetative phase contained the least amount of essential oil (1.39–1.51%). A decrease in oil content was observed in *S. montana* at the last stage of the study (in plants that had finished flowering). The oil content in this phase ranged from 1.47 to 1.55% (Table 1).

A total of 44 chemical compounds were identified (Tables 2–4), aligning closely with previously reported ranges of 29–47 [Čavar et al. 2013, Miladi et al. 2013, Hudz et al. 2020, Kovačević et al. 2021, Górska-Drabik et al. 2024]. The essential oil from winter savory herb was dominated by oxygenated monoterpenes and

Table 1. Essential oil content in winter savory herb depending on the plant development stage (%)

Plant development stage	2017	2018	2019
Vegetative stage	1.51 c	1.47 b	1.39 c
Onset of flowering	2.20 a	1.94 a	1.63 b
Full flowering	1.88 b	2.01 a	2.20 a
Senescence	1.54 c	1.47 b	1.55 b

Values marked with the same letter in column do not differ significantly

Table 2. Chemical composition (%) of essential oil from the herb of *Satureja montana* L. (2017)

Compounds	RI	2017			
		vegetative stage	onset of flowering	full flowering	senescence
α-thujene	856	1.16 ±0.02	1.02 ±0.08	0.95 ±0.03	1.29 ±0.06
α-pinene	862	0.73 ±0.00	0.65 ±0.03	0.61 ±0.01	0.88 ±0.03
camphene	877	0.25 ±0.00	0.29 ±0.01	0.26 ±0.00	0.53 ±0.02
sabinene	897	0.14 ±0.00	0.13 ±0.00	0.13 ±0.00	0.16 ±0.01
β-pinene	901	0.11 ±0.01	0.14 ±0.02	0.08 ±0.02	0.32 ±0.01
myrcene	909	1.48 ±0.04	1.16 ±0.05	1.47 ±0.00	1.27 ±0.10
α-phellandrene	923	0.26 ±0.06	0.27 ±0.04	0.25 ±0.02	0.25 ±0.04
δ-2-carene	924	tr	tr	–	–
α-terpinene	931	1.77 ±0.01	1.95 ±0.07	1.73 ±0.02	1.88 ±0.06
p-cymene	938	3.76 ±0.07	5.28 ±0.17	3.89 ±0.04	9.72 ±0.28
limonene	941	0.10 ±0.01	0.14 ±0.00	0.10 ±0.00	0.18 ±0.01
β-phellandrene	943	tr	tr	tr	tr
(Z)-β-ocimene	945	1.94 ±0.06	0.12 ±0.07	3.37 ±0.07	0.15 ±0.00
(E)-β-ocimene	954	0.33 ±0.03	0.45 ±0.01	0.56 ±0.05	0.50 ±0.01
γ-terpinene	964	9.39 ±0.13	9.98 ±0.26	9.51 ±0.15	9.23 ±0.22
cis-sabinene hydrate	977	0.54 ±0.01	0.63 ±0.03	0.52 ±0.01	0.88 ±0.01
terpinolene	988	0.08 ±0.00	0.06 ±0.01	0.07 ±0.00	0.09 ±0.01
trans-sabinene hydrate	1006	0.17 ±0.02	0.39 ±0.02	0.17 ±0.01	0.43 ±0.01
borneol	1092	0.68 ±0.01	0.89 ±0.01	0.83 ±0.02	1.67 ±0.01
terpinen-4-ol	1103	0.33 ±0.01	0.21 ±0.02	0.28 ±0.01	0.37 ±0.02
α-terpineol	1117	tr	tr	tr	0.10 ±0.01
thymol	1196	6.99 ±0.02	2.61 ±0.02	5.91 ±0.02	2.94 ±0.02
carvacrol	1206	63.58 ±0.54	70.36 ±0.58	62.36 ±0.37	63.71 ±0.86
α-copaene	1289	0.07 ±0.01	0.06 ±0.01	0.11 ±0.01	0.07 ±0.01
β-bourbonene	1299	tr	tr	tr	tr
α-gurjunene	1319	tr	tr	tr	tr
trans-caryophyllene	1331	2.64 ±0.05	0.20 ±0.01	2.76 ±0.02	0.31 ±0.01
β-gurjunene	1338	tr	tr	tr	tr
β-copaene	1340	tr	tr	0.06 ±0.00	tr
aromadendrene	1347	0.50 ±0.00	0.62 ±0.01	0.63 ±0.00	0.63 ±0.00
α-humulene	1362	0.10 ±0.00	0.10 ±0.00	0.10 ±0.00	0.10 ±0.01
allo-aromadendrene	1366	0.06 ±0.00	0.06 ±0.00	0.08 ±0.00	0.06 ±0.00
γ-muurolene	1377	0.22 ±0.01	0.18 ±0.02	0.33 ±0.01	0.15 ±0.00
germacrene d	1384	0.14 ±0.03	0.11 ±0.01	0.18 ±0.02	0.10 ±0.01
viridiflorene	1392	0.58 ±0.01	0.59 ±0.01	0.79 ±0.00	0.54 ±0.01
bicyclogermacrene	1396	0.51 ±0.01	0.55 ±0.01	0.63 ±0.01	0.46 ±0.01
epizonarene	1402	tr	tr	tr	tr
β-bisabolene	1407	0.32 ±0.00	tr	0.05 ±0.02	0.07 ±0.00
γ-cadinene	1414	0.16 ±0.00	0.14 ±0.01	0.20 ±0.01	0.10 ±0.00
δ-amorphene	1418	tr	tr	–	–
α-cadinene	1440	0.39 ±0.00	0.36 ±0.01	0.52 ±0.02	0.27 ±0.01
spathulenol	1486	0.14 ±0.00	0.17 ±0.02	0.20 ±0.00	0.24 ±0.02
caryophyllene oxide	1492	0.07 ±0.00	0.08 ±0.01	0.06 ±0.00	0.20 ±0.01
globulol	1496	tr	tr	0.06 ±0.00	0.06 ±0.00
Total	–	99.69	99.95	99.81	99.91

tr (trace) <0.05%

RI – non-isothermal Kovats'a retention indices (from temperature – programming using definition of Van Den Dool and Kratz [1963]), for series of n-alkanes C_6–C_{40}

Table 3. Chemical composition (%) of essential oil from the herb of *Satureja montana* L. (2018)

Compounds	RI	2018			
		vegetative stage	onset of flowering	full flowering	senescence
α-thujene	856	1.04 ±0.09	0.98 ±0.05	1.12 ±0.08	1.24 ±0.04
α-pinene	862	0.73 ±0.02	0.62 ±0.03	0.66 ±0.03	0.70 ±0.02
camphene	877	0.46 ±0.00	0.20 ±0.02	0.27 ±0.01	0.22 ±0.01
sabinene	897	0.14 ±0.00	0.14 ±0.00	0.15 ±0.00	0.15 ±0.00
β-pinene	901	0.24 ±0.01	0.12 ±0.09	0.29 ±0.01	0.10 ±0.07
myrcene	909	1.38 ±0.02	1.48 ±0.01	1.54 ±0.01	1.43 ±0.01
α-phellandrene	923	0.19 ±0.08	0.20 ±0.11	0.24 ±0.00	0.33 ±0.00
δ-2-carene	924	tr	tr	–	–
α-terpinene	931	1.49 ±0.01	1.68 ±0.01	1.34 ±0.03	1.81 ±0.03
p-cymene	938	6.05 ±0.07	4.01 ±0.07	3.61 ±0.07	4.82 ±0.08
limonene	941	0.13 ±0.00	0.09 ±0.00	0.08 ±0.00	0.14 ±0.01
β-phellandrene	943	tr	tr	tr	tr
(Z)-β-ocimene	945	3.31 ±0.02	2.76 ±0.06	3.07 ±0.04	0.93 ±0.01
(E)-β-ocimene	954	0.40 ±0.00	0.44 ±0.10	0.52 ±0.00	0.15 ±0.01
γ-terpinene	964	6.93 ±0.02	9.23 ±0.19	7.28 ±0.12	8.21 ±0.02
cis-sabinene hydrate	977	0.97 ±0.02	0.57 ±0.01	0.60 ±0.00	0.63 ±0.03
terpinolene	988	0.07 ±0.00	0.07 ±0.01	0.06 ±0.00	0.06 ±0.00
trans-sabinene hydrate	1006	0.23 ±0.01	0.24 ±0.02	0.16 ±0.05	0.32 ±0.02
borneol	1092	1.62 ±0.09	0.71 ±0.01	0.82 ±0.03	0.63 ±0.06
terpinen-4-ol	1103	0.39 ±0.08	0.36 ±0.03	0.29 ±0.01	0.27 ±0.04
α-terpineol	1117	0.10 ±0.01	0.08 ±0.00	0.07 ±0.00	0.09 ±0.04
thymol	1196	14.90 ±0.17	8.94 ±0.26	9.02 ±0.15	19.47 ±0.03
carvacrol	1206	51.43 ±0.79	60.97 ±0.69	62.37 ±0.72	52.58 ±0.19
α-copaene	1289	0.08 ±0.00	0.06 ±0.00	0.06 ±0.00	0.06 ±0.00
β-bourbonene	1299	0.09 ±0.01	tr	tr	0.06 ±0.04
α-gurjunene	1319	tr	tr	tr	tr
trans-caryophyllene	1331	2.56 ±0.14	2.75 ±0.00	3.13 ±0.04	2.61 ±0.10
β-gurjunene	1338	tr	tr	tr	tr
β-copaene	1340	0.08 ±0.01	0.05 ±0.00	0.06 ±0.00	tr
aromadendrene	1347	0.45 ±0.02	0.37 ±0.01	0.31 ±0.00	0.24 ±0.01
α-humulene	1362	0.10 ±0.00	0.11 ±0.00	0.12 ±0.00	0.10 ±0.00
allo-aromadendrene	1366	0.06 ±0.00	tr	–	–
γ-muurolene	1377	0.31 ±0.01	0.22 ±0.01	0.21 ±0.00	0.11 ±0.02
germacrene D	1384	0.36 ±0.00	0.18 ±0.00	0.19 ±0.01	0.15 ±0.01
viridiflorene	1392	0.54 ±0.00	0.43 ±0.02	0.39 ±0.02	0.27 ±0.00
bicyclogermacrene	1396	0.79 ±0.01	0.52 ±0.03	0.56 ±0.03	0.37 ±0.00
epizonarene	1402	tr	tr	tr	tr
β-bisabolene	1407	0.46 ±0.00	0.25 ±0.01	0.37 ±0.03	0.91 ±0.00
γ-cadinene	1414	0.22 ±0.01	0.16 ±0.02	0.16 ±0.02	0.13 ±0.01
δ-amorphene	1418	0.50 ±0.02	0.35 ±0.03	0.33 ±0.04	0.24 ±0.02
α-cadinene	1440	tr	tr	tr	–
spathulenol	1486	0.65 ±0.04	0.22 ±0.01	0.18 ±0.02	0.15 ±0.02
caryophyllene oxide	1492	0.35 ±0.01	0.22 ±0.01	0.22 ±0.04	0.16 ±0.03
globulol	1496	0.09 ±0.01	0.06 ±0.01	–	tr
Total	–	99.89	99.84	99.85	99.84

tr (trace) <0.05%

RI – non-isothermal Kovats' a retention indices (from temperature – programming using definition of Van Den Dool and Kratz [1963]), for series of n-alkanes C_6–C_{40}

Table 4. Chemical composition (%) of essential oil from the herb of *Satureja montana* L. (2019)

Compounds	RI	2019			
		vegetative stage	onset of flowering	full flowering	senescence
α-thujene	856	0.83 ±0.01	1.13 ±0.04	1.33 ±0.04	1.1 ±0.06
α-pinene	862	0.55 ±0.01	0.63 ±0.02	0.7 ±0.03	0.7 ±0.03
camphene	877	0.23 ±0.00	0.17 ±0.00	0.14 ±0.01	0.31 ±0.01
sabinene	897	0.13 ±0.00	0.14 ±0.00	0.16 ±0.01	0.15 ±0.00
β-pinene	901	0.21 ±0.01	0.22 ±0.01	0.22 ±0.01	0.36 ±0.00
myrcene	909	1.38 ±0.02	1.49 ±0.06	1.57 ±0.11	1.23 ±0.01
α-phellandrene	923	0.17 ±0.04	0.29 ±0.02	0.29 ±0.02	0.12 ±0.06
δ-2-carene	924	0.06 ±0.00	0.05 ±0.00	0.06 ±0.01	0.05 ±0.00
α-terpinene	931	1.37 ±0.01	1.44 ±0.04	1.45 ±0.13	1.11 ±0.03
p-cymene	938	5.16 ±0.10	3.37 ±0.10	4.05 ±0.36	5.46 ±0.01
limonene	941	0.08 ±0.00	0.07 ±0.00	0.07 ±0.01	0.07 ±0.01
β-phellandrene	943	tr	tr	tr	tr
(Z)-β-ocimene	945	4.08 ±0.15	1.86 ±0.07	1.6 ±0.12	1.72 ±0.01
(E)-β-ocimene	954	0.65 ±0.03	0.34 ±0.04	0.28 ±0.01	0.31 ±0.06
γ-terpinene	964	5.73 ±0.06	7.71 ±0.24	7.61 ±0.71	5.01 ±0.04
cis-sabinene hydrate	977	0.60 ±0.03	0.67 ±0.06	0.68 ±0.03	0.66 ±0.02
terpinolene	988	tr	tr	–	–
trans-sabinene hydrate	1006	0.48 ±0.01	0.16 ±0.04	0.14 ±0.06	0.48 ±0.01
borneol	1092	0.85 ±0.04	0.51 ±0.02	0.31 ±0.00	0.87 ±0.01
terpinen-4-ol	1103	0.30 ±0.01	0.32 ±0.01	0.34 ±0.01	0.27 ±0.03
α-terpineol	1117	0.12 ±0.03	0.12 ±0.00	0.11 ±0.01	0.12 ±0.05
thymol	1196	3.14 ±0.05	2.77 ±0.11	2.34 ±0.05	2.62 ±0.04
carvacrol	1206	65.49 ±0.19	72.03 ±0.73	73.54 ±1.18	68.8 ±0.06
α-copaene	1289	0.09 ±0.00	0.10 ±0.01	0.05 ±0.03	0.08 ±0.04
β-bourbonene	1299	0.10 ±0.00	tr	tr	0.15 ±0.02
α-gurjunene	1319	tr	tr	tr	tr
trans-caryophyllene	1331	3.20 ±0.11	0.51 ±0.01	0.57 ±0.06	4.5 ±0.04
β-gurjunene	1338	tr	tr	tr	tr
β-copaene	1340	0.08 ±0.00	0.05 ±0.00	–	0.06 ±0.00
aromadendrene	1347	0.43 ±0.01	0.44 ±0.01	0.25 ±0.00	0.17 ±0.00
α-humulene	1362	0.13 ±0.00	0.11 ±0.00	0.07 ±0.01	0.19 ±0.02
allo-aromadendrene	1366	0.06 ±0.01	0.07 ±0.00	–	–
γ-muurolene	1377	0.26 ±0.00	0.22 ±0.00	0.12 ±0.00	0.16 ±0.00
germacrene D	1384	0.23 ±0.00	0.15 ±0.00	0.15 ±0.02	0.30 ±0.01
viridiflorene	1392	0.49 ±0.01	0.50 ±0.01	0.27 ±0.01	0.23 ±0.00
bicyclogermacrene	1396	0.58 ±0.01	0.64 ±0.00	0.62 ±0.05	0.38 ±0.01
epizonarene	1402	tr	tr	tr	tr
β-bisabolene	1407	0.32 ±0.01	0.23 ±0.01	0.25 ±0.05	0.55 ±0.02
γ-cadinene	1414	0.23 ±0.00	0.18 ±0.00	0.11 ±0.01	0.15 ±0.01
δ-amorphene	1418	0.46 ±0.01	0.37 ±0.00	0.18 ±0.01	0.25 ±0.03
α-cadinene	1440	tr	tr	tr	–
spathulenol	1486	0.79 ±0.00	0.31 ±0.01	0.13 ±0.10	0.23 ±0.07
caryophyllene oxide	1492	0.77 ±0.02	0.53 ±0.07	0.11 ±0.09	0.89 ±0.22
globulol	1496	0.12 ±0.02	0.06 ±0.02	0.07 ±0.02	0.07 ±0.00
Total		99.95	99.96	99.94	99.88

tr (trace) <0.05%
RI – non-isothermal Kovats'a retention indices (from temperature – programming using definition of Van Den Dool and Kratz [1963]), for series of n-alkanes C_6–C_{40}

Table 5. The content of four main components of EO obtained from winter savory in different stage of vegetation in three years of the study

Compounds	Stage	2017	2018	2019
carvacrol	vegetative stage	63.58 b	51.43 b	65.49 c
	onset of flowering	70.35 a	60.97 a	72.03 a
	full flowering	62.36 c	62.37 a	73.54 a
	senescence	63.71 b	52.58 b	68.80 b
thymol	vegetative stage	6.99 a	14.90 b	3.14 a
	onset of flowering	2.61 b	8.94 c	2.77 b
	full flowering	5.91 a	9.02 c	2.34 b
	senescence	2.94 b	19.47 a	2.62 b
γ-terpinene	vegetative stage	9.39 b	6.93 b	5.73 b
	onset of flowering	9.98 a	9.23 a	7.71 a
	full flowering	9.51 a	7.28 b	7.61 a
	senescence	9.23 b	8.21 a	5.01 b
p-cymene	vegetative stage	3.76 b	6.05 a	5.16 a
	onset of flowering	5.28 b	4.01 b	3.37 b
	full flowering	3.89 b	3.61 b	4.05 a
	senescence	9.72 a	4.82 ab	5.46 a

Values marked with the same letter in the column (for each component separately) do not differ significantly

monoterpene hydrocarbons (Tables 2–4). In studies by many authors, the main components also belonged to these chemical groups [Čavar et al. 2013, Miladi et al. 2013, Hudz et al. 2020, Kovačević et al. 2021, Górska-Drabik et al. 2024]. According to Wesołowska et al. [2017], the distillation time does not affect the amount of components contained in the oil from *S. montana* or their content. In the present experiment, the oil hydrodistillation process lasted 3 hours.

The dominant compounds in all years and developmental stages of *Satureja montana* were: carvacrol (51.43–73.54%), γ-terpinene (5.01–9.98%), thymol (2.34–19.47%) and *p*-cymene (3.37–9.72); see Tables 2–4. Studies by Ćavar et al. [2013] showed that the main components of essential oil obtained from *S. montana* in Croatia were carvacrol (63.4%) and thymol (19.4%). In the study by Trifan et al. [2015], the carvacrol content in the oil from mountain savory grown in Romania was also 63.4%. In the study by Wesołowska et al. [2017], the essential oil isolated from *S. montana* grown in Poland and harvested during flowering contained 54.44–68.53% carvacrol. The present study showed the highest carvacrol content in fully flowering plants (73.54% in 2019) and at the beginning of flowering (72.03% in 2019 and 70.36% in 2017); see Table 5. The lowest carvacrol content was found in herb harvested from plants in the vegetative phase (51.43–65.49%). Essential oils from winter savory were characterized by a high carvacrol content in studies by Abbad et al. [2025] – 50.8% (cultivated in Morocco), Miladi et al. [2013] – 53.35% (natural sites in France) and Kavačević et al. [2021] – 55.01% (cultivated in Serbia). According to Trifan et al. [2015] winter savory, which contains significant amounts of carvacrol in its essential oil, belongs to the carvacrol chemotype. Carvacrol is a phenolic compound and is most often found in the company of its isomer, thymol [Schönknecht et al. 2016].

Essential oil from Montenegro extracted from *S. montana* before flowering contained thymol as the main component at a level of 37.36%, and the carvacrol content was 15.47% [Damjanović-Vratnica et al. 2011]. In the study by Górska-Drabik et al. [2024], thymol dominated in the oil from *S. montana* (40.04%). In the present study the oils extracted from plants in different phenological stages had a significantly lower thymol content in each year (Table 5). The herb collected from plants after flowering contained the highest amount of thymol (19.47% in 2018). The thymol content in essential oil from winter savory

in its natural site in Serbia during the flowering phase was 16.7% [Djordjevic et al. 2021], and in the studies by Dimitrijević et al. [2025] was 15.5%. The oil from winter savory, collected during the flowering phase in Ukraine and analyzed by Hudz et al. [2020], had an unusual chemical profile. *P*-thymol, an isomer of thymol and carvacrol, was dominant, with a level of 81.79%.

Based on the conducted research, it was demonstrated that γ-terpinene was present at the highest level at the beginning of *S. montana* flowering (7.71–9.98%) and at full flowering (7.61–9.51%); see Table 5. During the growing season, the γ-terpinene content changed slightly. In the study by Wesołowska et al. [2017], the γ-terpinene content in the oil during the flowering period ranged from 5.21 to 8.67%. In winter savory cultivated in Ukraine, γ-terpinene was present at a level of 0.9–6.6% [Pokajewicz et al. 2023]. In the studies by Górska-Drabik et al. [2024], γ-terpinene was one of the components present in high concentrations in the oil from *S. montana*. In the work of Kavačević et al. [2021], the level of γ-terpinene was 11.09%, in Abbad et al. [2025] was 18.5%, and in the studies of Dimitrijević et al. [2025] was only 3.1%.

The highest concentration of *p*-cymene was found in herbs obtained from plants that had finished flowering in 2017 (9.72%) and 2019 (5.46%); see Table 5. Damjanović-Vratnica et al. [2011] obtained different results. According to these researchers, the oil from *S. montana* herb before and during flowering contained *p*-cymene at levels of 7.86% and 31.37%, respectively. In the studies by Pokajewicz et al. [2023], *p*-cymene was also considered the dominant component of winter savory oil (5.0–8.8%). Similarly, Trifan et al. [2015] identified *p*-cymene (10.97%) as the main compound in *S. montana* oil.

Based on the conducted research, it was demonstrated that the α-terpinene content in *S. montana* essential oil remained relatively stable across all stages of plant development ranging from 1.11% to 1.95% (Tables 2–4). A similar trend was observed for myrcene. It concentration varied only slightly from 1.16% to 1.57%. Particularly noteworthy were the results concerning *trans*-caryophyllene in 2018. In the vegetative phase, *S. montana* essential oil contained 2.56% *trans*-caryophyllene. Its increased during full flowering, reaching 3.13%, followed by a decline in the post-flowering stage to 2.61% (Table 3). In the other years of the study, no such relationship was found in the *trans*-caryophyllene content (Tables 2 and 4).

Essential oil from *S. montana* from sites in Albania contained significant amounts of linalool (11.0%) [de Oliveira et al. 2011]. In the study by Milos et al. [2001], linalool was also the main component of the oil in winter savory at some sites in Croatia. The linalool content varied depending on the environment, with a maximum level of 62%. Čopra-Janićijević et al. [2020], due to the high linalool content in the EO, classified *S. montana* occurring in natural sites in Bosnia and Herzegovina as linalool type. In the present experiment, no linalool was found in cultivated plants (Table 2–4).

CONCLUSIONS

The essential oil obtained from winter savory cultivated in Lublin region was rich in phenolic compounds (carvacrol and thymol). The highest carvacrol content was found in essential oil obtained at the peak of flowering or at the beginning of flowering. However, the thymol content in the essential oil varied during the plant's developmental stages over the years. This proves the high quality of the essential oil and indicates the possibility of obtaining it from *S. montana* throughout the plant's growing season for medicinal purposes. The main components of the essential oil were also γ-terpinene and *p*-cymene, whose content also varied during the different stages of plant development. The results highlight the promising potential for introducing and expanding the cultivation of *S. montana* in Poland, especially for pharmaceutical and therapeutic applications. Further research should focus on optimizing agrotechnical factors in order to obtain a high-quality yield of *S. montana* raw material.

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KOKEDAMA – THE JAPANESE TECHIQUE OF PLANT CULTIVATION AND ITS POTENTIAL FOR THERAPEUTIC APPLICATIONS

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ABSTRACT

Kokedama is a Japanese art of growing plants using natural substrates, moss and ornamental plants, which is in line with trends of the bonsai art. It is a soilless and containerless cultivation method that allows reducing amounts of plastics and aids the care for the natural environment. It has also been proven that not only growing plants, but also just staying in their vicinity has a positive effect on human mental and physical health. These positive effects have been observed in ancient times when plants were introduced into the living space. Today, this is particularly important in the era of urbanization and spending more time indoors, which increases man's pursuit of contact with the natural environment. This work attempts to present the possibilities of using kokedama in horticultural therapy aimed at improving socialization of the populations.

Keywords: soilless cultivation, ornamental plants, containerless cultivation, human health

INTRODUCTION

In the modern world, more and more attention is paid to the aestheticization of the living environment of man. It pertains not only to residential premises and directly adjacent green spaces, but also to public buildings such as offices, hospitals, kindergartens or schools. Plants are one of the largest groups of living organisms accompanying humans and modeling their space, with plant-human relations dating back to the beginnings of the human evolution [Schaal 2019]. This is particularly noticeable in the Greek and Roman civilizations, where ornamental plants (*Hyacinthus* L., *Narcissus* L., *Hedera* L.) were used due to their reference to mythology [Francini et al. 2022]. The continuous need for closeness with nature in the man's environment does not change in the environment of a modern city [Kellert and Wilson. 1993] especially given the 68% increase expected in the urban population worldwide by 2050 [United Nations, 2025]. Many people cannot imagine life without plants in their environment [Gronostajska 2007, Selim 2021, Yerli and Sitemoglu 2024]. The beginnings of growing plants in closed spaces were recorded several thousand years ago in China [Zimny 2008]. In Egypt, plants were brought to houses in the 3rd century BC, and it is known from the ruins of Pompeii that indoor plants were already known more than 2,000 years ago

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[Bringslimark et al. 2009]. According to Deng and Deng [2018], city dwellers spend about 80–90% of their lives indoors, which is forced by the contemporary lifestyle. Therefore, increasing emphasis is being put on growing plants inside buildings. At home, plants contribute not only to environment purification or space aesthetics but also elicit beneficial effects on human health [Bermejo and Sparke 2019]. In the context of the COVID-19 pandemic, a significant improvement has been noted in the mental health of persons having contact with plants [Tu et al. 2020, Phillips and Schulz 2021, Pérez-Urrestarazu et al. 2021]. And this effect was even enhanced by the activities linked to the care, planting, watering or fertilizing plants and making plant compositions [Han 2018]. Such activities are part of a horticultural therapy, i.e., a form of treatment based on the natural relationship between humans and nature [Górska-Kłęk et al. 2009].

Given the above, programs aimed at improving human mental health are developed that integrate elements of the natural environment, such as landscape, natural light, plant scents, natural materials, the sound of water and birdsong with people's everyday lives [Mcsweeney et al. 2015, Chung et al. 2024]. Small areas or unfavorable habitat conditions are no longer bottlenecks to the cultivation of various plant species, and even persons who do not have large spaces can benefit from such a therapy. Modern technologies for controlling artificial light, self-irrigating containers as well as soilless cultivation methods including hydroponics, aeroponics or aquaponics are viable and helpful solutions in this case. Hanging compositions are popular as well, e.g., green vertical walls which impart a modern style to rooms [Azkorra et al. 2015, Venuh et al. 2023]. Minimalism, simplicity and elegance are highly valued. That is why, soilless and containerless cultivation becomes more and more common as it offers a great visual effect and simplicity, thereby bridging the indoor space and the natural environment of people's life [Mackoś-Iwaszko and Nowak 2017]. An example of the modern introduction of green vegetation to rooms is kokedama, namely, the Japanese art of plant cultivation harnessed also for the purposes of horticultural therapy. Growing plants in a substrate mixture wrapped in moss ("moss ball"), either suspended or standing, not only results in producing a decorative element, but also could be an example of horticultural therapy [Han 2018, Chung et al. 2024].

The aim of this work is to present the characteristics and possibilities of Japanese plant cultivation, such as kokedama, so that it can be successfully used in horticultural therapy, emphasizing its ease of creation and subsequent maintenance.

THE IMPACT OF ORNAMENTAL PLANTS ON HUMAN HEALTH AND WELL-BEING

In the contemporary world, where people are exposed to excessive stimuli, they need peace and quiet, which can be provided by their contact with nature. Ehrlich and Raven [1964] are creators of the "coevolution" concept, which refers to the process in which species interact with each other. Its definition also encompasses the relationship between plants and humans, including the beneficial ones. The biophilia hypothesis assumes that humans possess an innate need to seek connections with nature [Kellert and Wilson 1993].

The beneficial effect of potted plants on human general health, mental health and well-being has been proven in scientific research. Staying among potted plants is associated with mitigated feeling of pain, fear, unhappiness and aggression [Burchett et al. 2008]. In their survey conducted among 1,200 German consumers, Bermejo and Sparke [2019] showed that having ornamental plants at home not only resulted in satisfaction with the appearance of the living space but also generally improved the quality of life. Interiors with green plant elements allow for contact with nature, thereby satisfying people's longing for a natural environment and alleviating the feeling of stress compared to the interiors without plants [Thomsen et al. 2011, Deng and Deng 2018]. Studies conducted in the United States, Europe, Asia, and the Middle East confirm the positive impact of horticultural therapy on human health, in terms of reducing depression and anxiety, as well as increasing life satisfaction and quality [Soga et al. 2016, Zawiślak 2024]

Other authors [Grinde et al. 2009] also emphasized that potted plants can help reduce stress and suppress negative emotions. Improved well-being and even improved cognitive functions under the influence of contact with potted plants were demonstrated by Han et al. [2022]). Another experiment investigated the interac-

tion between plants and humans. To this end, 24 men were divided into 2 groups, and those from the first group were asked to replant a *Peperomia dahlstedtii* C.DC. plant, while those from the second group were given a task involving computer work. Men's feelings during replanting activities were different compared to those of men performing the computer task. Contact with plants provided a sense of calmness and comfort, as evidenced by the sympathetic nervous system examination and blood pressure measurement [Lee et al. 2015]. An empirical study conducted in China, which examined 430 participants, found correlations between plant care activities and psychological well-being [Ma 2022]. Persons in contact with nature had a low brain wave frequency and lesser brain activity in the frontal areas, which indicates regeneration and a sense of relaxation and relief [Norwood et al. 2019]. In addition, contact with nature can help suppress negative emotions resulting from stressful life events [Van den Berg et al. 2010].

Having plants in the man's environment is important not only in residential premises but also in offices or public utility buildings. An office with plants can have a positive effect on employees [Elzeyadi 2011], including their work comfort, mood, creativity, and stress reduction. This is partly due to increased humidity and the absorption of harmful substances by plants. Owing to their phytoremediating ability, green plants act like filters and are potent to absorb total volatile organic compounds (TVOC), carbon monoxide (CO), CO_2, formaldehyde and benzene [Hall and Knuth 2019, Liu 2022]. Having plants in the office is a viable means to increase overall productivity without too much extra spending [Husti et al. 2015]. Nieuwenhuis et al. [2014] proved that workers who had plants in their view completed a concentration test 19% faster compared to those who were not surrounded by plants. The Rural Development Administration of South Korea recommends placing one small potted plant and one large potted plant per 6 m^2 of floor space to improve the interior's quality [Kim et al. 2013]. Three small-sized or medium-sized plants in the interior have been shown to exert a positive effect on the mood, productivity, and reaction time of workers [Jumeno and Matsumoto 2013].

Contact with plants has also a positive effect on children's well-being, concentration, and reduces aggressive behaviors between peers [Nowak 2005]. ADHD children concentrate better after a walk in the park [Taylor and Kuo 2009]. In schools having green walls in classrooms, children received better scores in concentration tests and achieved 20–26% better academic results, learning the curriculum faster [Van Duijin et al. 2011, Van den Berg et al. 2017]. At universities, contact with plants and their care by students were reported to contribute to relieving stress, tension and anxiety, and also eliminate sleep problems [Yang et al. 2024]. Horticultural therapy is most commonly used among people with mental disabilities, after strokes, with sensory disorders, hearing and vision impairments, in depression, paralyzed individuals, as well as those excluded from society, alcoholics, drug

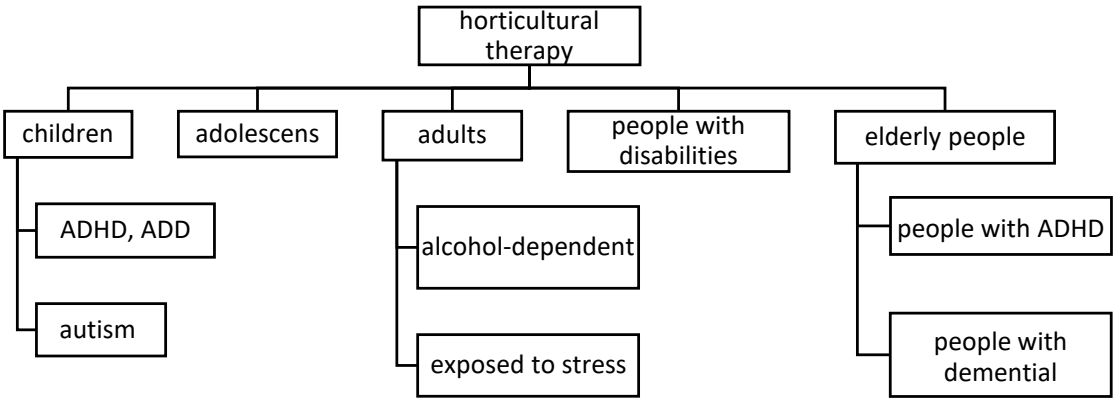

Fig. 1. Horticultural therapy as an element supporting the therapy of various groups of people

addicts, or people in prison (Fig. 1) [Zawiślak 2015, Mackoś-Iwaszko and Nowak 2017].

KOKEDAMA CONCEPT

Kokedama is an art of plant cultivation originating from Japan. Like bonsai, it is created using ornamental plants in minimalist arrangements. The exact origins of kokedama are inexplicit, but it probably began in Japan's Edo era (1608–1868), based on the Nearai Bonsai method, and was propagated by persons from lower social classes as a substitute for bonsai. Therefore, some authors call it "bonsai for the poor" because the plants do not require specialist pruning nor appropriate containers [Oshima and Kimura 2017, Lokare and Keshamma 2021]. It is believed that kokedama fits into the Kawai style that has been prevailing in Japan for 40 years [Esentürk and Yerli 2019] and pertains to a container-free and soil-free cultivation of plants. The roots of the plant are covered with a special substrate mixture, which is formed in the shape of a ball. In order to maintain optimal conditions for the root system development and thus for plant growth, the ball is coated with a layer of moss and wrapped with a string. Therefore, the term kokedama comes from the Japanese words "koke" meaning a ball, and "dama" meaning moss. Kokedama is used to grow many species of plants, including herbs, grasses and ferns, but most often it serves to grow ornamental plants [Sunamori 2012, Ibrahim 2025]. Kokedama can be in a hanging or a standing form. A properly composed substrate provides stable air-water conditions and prevents moisture loss. The moss ball is watered once a week or less frequently by immersing it in water when the substrate had dried out [Putra et al. 2021]. As the authors report, kokedama is an ecological and social plant cultivation method since it uses organic substrates and natural materials, and there is a complete elimination of plastics [Wiyatasari 2019, Panglipurningrum et al. 2024, Afnan et al. 2025].

Kokedama substrates

Kokedama is a cultivation system where a specially composed mixture of substrate with added water is stuck to the roots of plants and wrapped with moss [Oshima and Kimura 2017, Mitarai 2021]. The mixture consists of keto, akadama, and zeolite. Keto is a Japanese substrate used for growing bonsai. It is

a) b)

Fig. 2. Orchids (a) – *Phalaenopsis* Blume, and crocus (b) – *Crocus* L. in kokedama cultivation. Photo by K. Pitura

a mixture of peat and soil from swampy areas of Japan. It has very good sorption properties, retains moisture and nutrients well, and has a dark brown color [Pietraszko and Sobota 2008].

Akadama is a slightly acidic volcanic soil, commonly found in Japan and called mud. It has a wide range of applications as an additive to the substrate due to its osmotic properties for oxygen and water [Budinova et al. 2009].

Zeolite is a naturally occurring, alkaline, hydrated aluminosilicate with a broad range of applications. It is of great importance in horticulture. Natural zeolites improve soil quality as they exhibit good water and nutrient retention capacity. They also possess chelating capacity, improving the sorption properties of soils [Elliot and Zhang 2005, Mondal et al. 2021].

HORTITHERAPEUTIC PROPERTIES OF KOKEDAMA

Horticultural therapy is a form of therapy related to the mental and physical regeneration of a person based on the genuine relationship between man and nature. This kind of therapy can be passive, aimed at observing nature and deriving health benefits from it, or it may take an active form including gardening work [Zawiślak 2015].

Kokedama fits into both passive and active parts of horticultural therapy. The passive aspect appreciates the aesthetic dimension and the green color, which has a calming effect on people. According to Eliot and Maier [2014] and Liu [2022], the color green has a soothing and relaxing effect, and makes people focus more on their inner experiences. The manual activity of mixing the substrate and shaping the ball could potentially support motor coordination and may enhance sensory stimulation. Caring for a finished kokedama, such as watering, fertilizing, or replenishing the moss, might constitute an element of active horticultural therapy, as well.

Plant-related activities complement conventional rehabilitation methods. They can be implemented in every age group, among patients with physical, mental and psychological disorders. Horticultural therapy helps in social integration and addiction therapy [Catlin 1998, Górska-Kłęk et al. 2009, Reis et al. 2020]. Kokedama is easy to grow; hence, it can become a substitute for a garden or a minimalist green form in the man's environment, which will contribute to health improvement in times of the growing ecological crisis [Pyurko et al. 2024].

Research conducted in the city of Batu allowed concluding that kokedama has a high aesthetic value,

a) b)

Fig. 3. Keto (a), and akadama (b) substrates. Photo by K. Pitura

attracts the attention of recipients, and ensuring pleasure in perceiving green vegetation. The care over plants is not troublesome as they require watering twice a week. Therefore, this form of cultivation is easy and convenient even for persons inexperienced in gardening [Putri and Siswadi 2024]. Yerli and Sitemoglu [2024] proves that kokedama has a positive effect on both the persons and the space they function in. In interior design, it enlivens the space, eliminates monotony, ensures a balance of colors and textures, and affects the aesthetics of the entire room, which facilitates man's functioning in this space. The appearance of kokedama has also an artistic dimension and therefore this type of plant cultivation is often called the "art of kokedama".

Kokedama was promoted in Indonesia as a social activity. During the COVID-19 pandemic, an online program was developed with training materials that precisely showed the planting method using this cultivation technique so that entire families locked in their homes could create unique decorations from plants and then take care over them [Sinaga et al. 2020]. Kokedama cultivation can be implemented as a method of therapeutic socialization. The Family Welfare Movement introduced kokedama-making workshops for mothers in Malaysia. In small villages, 30 women were taught to grow plants using this technique. The aim was to unite these women, and teach them how to grow plants appropriate to the environment and opportunities in which they live [Wiyatasari and Hum 2019]. The kokedama concept is environmentally friendly because it eliminates the use of plastic pots. Training in this technique was carried out among a group of Indonesian women and strengthened their social position and helped them understand the idea of recycling [Pintakami et al. 2024].

The psychological aspect of kokedama was investigated by Mitarai [2021]. A group of elderly and unemployed persons reported to experience peace and joy just by looking at kokedama installations. In turn, training in kokedama-making was conducted in the village of Tabang Kacang (Indonesia), to empower the position of village's housewives [Zulfita and Budi 2024]. In Sidomulyo (Indonesia), i.e., a village famous for growing ornamental plants and frequently visited by tourists, kokedama cultivation was introduced to increase their sales and help local residents make a living from tourism [Batu et al. 2021].

The research by Tu et al. [2020] noted the impact of kokedama on human health. A group of 27 people aged between 60 and 75 years underwent studies on the effects of horticultural activities as two plant cultivation methods Grass Doll and Kokedama, artistic creation Rocky Leaf Prints, and activity combined

a) b)

Fig. 4. Zeolit substrates (a), materials needed for kokedama preparation (b). Photo by K. Pitura

a) b)

Fig. 5. Marantha's (*Maranta leuconeura* Kerchoveana) in kokedama. Photo by K. Pitura

tasting and smelling of herbs. Based on the results of the study, engaging in a kokedama activity significantly reduced salivary amylase activity (SAA) among elderly participants, indicating a marked reduction in acute stress response. Moreover, kokedama contributed to a downward trend in pulse rate and in the ratio of low-frequency to high-frequency heart-rate variability (LF/HF), suggesting a shift toward increased parasympathetic (i.e., relaxation) nervous system activity. Participants also reported a significant decrease in "anger" (a negative mood subscale) and a significant increase in "vigor" (a positive mood subscale) after kokedama, demonstrating improved emotional well-being [Tu et al. 2020]. Moreover, kokedama increases creativity, and self-satisfaction that encourages students to take responsibility for caring for the plants, nature and environement [Afnan et al. 2025]. Kolański and Warachim [2023], conducting horticultural therapy among individuals with brain injury accompanied by cognitive dysfunctions and motor impairments. Authors observed an increase in muscle strength of the patients, improvement in their physical endurance, and an increase in the range of joint mobility. The horticultural therapeutic significance of kokedama can be emphasized during the preparation of the substrate. Mixing the blend with water helps increase the mobility of the wrist joints and allows the senses to perceive different textures.

In Poland, kokedama is less known plant cultivation method. Kokedama could be made with various plants e.g. *Chlorophytum* Ker Gaw, *Hedera helix* L., *Chamaedorea* Willd, *Epipremnum* Schott. (Fig. 5). The authors of this article conducted numerous kokedama-making workshops in various social welfare centers (Figs 6–7) during which they observed specific effects of this activity on participants. Residents consistently demonstrated noticeable satisfaction and engagement when provided the opportunity to interact directly with plants. In addition, persons with intellectual disabilities within the ADHD spectrum appeared to experience enhanced calmness, likely facilitated by the sensory stimulation associated with substrate mixing together in the group, and the manual formation of moss balls.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Although kokedama represents a relatively old art of plant cultivation, its therapeutic properties remain insufficiently elucidated. Existing scientific publications acknowledge its horticultural relevance, with the majority of studies and practical implementations

Fig. 6. Crokus (*Crocus* L) kokedama making workshops in Warsaw (Poland). Photo by K. Pitura

a) b)

Fig. 7. Kokedama making workshops in school in Poland with disabled persons (a and b). Photo by K. Pitura

reported in Indonesia. In contrast, no documented applications of kokedama as a therapeutic method have been identified in Europe or the Americas, where it has predominantly been described in decorative or amateur contexts. Until now, it has been considered only as a decorative element, but increasing body of literature suggests its potential applicability within horticultural therapy. However, its potential therapeutic benefits require further systematic and comprehensive research. Due to its simplicity of preparation, and the availability of required materials, kokedama may hold substantial promise as an accessible horticultural therapy tool across diverse age groups. Its ecological character, relying exclusively on natural materials, further enhances its suitability for contemporary therapeutic and educational practices.

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IMPROVING YIELD AND QUALITY OF RED PEPPER (*Capsicum annum* L.) USING CHITOSAN ELICITATION UNDER DEFICIT IRRIGATION

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ABSTRACT

In semiarid and arid climates in the Middle East, environmental stresses such as drought and water deficit result in significant reductions in the growth and productivity of horticultural and agronomic crops. To improve crop tolerance to water-deficient conditions, applying chitosan can be a practical approach. The foliar-spraying of medium molecular weight chitosan (450 kDa, 95–98% degree of deacetylation, and 30 mPa.s viscosity) under different irrigation regimes on the yield and phytochemicals of the Iranian landrace red pepper were studied. Experimental factors were irrigation system included drip and flood irrigation; irrigation frequencies included optimal irrigation (irrigation every 5 to 7 days based on irrigation at 85–90% field capacity or F.C.), deficit irrigation or 50% optimum irrigation (irrigation every 13 to 15 days based on irrigation at 45–50% F.C.); the foliar applications included negative control (no spraying), positive control (the foliar spraying by water as solvent), and foliar spraying by chitosan at 2 and 4 g L^{-1}. The highest yields of the fruit were obtained from the pepper plants treated with foliar-sprayed chitosan under a drip irrigation system and optimal irrigation conditions. However, the maximum values of capsaicin (11.49 mg g^{-1} DW), dihydrocapsaicin (4.99 mg g^{-1} DW), capsaicinoids (16.48 mg g^{-1} DW), vitamin C (1.26 mg g^{-1} DW), total phenolic content (2.15 mg GAE 100 g^{-1} DW), and antioxidant capacity (55%) were achieved in the plants sprayed by chitosan at 2 g L^{-1} under 50% optimum irrigation (deficit irrigation) and drip irrigation system. The use of chitosan under water-deficit conditions (471 g capsaicin m^{-2}) resulted in the highest capsaicin yield. In conclusion, chitosan foliar application under deficit irrigation is recommended to maintain and stabilize red pepper's quantitative and qualitative performance.

Keywords: capsaicin, dihydro-capsaicin, deficit irrigation, drip irrigation, total phenol, vitamin C

INTRODUCTION

Red pepper (*Capsicum* spp.), a member of the Solanaceae family, is a globally grown and consumed vegetable crop. Besides their use as food and in the food additive industries, pepper species are also exploited in the pharmaceutical and medicinal industries [Liu et al. 2022]. Capsaicinoids, carotenoids (capsanthin,

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α-carotene, and fatty acid esters), flavonoids, steroidal saponins, and essential oils are the main components of the red pepper fruits [Hernández–Pérez et al. 2020]. The spicy taste of the fruit of this plant is related to capsaicin [Sanati et al. 2018]. The high consumption of this horticultural crop is related to its spicy properties, conferred by capsaicinoids. Capsaicin and dihydrocapsaicin, as two main constituents, make up more than 80% of capsaicinoids [Arce-Rodriguez and Ochoa-Alejo 2019]. In addition, pepper fruits contain biologically active compounds, including vitamins A, C, B_2, and B_{11}, as well as potent antioxidants such as carotenoids [Khamoushi et al. 2021, Olatunji and Afolayan 2018].

Water is one of the environmental factors that significantly affect plant growth and the biosynthesis of secondary metabolites [Mosaedi et al. 2024]. The decrease in soil moisture is a limiting factor for biomass and dry matter yields of the herbs; however, it is possible that increasing some secondary metabolites under deficit water stress [Danesh-Shahraki et al. 2023]. This condition can depend on the duration, type, time, and growth stage under stress, genotype, agronomic management, etc. [Shaykh-Samani et al. 2023, Rezaei-Adl et al. 2025]. Reactions to water deficit stress differ among plant species. This environmental stress causes reduced available water, decreased photosynthesis rate, and increased reactive oxygen species (ROS), as well as degradation of the plasma membrane (lipid peroxidation), pigments, proteins, and DNA [Rabêlo et al. 2019, Rezaei-Adl et al. 2025].

The biosynthesis of secondary metabolites in some plants is influenced by genetic and agronomic practices and ecological factors, and their interaction impacts [Danesh-Shahraki et al. 2023]. Environmental stresses, particularly abiotic stresses, have significant impacts on the biosynthesis of biologically active substances [Alavi Samany et al. 2022]. To achieve stable quantitative and qualitative plant functions under environmental stress, such as water deficit, and to enhance secondary metabolite production, it is necessary to adopt novel horticultural management practices. One solution to this challenge could be to increase plant tolerance by utilizing elicitors such as chitosan [Ghasemi Pirbalouti et al. 2017]. Chitosan is a glucosamine polysaccharide [Hafez et al. 2020, Darani et al. 2025, Rezaei-Adl et al. 2025] which, through the induction of the defense system, will cause the secondary metabolites biosynthesis, the activity of antioxidant enzymes, and cell-compatible substances, so that it sends a series of chemical messages to the plant, which will result in increased growth and yield, as well as improving the physiological and morphological processes of many plants under stress or non-stress conditions [Hidangmayum et al. 2019, Alavi Samany et al. 2022]. Previous studies have shown that chitosan stimulates the biosynthesis of secondary metabolites in various herb species. Khodadadi et al. [2023] found that the application of chitosan could enchain the biosynthesis and accumulation of phenolic compounds via the phenylpropanoid pathway, as well as reduce the effectiveness of drought stress in sage.

It seems that the use of chitosan in plants under water-deficit conditions, which induces the production of free radicals, can reduce the adverse effects of water deficit stress on some yield traits by boosting the biosynthesis of phenolic compounds and antioxidant substances such as vitamin C and capsaicinoids. Some investigations have been conducted on the impacts of chitosan use on the growth and phytochemical properties of some herbs under varying moisture conditions. However, the interaction effects of chitosan application under different irrigation regimes on red pepper performance are not well documented. Therefore, this study was conducted to determine the effects of foliar-spraying with chitosan under different irrigation conditions on yield and selected phytochemical traits of red pepper cultivated in arid and semiarid climates.

MATERIALS AND METHODS

Experimental site description. This research was done at the research farm of Goldaru Herbal Pharmaceutical Company of Isfahan, Iran (latitude 32° 51' N, longitude 51° 52' E, altitude 1600 m) during 2018–2019. According to Koppen–Geiger's climate classification, Isfahan has a characteristic dry climate [Kottek et al. 2006]. Dry weather and very low rainfall are the prominent features of this classification, with minimum and maximum temperatures of −10.6 °C and 40.6 °C, respectively. The average annual precipitation over the city is 116.9 mm [Karimi et al. 2021]. Some meteorological parameters of the study area during the growth season are presented in Table 1.

Plant material and soil analysis. Seeds of the landrace of red chili pepper (*Capsicum annum* L.) were provided from the Research & Development Section, Goldaru Herbal Pharmaceutical Laboratories, Isfahan, Iran. Firstly, the seeds were sterilized by 1% sodium hypochlorite for 10 min and sown in 8 cm plastic pots on 8 March 2018. The pots were maintained in a glass greenhouse under controlled conditions: 25 °C ±2 / 15 ±1 °C (day/night) air temperatures, 65–70% humidity, and 12/12 hours (light/light less). The pots were filled with the same combination of farm soil (Table 2), sand, and peat moss. Soil samples of the experimental field were taken before the experiment from three random parts of each plot from 0 to 30 cm depth (Table 2). Organic carbon, soil texture, pH, and electrical conductivity (EC) were determined using the sulfuric acid method, hydrometer assay, pH meter, and EC meter [AACC 2000]. Total nitrogen was measured using micro-Kjeldahl digestion and distillation techniques [Page 1982]. Available phosphorus content (in extraction) was measured using a spectrophotometric method with a spectrophotometer Perkin-Elmer Inc. (Waltham, MA) [Olsen et al. 1954]. Potassium (extracted with ammonium acetate) was determined using a flame photometer [Black 1965]. The main micronutrients, including manganese (Mn), iron (Fe), copper (Cu), and zinc (Zn), were measured using an atomic absorption spectrophotometer (PerkinElmer Analyst 400, Waltham, United States of America) [AACC 2000].

About 45–50 days after sowing on 22–28 May 2018, when the seedlings had 4–6 true leaves and were 10–15 cm tall, they were transferred to the experimental field. The planting density was 25 cm between plants and 50 cm between the rows (~8 plant m^{-2}). The thoroughly reddened peppers were hand-harvested between 9 and 15 September 2018.

In winter 2017, the field soil was ploughed with a moldboard plow (up to a depth of 30–35 cm). Cow dung manure (10 tons ha^{-1}) and chemical fertilizers (20–20–20 kg ha^{-1} N, P, and K, respectively) were applied to the soil before transplanting as urea, triple superphosphate, and potassium sulphate, respectively. The ratio of cow manure and chemical fertilizer was determined based on the optimal plant nutrient requirements, soil characteristics, and cow manure

Table 1. Some of the meteorological parameters of the study area during the growth season

Parameters	April	May	June	July	August	September
Minimum temperature (°C)	10.7	12.9	19.7	21.4	38.2	36.5
Maximum temperature (°C)	23.7	25.0	33.9	37.1	30.9	29.8
Average temperature (°C)	17.2	18.9	26.8	29.2	34.5	33.2
Average precipitation (mm)	5.4	35.9	7.0	0.0	0.0	0.0
Relative humidity (%)	37.2	45.5	27.4	16.3	15.0	15.7
Potential evapotranspiration PET (mm/day)	5.17	5.57	7.64	8.91	7.42	6.47

Table 2. Some physicochemical properties of the soil of the study area

Depth (cm)	Soil texture	pH	EC (dS/m)	Organic carbon (%)	N (%)	P	K	Zn	Mn	Fe	Cu
						(mg/kg)					
0–30	silt loam	7.42	2.06	0.58	0.05	7.52	219.0	1.08	0.86	1.28	1.20

element concentrations. No systemic pesticides or herbicides were used during the research. Weeds were controlled manually.

Treatments and experimental design. The experimental factors were conducted using a split-split plot design based on a randomized complete block design (RCBD) in three replications. Three factors were irrigation system included drip using a seam drip irrigation tape 20 cm with a flow rate of 1.5 L ha^{-1} and flood irrigation; irrigation frequencies included optimal irrigation (irrigation every 5 to 7 days based on irrigation at 85–90% field capacity or F.C.), deficit irrigation (irrigation every 13 to 15 days based on irrigation at 45–50% F.C.); the foliar applications included negative control (no spraying), positive control (the foliar spraying by water as solvent), and foliar-spraying of chitosan at 2 and 4 g L^{-1} according to results of previous investigations by our and other researchers [Valletta et al. 2016, Alavi-Samany et al. 2022].

Deficit irrigation by changing the irrigation intervals to about 13 to 15 days, once based on moisture discharge from F.C. conditions and evaporation rate from Class A pan, calculated after 45 days of the establishment of seedlings. Irrigation time and volume were determined based on the soil moisture curve. For this purpose, soil moisture was determined daily using a TDR device (PMS-714, Lutron, Taiwan) according to the manufacturer's guidelines. Field capacity and permanent wilting point (PWP) were –33 and –1500 kPa, respectively. In semiarid and arid regions, a soil matric potential threshold range of –30 to –40 kPa at a 20 cm depth was recommended for chili pepper irrigation under a drip irrigation system [Liu et al. 2012]. To prevent water leakage between irrigation treatments, a distance of 2 m was maintained between the moisture levels. Medium molecular weight chitosan with 450 kDa molecular weight, 95–98% degree of deacetylation, and 30 mPas viscosity (Sigma–Aldrich Co., Steinheim, Germany) was dissolved in acetic acid (5%) diluted with water. The solutions were sprayed onto the entire pepper plants in three growth stages (before flowering, early flowering, and 25% flowering) at dew point (150–160 mL per plant) using a handheld garden pump sprayer.

Morpho-physiological measurements. When the pepper fruits were fully ripe and red, three plants were randomly selected from the center of each plot, after removing the effects of the margins, to evaluate their morphological characteristics. The number of fruits in each plant and the primary branches in each experimental unit were counted and recorded. The plant height and fruit length were measured using a ruler and a digital vernier caliper (0–150 mm, accuracy 0.01 mm), respectively. The harvested fruits were weighed using a sensitive digital scale (Sartorius, Germany) with an accuracy of 1 mg. Then, the fruits were dried using an oven at 55 °C for 48 hours and weighed again.

Phytochemical measurements. The total phenolic content of the fruits was determined using the Folin-Ciocâlteu colorimetric method [Menichini et al. 2009]. For this purpose, the standard curve was prepared using gallic acid. Then, 1.5 g of the extract was diluted with 1 mL of methanol, mixed with 0.2 mL of Folin-Ciocâlteu reagent, 2 mL of distilled water, and 1 mL of 15% Na_2CO_3. The blue solution formed was kept at room temperature for 2 hours. The absorbance was then measured at 765 nm using a UV–Vis spectrophotometer (Perkin-Elmer Inc., Waltham, MA). The absorption values were measured using the standard curve. Afterward, total phenolic content was determined based on mg of gallic acid as a standard in dry weight (DW). To measure the content of C in the red pepper fruits, 0.5 g of the dried samples was pounded in a mortar, and 5 mL of 4% oxalic acid was added to it and homogenized. The homogenates were centrifuged at 5000 rpm for 10 min, and then the supernatants were filtered with 541 Whatmann filter paper the obtained residues were made up to 25 mL with 4% oxalic acid. The amount of vitamin C was determined using the 2,4-dinitrophenylhydrazine reagent on a spectrophotometer (Perkin-Elmer Inc., Waltham, MA) at 540 nm [Aniel Kumar and Subba Tata 2009]. The antioxidant activity of the extracts was measured using the DPPH assay [Brand-Williams et al. 1995]. To determine the amount of capsaicin in red pepper fruit, 2.5 g of dry pepper powder was extracted with 80 mL of acetonitrile using the Soxhlet extraction technique for 6 h, until the solution became colorless. The extract was concentrated to 40 mL under vacuum and transferred to a 50 mL volumetric flask. Then, 10 mL of acetonitrile (CH_3CN) was added. Subsequently, 20 µL of supernatant was injected into the HPLC system in triplicate. In this study, an HPLC (Knauer Corp., Berlin, Germa-

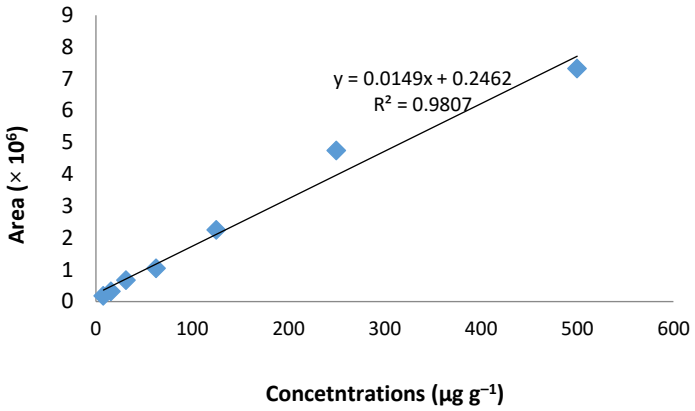

Fig. 1. Calibration curve for capsaicin

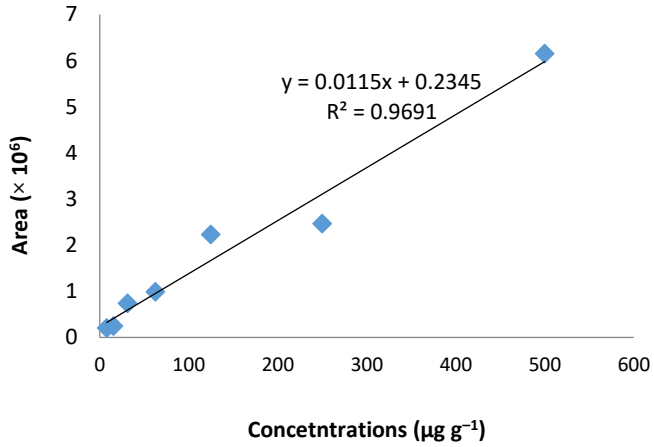

Fig. 2. Calibration curve for dihydrocapsaicin

ny) equipped with a K-1001 pump, a vacuum degasser, a tunable UV/Visible detector, and a ProntoSIL 120-5 C18 H (25 × 0.4 cm ID) column was used. Water and 0.1% phosphoric acid (A) and methanol (B) at a 30:70 (v/v) ratio were used as mobile phases at a flow rate of 1.0 mL min^{-1}. Capsaicin was monitored at 290 nm. The column and sample temperatures were 60 °C and 20 °C, respectively [Dang et al. 2014]. During HPLC sample analyses, a standard solution was injected to evaluate retention time reproducibility and instrument calibration [Othman et al. 2011]. Capsaicin and dihydrocapsaicin in red pepper were measured by comparison to external reference standards injected under the same conditions [Othman et al. 2011]. Standard solutions were prepared from stock solutions of capsaicin and dihydrocapsaicin with various concentrations (500, 250, 125, 62, 31, 15.5, and 8 µg g^{-1}). After injection of the standard solutions into the HPLC, standard curves of peak area versus concentration were plotted (Figures 1 and 2). The capsaicinoid concentrations in the samples are expressed as mg g^{-1} dry matter or dry weight.

Statistical analysis. Firstly, the experimental data were tested for normality (Kolmogorov–Smirnov test) and homogeneity of variance (Levene's test). Then, the data were statistically analyzed using the GLM

procedure in SAS (SAS/STAT® v.9.2; SAS Institute Inc., Cary, NC). The comparison of means was done using Duncan's multiple range test ($p \leq 0.05$). All tests were done in triplicate. Excel software was used to draw the graphs.

RESULTS

Morpho-physiological traits. Based on the ANOVA results, the simple effects of treatments – foliar spraying, irrigation frequency, and irrigation system – did not significantly affect plant height (Table 3) or the number of primary branches (data not shown). However, the interaction influence of the foliar spraying × irrigation frequency × irrigation system on the plant height was highly significant ($p \leq 0.01$), see Table 3. The highest plant height was observed for plants treated with chitosan (4 g L^{-1}) under a flood irrigation system and optimal moisture conditions. In contrast, the lowest plant height was observed in plants grown under the no-spraying (control) × optimal irrigation condition (control) and the drip irrigation system (Table 3). On the other hand, plant height increased by 6% and 15% under chitosan spraying at 2 and 4 g L^{-1}, respectively, in deficit irrigation conditions (Table 3). The results indicated that foliar spraying significantly influenced ($p \leq 0.05$) the number of fruits per plant (Table 3). The application of chitosan at 4 g L^{-1} could produce the highest number of fruits on the pepper plant. In addition, the interaction effect of foliar spraying × irrigation frequency × irrigation system significantly ($p \leq 0.05$) affected the number of fruits per plant (Table 3). The highest value for the number of fruits per plant was observed with chitosan (4 g L^{-1}) under optimal irrigation conditions and flood irrigation (Table 3).

As shown in Table 3, the simple effect of irrigation frequency was significant ($p \leq 0.05$) on the fresh and dry weights of the pepper fruits. However, the use of chitosan did not have a significant effect on the fresh and dry weights of the pepper fruit (Table 3). Increasing the irrigation interval or applying deficit irrigation significantly reduced both traits (Table 3). Our results indicate that the simple effect of the irrigation system had a significant ($p \leq 0.05$) impact on fresh and dry fruit weights (Table 3). The drip irrigation system increased fresh and dry fruit weights by 11% and 38%, respectively, compared with the flood irrigation system (Table 3).

According to the results shown in Table 3, the interaction effect of irrigation system × irrigation frequency × foliar spraying ($p \leq 0.01$) on the fresh and dry weights of fruit was significant. The maximum fresh fruit weight (5.50 kg m^{-2}) and dry fruit weight (1.92 kg m^{-2}) were observed for plants treated with chitosan (4 g L^{-1}) under a drip irrigation system in both moisture conditions (Table 3). The minimum amounts of the fresh fruit weight (2.91 kg m^{-2}) and dry fruit weight (0.61 kg m^{-2}) were obtained in the plants treated by flood irrigation system × deficit irrigation × no foliar spraying (Table 3). In deficit irrigation conditions, the use of chitosan at 2 and 4 g L^{-1} could improve dry fruit weight by 85% and 97%, respectively, compared with the optimum irrigation condition or the control.

Phytochemical traits. The chromatograms of the standard and extracted solutions are shown in Figures 3 and 4, respectively. Results of HPLC analyses showed that the capsaicin content ranged from 4.46 to 11.49 mg g^{-1} dry weight, and the dihydrocapsaicin content ranged from 1.84 to 4.99 mg g^{-1} dry weight (Table 3). According to the results shown in Table 3, the simple influences of the foliar spraying, irrigation frequency, and irrigation system on the concentrations of capsaicin and dihydrocapsaicin were significant (Table 3). The highest levels of capsaicin and dihydrocapsaicin were observed in plants treated with chitosan spray at 2 g L^{-1}, under deficit irrigation, and in the drip irrigation system (Table 3). The foliar spraying × irrigation frequency × irrigation system had significant influences ($p \leq 0.01$) on the capsaicin and dihydrocapsaicin concentrations (Table 4). The maximum values of capsaicin (11.49 mg g^{-1} DW) and dihydrocapsaicin (4.99 mg g^{-1} DW) were obtained from pepper plants sprayed with chitosan at 2 g L^{-1} under deficit irrigation conditions and a drip irrigation system (Table 3). Moreover, the highest concentration of capsaicinoids (16.48 mg g^{-1} DW) as unique spicy characteristic, which two main constituents i.e. capsaicin and dihydrocapsaicin was detected in the plants treated by 2 g L^{-1} chitosan and deficit irrigation condition and drip irrigation system (Fig. 5). The interaction impact of irrigation system × irrigation frequency ($p \leq 0.05$) demonstrated that this treatment signifi-

Table 3. The main and interaction effects of foliar spraying × irrigation frequency × irrigation system on some studied characteristics

Experimental factors	Plant height (cm)	Fruit fresh eight (kg m^{-2})	Fruit dry weight (kg m^{-2})	Number of fruits per plant	Total phenol (mg GAE per 100 g DW)	Antioxidant capacity (%)	Capsaicin (mg g^{-1} DW)	Dihydrocapsaicin (mg g^{-1} DW)
Foliar spraying:								
control – (no foliar): NOF	50.5	5	1.3	41.2 b*	1.3 b	48.9	5.8 b	2.5 b
control + (water). WAF	52.5	4.9	1.2	37.2 b	1.1 b	48.4	5.2 b	2.5 b
chitosan 2 g/L: CHF1	54.7	4.7	1.4	47.2 ab	1.2 b	49.8	8.1 a	4.0 a
chitosan 4 g/L: CHF2	58	5.2	1.6	55.6 a	1.8 a	47.5	6.0 b	2.8 b
ANOVA	n.s¶	n.s	n.s	$p \leq 0.05$	$p \leq 0.05$	n.s	$p \leq 0.05$	$p \leq 0.05$
Irrigation frequency:								
optimal irrigation: OPI	54.2	5.1 a	1.61 a	47.3	1.34 b	48.64	5.74 b	2.5 b
deficit irrigation: DEI	52.3	4.3 b	1.26 b	51.4	1.56 a	46.80	8.47 a	3.9 a
ANOVA	n.s	$p \leq 0.05$	$p \leq 0.05$	n.s	$p \leq 0.05$	n.s	$p \leq 0.05$	$p \leq 0.05$
Irrigation system:								
drip irrigation: DRI	54.6	5.0 a	1.54 a	51.4	1.4	48.5	7.7 a	3.4 a
flood irrigation: FLI	51.9	4.2 b	1.20 b	46.5	1.3	46.9	6.6 b	2.8 b
ANOVA	n.s	$p \leq 0.05$	$p \leq 0.05$	n.s	n.s	n.s	$p \leq 0.05$	$p \leq 0.05$
Interaction of experimental factors:								
FLI × OPI × NOF	52.3 g	4.6 d	1.3 d	45.3 f	1.2 e	48 b	5.5 f	2.3 f
FLI × OPI × WAF	52.3 g	4.5 e	1.2 d	37.7 gh	1.5 cd	48 b	5.1 f	2.2 f
FLI × OPI × CHF1	53.7 f	4.7 cd	1.3 d	47.7 e	1.7 c	50 b	7.5 cd	3.1 d
FLI × OPI × CHF2	61.3 a	5.0 c	1.4 c	67.7 a	1.9 b	47 bc	4.5 g	1.8 f
FLI × DEI × NOF	52.3 g	2.9 h	0.6 f	53.3 cd	1.2 e	49 b	8.1 c	3.3 cd
FLI × DEI × WAF	56.3 d	4.2 f	1.1 e	43.3 fg	1.5 cd	45 c	6.7 d	2.8 d
FLI × DEI × CHF1	60.3 ab	3.6 gh	1.3 d	54.7 c	0.8 f	40 d	8.0 c	3.4 c
FLI × DEI × CHF2	56.3 d	4.3 ef	1.5 c	61.3 b	1.3 d	45 c	8.7 c	3.8 c
DRI × OPI × NOF	48.7 h	5.3 b	1.3 d	37.0 gh	1.4 d	50 b	6.0 e	2.7 e
DRI × OPI × WAF	52.7 g	5.3 ab	1.5 c	36.7 h	0.8 f	48 b	6.3 e	2.7 e
DRI × OPI × CHF1	55.7 e	4.6 d	1.5 c	46.7 ef	0.8 f	49 b	7.0 d	3.0 d
DRI × OPI × CHF2	56.7 d	5.5 a	1.92 a	43.77 fg	1.3 d	49 b	6.1 e	2.7 e
DRI × DEI × NOF	52.70 g	3.7 g	1.0 e	47.3 e	1.4 d	46 bc	7.2 d	3.0 d
DRI × DEI × WAF	57.00 c	4.8 cd	1.4 c	42.30 g	1.1 e	40 d	7.1 d	2.9 d
DRI × DEI × CHF1	56.00 d	5.5 ab	1.8 b	65.7 ab	2.2 a	55 a	11.5 a	5.0 a
DRI × DEI × CHF2	56.70 d	5.4 ab	1.9 a	52.3 d	1.5 cd	44 c	10.0 b	4.6 b
ANOVA	$p \leq 0.01$	$p \leq 0.01$	$p \leq 0.01$	$p \leq 0.05$	$p \leq 0.01$	$p \leq 0.01$	$p \leq 0.01$	$p \leq 0.01$

* The averages with at least a standard alphabet are not statistically significant at the 5% level.
¶ Not significant.

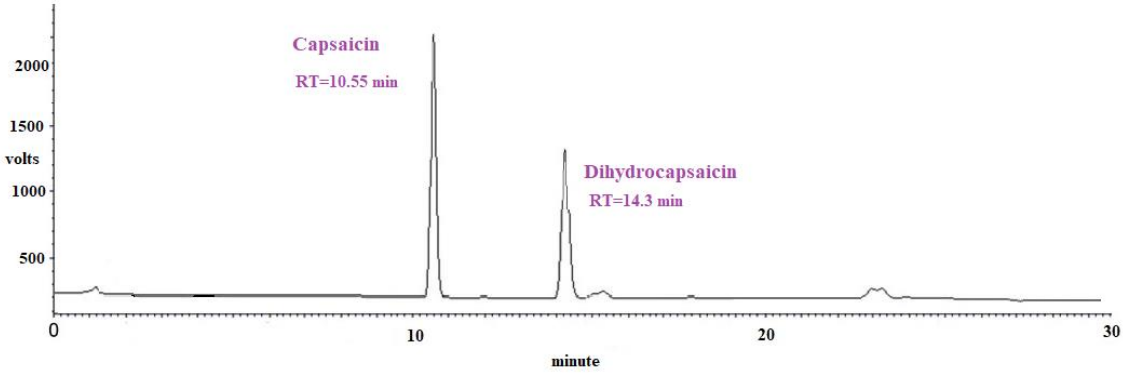

Fig. 3. Chromatogram of the standard solution of capsaicin and dihydrocapsaicin

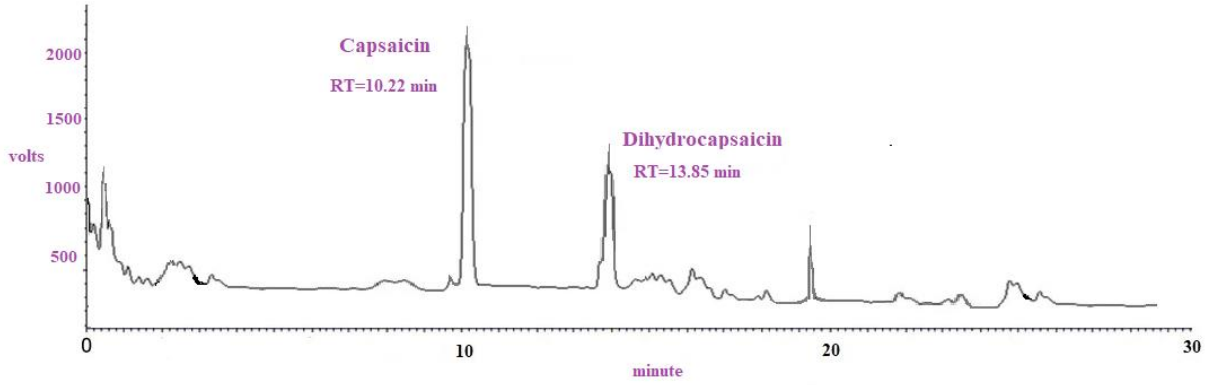

Fig. 4. Chromatogram of the extract from the red pepper fruits

cantly changed the capsaicin yield per area unit (m^2), see Figure 6. However, the interaction effect of foliar spraying × irrigation frequency × irrigation system did not significantly impact this agronomic parameter. In this investigation on irrigation system × irrigation frequency, the maximum capsaicin yield (384 g capsaicin m^{-2}) was observed in the drip irrigation system under deficit irrigation conditions (Fig. 6). In this research, the foliar spraying × irrigation frequency treatment greatly influenced capsaicin yield ($p \leq 0.01$). The maximum capsaicin yield (471 g capsaicin m^{-2}) was obtained from foliar application of chitosan × deficit irrigation (Fig. 7). Interestingly, this improvement under the applied chitosan was about 95% compared to controls. The simple effects of the foliar spraying and irrigation frequency significantly changed the amount of vitamin C in the red pepper fruit (Table 3). However, there was no significant difference between the two irrigation systems in vitamin C concentration (Table 3). The interaction effect of foliar-spraying × irrigation frequency × irrigation system ($p \leq 0.01$) on the ascorbic acid content in red pepper fruit was significant (Fig. 8). The effects of the foliar application and irrigation frequency significantly affected the total phenolic content in the red pepper fruit (Table 3); however, the irrigation system treatment had not significantly effect on the total phenolic content (Table 3). The highest content of total phenolic was recorded once plants were treated with 4 g L^{-1} chitosan, compared to the untreated plants (1.81 mg GAE 100 g^{-1} DW), see Ta-

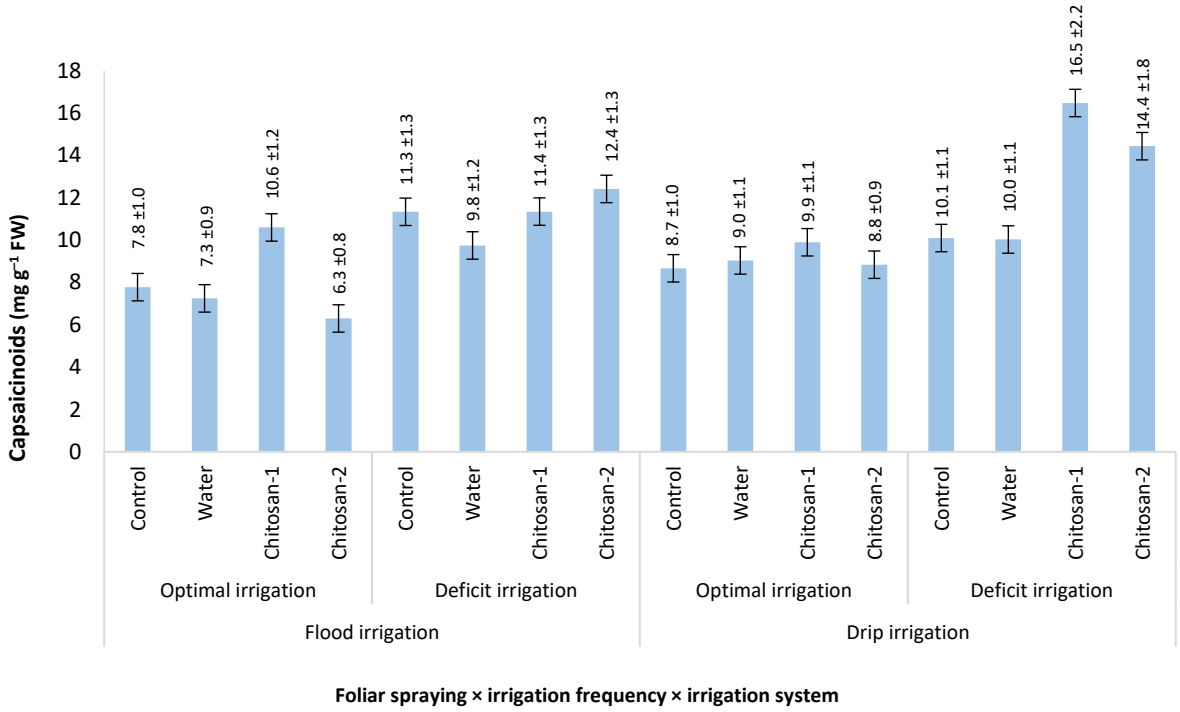

Fig. 5. Interaction effect of foliar spraying × irrigation frequency × irrigation system on the amount of capsaicinoid

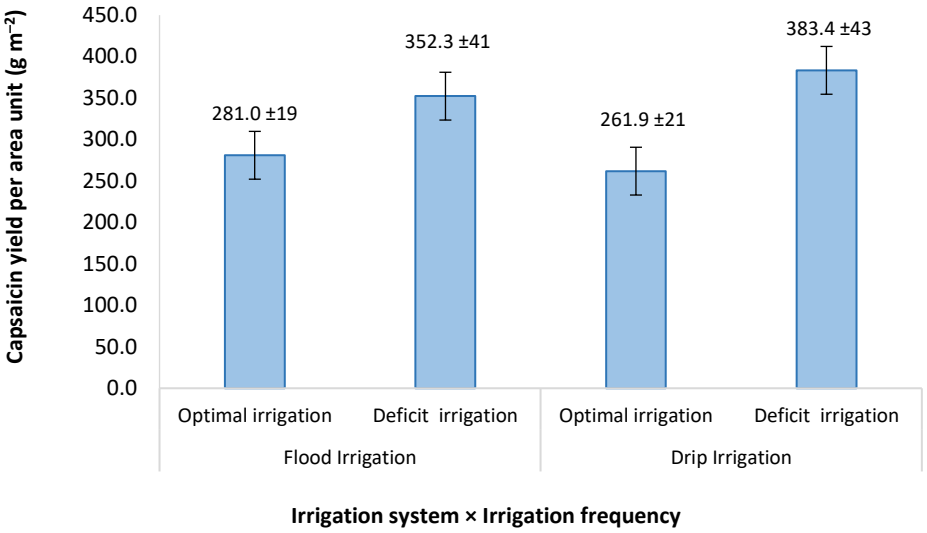

Fig. 6. Interaction effect of irrigation system × irrigation frequency on the capsaicin yield per area unit

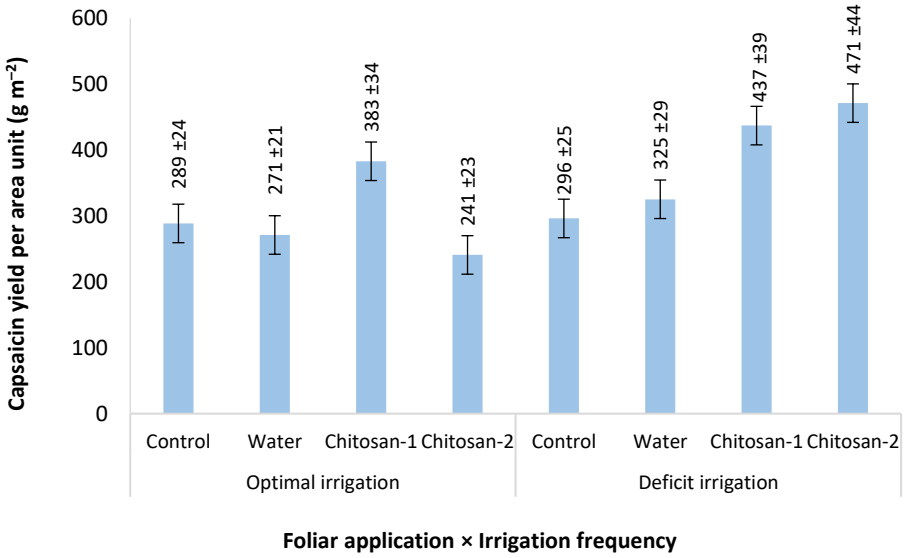

Fig. 7. Interaction effect of foliar spraying × irrigation frequency on the capsaicin yield per area unit

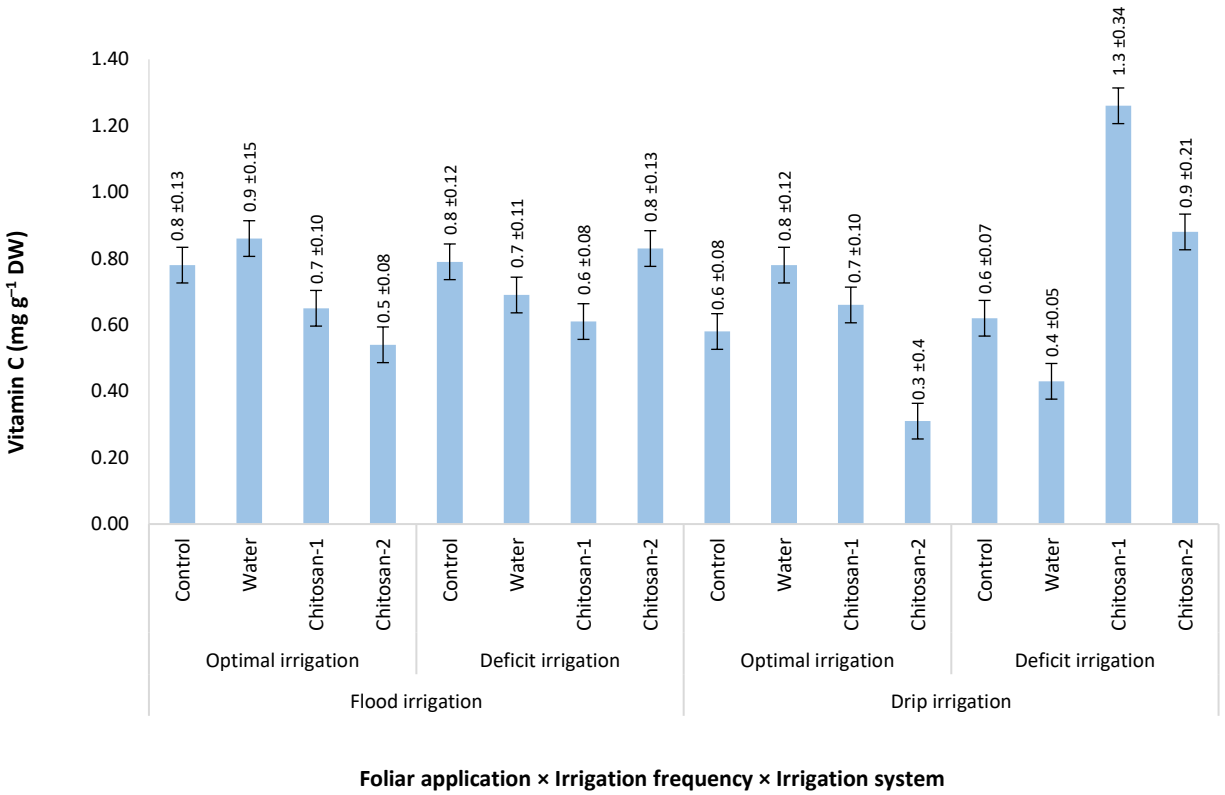

Fig. 8. Interaction effect of foliar spraying × irrigation frequency × irrigation system on the vitamin C content

ble 3. On the other hand, deficit irrigation significantly increased (1.56 mg GAE 100 g^{-1} DW) the total phenol content compared to the optimum irrigation condition (Table 3).

The results of this study show that there were no differences in the simple effects of the experimental treatments on the antioxidant activity of red pepper fruit (Table 3). At the same time, the simple effects of the experimental treatments had significant effects on antioxidant activity, with the maximum antioxidant activity observed in plants under chitosan utilization × optimal irrigation conditions × drip irrigation system (55%), see Table 3.

DISCUSSION

According to the results of the experimental factors including the chitosan utilization, irrigation frequency and system on some traits of red pepper showed that the applied of chitosan elicited the biosynthesis and accumulation of secondary metabolites, the activity of antioxidant enzymes and cell compatible substances, the growth and productivity as well as improve the physiological and morphological processes of plants and improved the accessibility and absorb of indispensable elements and water [Farouk and El-Metwally 2019, Hidangmayum et al. 2019]. Additionally, the antiperspirant properties of chitosan reduce water loss from the plant, and the plant cools down, especially under stress [Ghasemi Pirbalouti et al. 2017]. The results showed that the maximum number of fruits was obtained from plants sprayed with chitosan under optimal irrigation conditions and under flood irrigation. In this regard, Ghanbari et al. [2021] reported that the number of bell pepper fruits significantly decreased with increasing irrigation interval. Chitosan enhances the absorption of water and nutrients, stimulates photosynthesis and CO_2 stabilization, decreases the generation of free radicals by increasing the activity of antioxidant enzymes, and improves plant performance and growth, such as fruit length [Ghasemi Pirbalouti et al. 2017]. Also, the maximum fresh and dry weights of pepper fruit in this study were observed under optimal irrigation conditions, which were attributed to the plant's higher growth rate due to the availability of water [Babaei et al. 2021]. The reason for the improvement in yield under the chitosan treatment in water-deficient conditions could probably be related to the positive effects of the chitosan utilization on enhancing photosynthesis rate, chlorophyll content, and adequate supply of nutrients by increasing the activity of enzymes involved in nitrogen metabolism (nitrate reductase, glutamine synthetase, and protease) [Bistgani et al. 2017a]. We found that the fresh and dry fruit weights in the drip irrigation system were higher than those in the flood irrigation system. More moisture available to the plant and proper soil moisture supply in the drip irrigation system improved fresh and dry fruit weights compared with flood irrigation [Peyrov et al. 2017]. Agronomic and horticultural crops are typically irrigated at close intervals with small amounts of water via drip irrigation [Zamljen et al. 2020]. In the present study, the interaction effects of the experimental factors on fruit weight indicated that plants grown under chitosan × drip irrigation system × optimal and deficit irrigation had the highest fruit yields. Similar to our results, Farouk and El-Metwally [2021] reported that deficit irrigation with a drip irrigation system, combined with chitosan, increased wheat biomass and dry matter yields compared to the control.

In contrast, deficit water stress reduces the absorption of available water and nutrients through the plant roots and also decreases the transfer of the elements from the roots to the branches [Gharakhani-Beni et al. 2021, Rezaei-Adl et al. 2025]. In this study, it appears that chitosan reduced the effects of deficit moisture stress and improved the performance of the aerial parts by enhancing plant physiological processes, such as photosynthetic tissue activity [Ghasemi Pirbalouti et al. 2017, Bistgani et al. 2017a]. In this research, a drip irrigation system could significantly increase plant growth and crop productivity, water and nutrient use efficiency, and reduce evaporation, plant stress, the occurrence of diseases and weed competition, fertilizer leaching, and soil salinity [Yang et al. 2023].

Capsaicinoids, as natural organic components, are responsible for the pungency of pepper fruits [Koleva-Gudeva et al. 2013]. In our experiment, the use of chitosan, a water-deficient condition, and a drip irrigation system could, by increasing the active substances, especially capsaicin, improve the commercial quality of red pepper [Perucka and Materska 2001]. In line with our results, Zamljen et al. [2020] reported increased capsaicin and dihydrocapsaicin concentra-

tions in pepper plants under water-deficit conditions, attributing the increase to four key enzymes of the capsaicinoid biosynthesis pathway [Sung et al. 2005].

Based on our results, deficit irrigation increased capsaicin yield by 20% compared with optimal irrigation. The research results have shown that although soil moisture is a limiting factor for plant production, under deficit water conditions, through metabolic pathways, by stimulating the secondary metabolites biosynthesis, can increase the active biological compounds in the medicinal and aromatic plants [Danesh-Shahraki et al. 2023, Shaykh-Samani et al. 2023, Mosaedi et al. 2024, Darani et al. 2025, Rezaei-Adl et al. 2025]. In fact, the production of this metabolite group is considered a survival strategy against environmental stress [Maghsoudi et al. 2023]. The deficit in moisture triggers various metabolic reactions and the activity of specific genes [Maghsoudi et al. 2023]. The most important biologically active compound among the secondary metabolites of pepper species is capsaicin, which is derived from benzylamine [Koleva-Gudeva et al. 2013]. This active substance is a unique alkaloid that gives pepper its spicy taste. Similarly, Zamljen et al. [2020] reported that the capsaicin and dihydrocapsaicin contents or pungency of the *C. annuum* Chili-AS Rot fruits under deficit irrigation conditions were higher than optimum irrigation.

Additionally, the results of this research illustrated the positive impact of the utilization of chitosan on the levels of capsaicinoides. Probably, chitosan leads to the activation of new genes that stimulate enzymes and metabolic pathways involved in the biosynthesis of secondary metabolites [Bistgani et al. 2017a, Darani et al. 2025, Rezaei-Adl et al. 2025]. Elicitors such as chitosan are first recognized by plant receptors, which activate ion channels, GTP-binding proteins, and protein kinases.

According to the results of the experimental factors on vitamin C concentration, the highest vitamin C concentration was obtained from pepper plants sprayed with chitosan. This finding concurs with the results of a previous investigation [Metwaly et al. 2023], they found that the foliar application of chitosan (0.5 and 1 g L^{-1}) significantly improved fruit quality. Similar to our results, Zamljen et al. [2020] reported that ascorbic acid content in the *C. annuum* Chili-AS Rot fruit decreased by 20% under deficit irrigation. Vitamin C or ascorbic acid is the most abundant and strongest water-soluble antioxidant that prevents or reduces damage caused by reactive oxygen species in plants. Ascorbic acid plays a role in removing reactive species *via* the ascorbate peroxidase reaction. The increase in ascorbic acid synthesis is one of the positive aspects under water-deficit stress conditions. In this study, it seems that the application of chitosan under deficit irrigation conditions could increase ascorbic acid levels, which have a protective role against stress. Li et al. [2017] reported that the use of chitosan significantly increased chitosan content under water-deficit conditions by maintaining higher accumulation of metabolites related to osmotic regulation, antioxidant defense, stress signaling, and energy metabolism. They reported that chitosan-induced drought tolerance was associated with the accumulation of stress-protective metabolites, increased polyamine synthesis, and flavonoid metabolism [Li et al. 2017].

Regarding the interaction effects, the highest total phenolic content was observed with foliar chitosan spraying under deficit irrigation and drip irrigation conditions. Similar to the present research, Maghsoudi et al. [2023] reported that phenolic compounds accumulated in the plant under water deficiency stress. Moreover, Bistgani et al. [2017b] reported that varying levels of chitosan increased total phenolic content in thyme (*Thymus daenensis* Celak.) compared with the control under deficit irrigation conditions. Some plants have different protective mechanisms, such as enzymatic and non-enzymatic antioxidant systems (phenolic compounds and flavonoids), under environmental stress [Babaei et al. 2021].

Water deficit stress causes oxidative stress by disrupting the balance between the production of reactive oxygen species and the plant's antioxidant defense activities. Antioxidants are molecules that neutralize reactive oxygen species and help prevent damage to plant cells [Shaykh-Samani et al. 2023]. It seems that chitosan dissolution has significantly increased the activity of the antioxidant defense system and reduced lipid peroxidation. Results from several studies have shown that the main components of the cell wall of the biological elicitor chitosan may be able to scavenge free radicals and stimulate plant defense mechanisms and secondary metabolite production [Ghasemi Pirbalouti et al. 2017]. Based on our hypotheses and re-

sults, the use of medium molecular weight chitosan at 2 g L^{-1} in red pepper plants could mitigate the negative effects of water deficit stress on fruit yield traits by boosting the biosynthesis of phenolic compounds and antioxidant substances such as capsaicinoids.

Additionally, the foliar application had positive impacts on pepper performance and alleviated the adverse effects of water deficit stress on phytochemical and antioxidant properties. Complex processes are activated in plants in response to drought stress, including hormonal modulation, transcription factor-mediated signaling, and biosynthesis of secondary metabolites [Arabsalehi et al. 2022]. Among drought stress resistance traits, secondary metabolites are of great importance because these compounds play an important role in regulating plant-environment interactions and subsequent adaptive responses [Babaei et al. 2021, Arabsalehi et al. 2022]. Water shortage affects secondary metabolites by altering gene expression or the activity of enzymes involved in their biosynthesis pathways [Arabsalehi et al. 2022]. The mechanism of chitosan effects on the biosynthesis pathways of capsaicin, dihydrocapsaicin, and vitamin C in pepper fruit is not currently understood. In general, molecular-level analysis, identification of secondary metabolite biosynthesis pathways, and the nutritional quality of the fruit need to be studied in red pepper under foliar-spraying with chitosan and under water-deficit stress conditions.

CONCLUSIONS

Based on the results of this investigation, the active biologically compounds, especially the capsaicin and dihydrocapsaicin concentrations in the pepper fruits under deficit irrigation, improved significantly. The foliar application of chitosan could, by stimulating biosynthetic pathways, increase the biosynthesis of secondary metabolites such as capsaicin, dihydrocapsaicin, vitamin C, and total phenols in pepper fruit. Interestingly, the quality yield of red pepper was improved by increases in the concentrations of capsaicin, dihydrocapsaicin, vitamin C, total phenolic content, and capsaicin yield, as well as antioxidant capacity, with the utilization of chitosan under water deficit stress showing the highest amounts. In conclusion, foliar spraying of medium molecular weight chitosan, particularly at 2 g L^{-1}, could mitigate the adverse effects of water deficit stress and improve active substances such as capsaicin, dihydrocapsaicin, vitamin C, and total phenolic content under water deficit stress, particularly in arid and semiarid conditions. Future studies should investigate the molecules, genes, proteins, and specific metabolic pathways underlying capsaicin and vitamin C biosynthesis in response to chitosan application under dehydration conditions in red pepper, and optimize its use in combination with deficit irrigation strategies.

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REVIEW PAPER

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EXPLORING THE ROLE OF ABIOTIC ELICITORS IN THE BIOSYNTHESIS OF SECONDARY METABOLITES IN PEPPERMINT (*Mentha piperita* L.)

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ABSTRACT

Secondary metabolites are a large group of organic compounds generally biosynthesized by medicinal and aromatic plants, which have a wide range of uses in human life today. Peppermint (*Mentha piperita* L.) is widely used as a traditional medicinal plant worldwide due to its high content of secondary metabolites, including menthol, limonene, pulegone, rosmarinic acid, cinnamic acid, eriocitrin, narirutin and hesperidin. It has different medicinal and culinary uses, such as food flavoring and treating rheumatoid arthritis pain, sinusitis headache and breathing problems. Given the chemical synthesis of plant secondary metabolites under laboratory conditions is considerably expensive and complicated, some alternative methods have been developed. Applying abiotic elicitors such as UV-radiation, abiotic stresses, and phytohormones during peppermint cultivation is an effective strategy to modify secondary metabolite content and components. Therefore, in this review, the most important secondary metabolites of peppermint and their uses are first described, and the abiotic elicitors used to influence the secondary metabolites profile of peppermint and their reaction mechanisms are then explained.

Keywords: elicitation, essential oil, chemical elicitors, menthol, mint

INTRODUCTION

Plants produce a large group of organic compounds, called secondary metabolites, with generally low molecular weight (lower than 150 kDa), which, according to reports, have considerably low concentrations in plant tissues (lower than 1% of plant dry weight) [Abdi and Karami 2020]. To date, more than 100,000 secondary metabolites have been identified, and more are still being identified [Haydari et al. 2019]. Secondary metabolites vary depending on the species and growth stage and play important ecological roles in plants. For instance, protection against herbivores and microbial agents, attraction of pollinators and improvement of plant-microbe symbiosis are some of the vital ecological roles of secondary metabolites [Abdi

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and Karami 2020, Ahmad et al. 2022]. Currently, secondary metabolites are widely used in human life as medicine, biotic herbicides, flavoring agents, natural colors, pesticides, hallucinogenic substances (such as cocaine, heroin and morphine) and perfumes [Nazerieh et al. 2018, Abdi and Karami 2020]. There are three main pathways for the biosynthesis of secondary metabolites in plant tissues including: the mevalonic acid pathway, the malonic acid pathway and the shikimic acid pathway [Nazerieh et al. 2018, Haydari et al. 2019, Tabbert et al. 2022]. Secondary metabolites are generally grouped based on their biosynthesis pathway: nitrogen-containing secondary products (such as nicotine and polyamines), phenolic compounds (such as flavonoids), oxylipins (such as jasmonic acid) and terpenes (such as steroid alkaloids) are four main groups of plant secondary metabolites [Abdi and Karami 2020].

Peppermint (*Mentha piperita* L.) is a perennial herb belonging to the Lamiaceae family [Afkar et al. 2013], a natural hybrid from watermint (*Mentha aquatic* L.) and spearmint (*Mentha spicata* L.) [Askary et al. 2016]. Peppermint is widely used as a traditional medicinal plant worldwide due to its high content of secondary metabolites. Ancient Egyptian people cultivated this plant for different medicinal purposes, such as better food digestion and treatment of stomach ailments [Askary et al. 2016, Abdi and Karimi 2020]. Currently, several products with a wide range of medicinal and culinary uses, such as food flavoring and treating rheumatoid arthritis pain, sinusitis headache and breathing problems, are produced from this plant [Cappellari et al. 2020]. The aerial parts of peppermint are enriched sources of phenolic compounds, flavonoids, fatty acids, vitamins (A, C and B_6), nutrients (K, Ca, Mg, Fe, Mn, Zn and Cu) and salicylic acid. Essential oil is the most important phytochemical compound of this plant, mainly extracted from leaves through the steam distillation method. Monoterpenes such as menthol, menthyl acetate and menthone are considered the main components of peppermint essential oil [Nazerieh et al. 2018, Haydari et al. 2019, Cappellari et al. 2020, Tabbert et al. 2022]. Studies have shown that the ethanol extract of peppermint contains tannins and flavonoids, while glycosides, saponins and alkaloids are not extracted by methanolic extract [Nemati Lafmejani et al. 2018]. More than 40 phenolic compounds such as rosmarinic acid, cinnamic acid, caffeic acid and salvianolic acid and also some flavonoid glycosides such as eriocitrin, narirutin, hesperidin, isorhoifolin and diosmin were extracted from the aerial parts of peppermint. The amount of phenolic and flavonoid compounds in aerial parts of peppermint has been reported to range from 2.8 to 17.8% and 0.71 to 3.86%, respectively [Nazerieh et al. 2018].

Given the chemical synthesis of plant secondary metabolites under laboratory conditions is considerably expensive and complicated, some alternative methods have been developed. Applying abiotic elicitors such as UV-radiation, abiotic stresses and phytohormones during medicinal plant cultivation is an effective strategy to change their secondary metabolite content and components. The word elicitor comes from elicit, which means extraction [Cappellari et al. 2020, Abdi and Karami 2020]. Elicitors are factors that directly or indirectly induce defensive changes in plants, leading to the activation of protective mechanisms and the biosynthesis of useful chemical compounds involved in plant adaptation to stress conditions. These factors are considered physical stimuli or chemical compounds with biotic or abiotic origins, which can induce different responses in plants, resulting in biosynthesis and accumulation of secondary metabolites in cells. Elicitors send some chemical messages to plant cells, which lead to physiological and morphological responses and the accumulation of phytoalexins. The antioxidant and defensive systems of plants are activated during the cell response to the elicitor's signals, leading to the expression of genes involved in secondary metabolite biosynthesis and accumulation [Merely et al. 2014, Abdi et al. 2018, Tabbert et al. 2022]. Using elicitors in limited amounts and low concentrations improves the biosynthesis of some compounds and generally reduces the time to achieve high amounts of metabolites. Elicitation is among the most effective practical methods to increase the biosynthesis of secondary metabolites in plant cells and tissues [Ahmad et al. 2022]. Elicitors may activate some new genes, leading to the production of new enzymes and activating new pathways, resulting in the accumulation of secondary metabolites. One of the most common groupings of elicitors is based on their nature, and according to that, the elicitors are divided into two different groups, including biotic and abiotic elicitors

(Fig. 1). Biotic elicitors are certain molecules from pathogens or host plants that can induce defensive responses. They are produced by plant enzymes on the cell membrane of microorganisms [Malik et al. 2020]. Moreover, biotic elicitors include some organic compounds produced by plant cells in response to various stimuli. Yeast extract, cell wall polysaccharides, oligosaccharides, proteins, glycoproteins and fatty acids are considered some of the most important biotic elicitors. Abiotic elicitors generally induce the production of phytoalexin in plant cells, and there are many reports on applying these factors to increase the secondary metabolite content of different plants [Nazerieh et al. 2018, Abdi et al. 2018, Cappellari et al. 2020, Tabbert et al. 2022].

The purpose of the current study was to investigate the roles of different abiotic elicitors in the biosynthesis and accumulation of different secondary metabolites in peppermint with the aim of better understanding the induction of biosynthesis pathways and the response mechanisms of plant cells to different abiotic stimuli.

MATERIALS AND METHOD

In this study, articles published, from 1999 to 2023, on the influence of abiotic elicitors on secondary metabolites of peppermint were also analyzed. Keywords, including "UV-radiation", "heavy metals", "chemical elicitors", "Peppermint", "*Mentha piperita*" and "secondary metabolites", were searched for in the Science Direct, Scopus and Google search engines. Sixty-one articles were collected, and their contents were carefully analyzed for the purposes of this study.

RESULT

Most important secondary metabolites of peppermint

The amounts of monoterpenes, phenolic compounds and flavonoids are higher than the other secondary metabolites in peppermint [Atanassova et al. 2011, Bodalska et al. 2019, Mahendran and Rahman 2020] (Table 1). The most important monoterpenes extracted from peppermint leaves are menthol

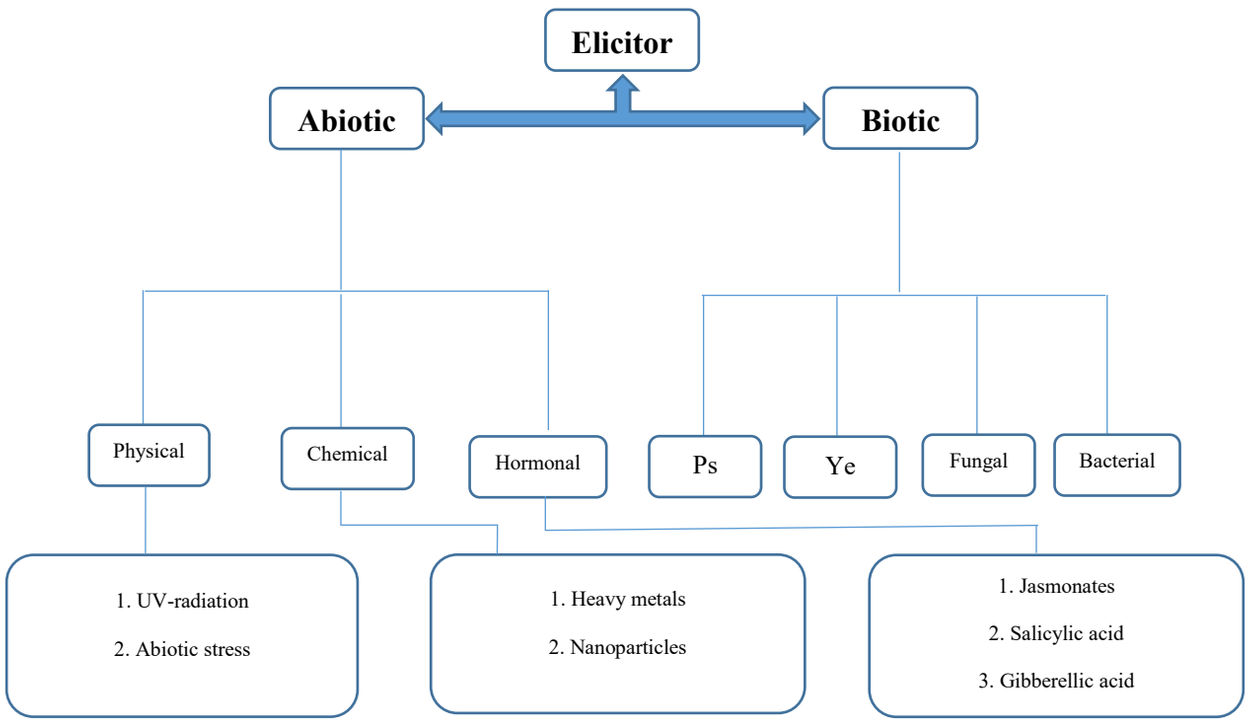

Fig. 1. Elicitors grouped based on their nature. Ps: polysaccharide, Ye: yeast extract

(30–55%), menthyl acetate (2.8–10%) and menthone (14–32%) [Croteau et al. 2005, Davis et al. 2005, Zhao et al. 2022]. Limonene (cyclic monoterpene), pulegone (monoterpene ketone), menthofuran, isomenthone (menthane monoterpene) and caron are the other monoterpenes found in peppermint leaves [Davis et al. 2005, Atanassova et al. 2011]. About 40 phenolic compounds, such as rosmarinic acid, cinnamic acid, caffeic acid and salvianolic acid, were extracted from the different aerial parts of peppermint [Sroka et al. 2005, Park et al. 2019, Naksawat et al. 2023]. Eriocitrin, narirutin, hesperidin, isorhoifolin, diosmin

Table 1. The most important secondary metabolites of peppermint

Secondary metabolite	Major compounds	Uses	Reference
Monoterpene	menthol	cosmetics, pain relief, improve the respiratory system	Mimica-Dukić et al. 2003, Croteau et al. 2005
	menthyl acetate	solvent for varnishes, lacquers, dry cleaning, stains, fats and nitrocellulose	Davis et al. 2005, Croteau et al. 2005
	menthone	treatment of stone formation in the gallbladder and liver	Davis et al. 2005, Croteau et al. 2005
	limonene	in medicinal ointments and creams penetrating the skin, fragrance, cleaner (solvent), and as an ingredient in household cleaning products, cosmetics, and personal hygiene products	Croteau et al. 2005, Bupesh et al. 2007, Zhao et al. 2022
	pulegone	flavoring agents, in perfumery, and in aromatherapy	Mahendran and Rahman 2020
	menthofuran	carminative and antispasmodic for esophageal spasm and irritable bowel syndrome	Davis et al. 2005, Croteau et al. 2005, Singh et al. 2015
	isomenthone	artificial flavorings in food, cosmetics, and pharmaceuticals, and even as pharmaceuticals themselves, e.g. camphor and eucalyptol	Davis et al. 2005, Croteau et al. 2005
	caron	treatment and prevention of iron deficiency anemia; for preventing iron, folic acid and zinc deficiencies during pregnancy, breastfeeding, after surgery or in conditions of nutritional malabsorption	Davis et al. 2005, Croteau et al. 2005, Mahendran and Rahman 2020
Phenolic compound	rosmarinic acid	treatment of inflammatory conditions such as arthritis, asthma, and atopic dermatitis	Aldoghachi et al. 2021
	cinnamic acid	flavorings, synthetic indigo, and certain pharmaceuticals	Sroka et al. 2005, Park et al. 2019
	caffeic acid	decreasing inflammation, preventing cancer, preventing toxicity associated with chemotherapy and radiation, preventing diabetes and premature aging, decreasing exercise-related fatigue	Lv et al. 2012, Park et al. 2019
	salvianolic acid	anti-oxidative activity	Lv et al. 2012
Flavonoid	eriocitrin	anti-atherosclerotic activity	Bodalska et al. 2019
	narirutin	anti-inflammatory and anti-oxidant	Bodalska et al. 2019
	hesperidin	treatment of blood vessel conditions such as hemorrhoids, varicose veins, and poor circulation (venous stasis)	McKay and Blumberg 2006, Bodalska et al. 2019
	isorhoifolin	antimutagenic	Bodalska et al. 2019
	diosmin	treatment of blood vessel disorders, such as hemorrhoids and chronic venous insufficiency	Bodalska et al. 2019, Soheilikhah et al. 2021
	Luteolin--glucuronide	depression-like and stress coping behaviors in sleep deprivation	Bodalska et al. 2019

and luteolin-glucuronide are the most important flavonoids extracted from peppermint leaves [Atanassova et al., 2011; Bodalska et al., 2019; Naksawat et al., 2023]. The amounts of phenolic compounds and flavonoids in peppermint aerial parts were reportedly about 2.8–17.8% and 0.71–3.86%, respectively [Ćavar Zeljković et al. 2021]. Atanassova et al. [2011] noted that the amount of phenolic compounds and flavonoids in aerial parts of peppermint is about 45.3 and 25.2 mg 100 g DW, respectively.

The reaction mechanisms of abiotic elicitors in peppermint

Abiotic elicitors act as stimulants and lead to morphological and physiological responses and the accumulation of phyto-alexins in different tissues of peppermint [Halder et al. 2019]. Research has shown that the treatment of peppermint with abiotic elicitors simulates a pathogen's attack, leading to a set of defensive reactions and accumulation of secondary metabolites [Baenas et al. 2014]. Although the reaction mechanisms of abiotic elicitors in peppermint are not yet clear, different mechanisms have been suggested, such as Ca^{2+} second messenger, factors involved in membrane integrity, inhibition or stimulation of intra- and inter-cellular pathways and changes in osmotic factor amounts and activity [Afkar et al. 2013, Askary et al. 2016, Nazerieh et al. 2018, Haydari et al. 2019, Abdi and Karami 2020, Ahmad et al. 2022]. Based on reports, the elicitors bind to membrane receptors, resulting in a change in cytosol pH and biosynthesis of reactive oxygen species (ROS) and increasing their activity rate, leading to up-regulating of the expression of genes involved in defensive responses [Afkar et al. 2013, Haydari et al. 2019]. Biological signals produced by abiotic elicitors induce the synthesis of the second messengers in plant cells, leading to kinase protein biosynthesis and subsequently enhancing secondary metabolite accumulation [Haydari et al. 2019]. Based on the studies conducted, the reaction mechanism of abiotic elicitors in peppermint can be explained based on Figure 2 [Afkar et al. 2013, Abdi

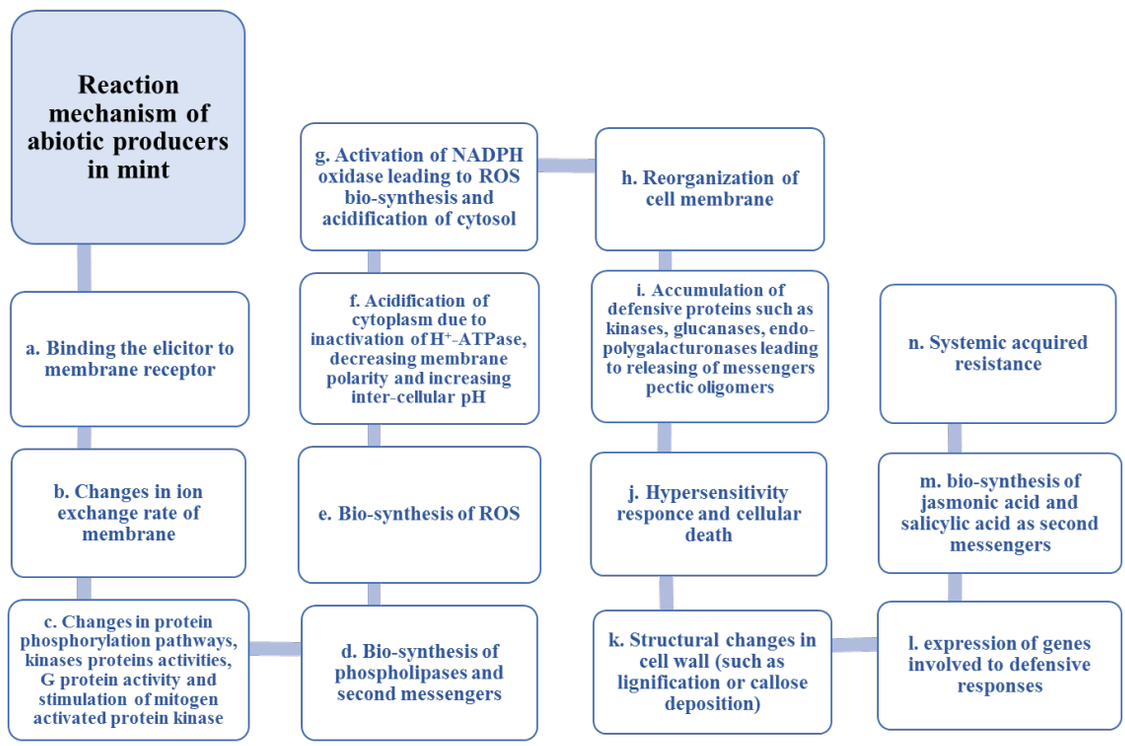

Fig. 2. The reaction mechanism of abiotic elicitors in peppermint; NADPH: nicotinamide adenine dinucleotide phosphate, ROS: reactive oxygen species

et al. 2018, Cappellari et al. 2020, Abdi and Karami 2020, Tabbert et al. 2022, Ahmad et al. 2022].

Effect of abiotic elicitors on secondary metabolites of peppermint

Physical elicitors. *UV-radiation.* Industrial crops such as peppermint show considerable metabolic changes in response to changes in environmental factors such as light quality and quantity [Farooqi et al. 1999, Croteau et al. 2005]. According to conducted studies, UV-B (280–315 nm), UV-A (315–400 nm) and PAR, meaning Photosynthetic Active Radiation (400–700 nm), as well as their respective ratios and interactions, are the most important radiation factors that can significantly influence secondary metabolite biosynthesis in peppermint [Maffei et al. 1999, Croteau et al. 2005, Behn et al. 2010, Dolzhenko et al. 2010, Mahendran and Rahman 2020, Tabbert et al. 2022].

UV-B radiation can affect the essential oil content of peppermint, and according to Behn et al. [2010], applying UV-B at 290 nm leads to a remarkable increase in peppermint essential oil content. Based on reports, UV-B radiation can remarkably affect the monoterpene content of peppermint leaves. The absence of UV-B radiation during the growth and development of the peppermint plant leads to lower menthol and higher menthone content, resulting in a significant reduction in oil quality (menthol is one of the main final products of a menthone reduction reaction catalyzed by menthone reductase enzyme) [Behn et al. 2010, Dolzhenko et al. 2010]. The influence of UV-B radiation on essential oil quality and content is correlated with the growth stage of the pepper plant. Measurements clearly demonstrate that the effect of UV-B radiation on essential oil components of pepper mint is more pronounced in the flowering stage, while this effect is lower in the vegetative stage and is not significant during bud formation [Behn et al. 2010]. The results of some studies have shown that UV-B radiation can affect the monoterpene and oil quality of peppermint in interactions with PAR radiation. In low PAR conditions (regions with mostly cloudy weather), using supplemental UV-B radiation can increase secondary metabolites, monoterpene content and oil quality of peppermint leaves, while when the PAR radiation is sufficient, changes in UV-B radiation have no significant effect on oil quality or secondary metabolite contents [Croteau et al. 2005, Mahendran and Rahman 2020]. The effect of UV-B radiation on the secondary metabolites of peppermint could be induced by changes in gene expression, enzymatic activities and defense responses [Saharkhiz and Goudarzi 2014]. Supplemental UV-B radiation in peppermint cultivation under greenhouse and open field conditions up-regulated the expression of five genes, including *dxs* (involved in early steps of terpenoid biosynthesis), *gpps* (involved in monoterpenes biosynthesis), *mr* (the most important gene involved in menthol biosynthesis) and *fpps* (involved in the biosynthesis of essential isoprenoids such as sterols and brassinosteroids, cytokinins, ubiquinone, dolichols, and prenylated proteins and some sesquiterpenes such as E-βcaryophyllene and germacrene-D); see Figure 3. Generally, this supplemental light treatment led to an increase in limonene, 1,8-cineol, E-(β)-ocimene, sabinene hydrate, linalool, menthone, menthofuran, pulegone, piperitone, piperitenone, (E)-β-caryophyllene and germacrene D. In addition, the content of eriocitrin, kaempferol 7-O-rutinoside and hesperidin is considerably increased in peppermint by induction of UV-B treatment [Dolzhenko et al. 2010].

As mentioned, UV-A (360 nm) radiation could be effective on peppermint metabolites. The results of a study regarding the influence of UV-A radiation on peppermint secondary metabolites demonstrated that the time of light treatment induction is effective in determining metabolite content and composition. Using supplemental UV-A radiation during the days led to a remarkable increase in phenolic compounds, essential oil content and menthol and menthofuran content, although light treatment during the nights led to the appearance of shade-avoidance syndrome, resulting in lower phenol, essential oil and menthol content. UV-A modulates the essential oil content of peppermint by regulation of enzymatic activity (menthone reductase enzyme) and gene expression (mainly the *mr* gene) [Maffei et al. 1999].

Abiotic stress. Research demonstrated that abiotic stresses such as drought stress, salt stress and heat stress change the content and ratio of the secondary metabolite components in medicinal plants [Charles et al. 1990, Khorasaninejad et al. 2011, Heydari et al. 2018, Alhaithloul et al. 2019, Hosseini et al. 2021]. Therefore, induction of abiotic stresses at low levels

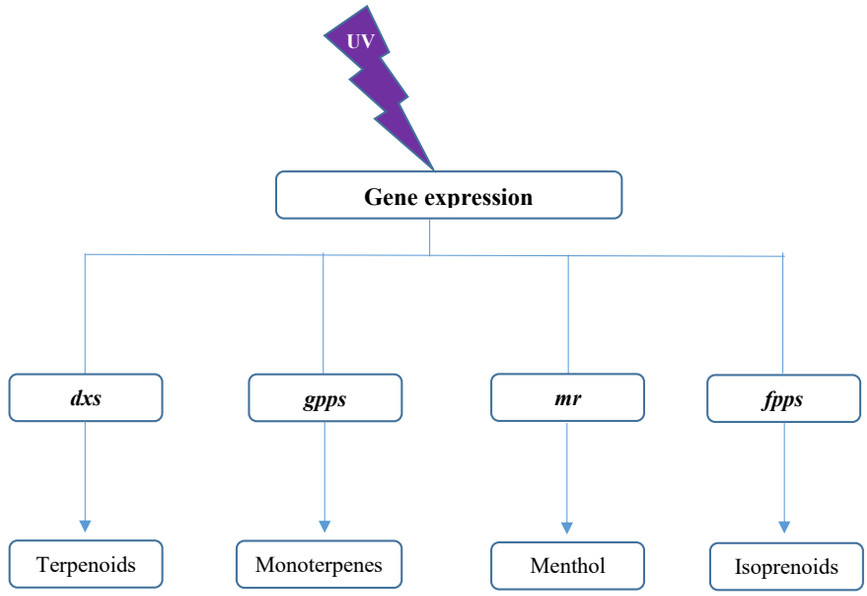

Fig. 3. The UV radiation effect on gene expression and metabolite biosynthesis in peppermint

during different stages of preharvest could be considered a widely used strategy to change the amounts of desired compounds in medicinal plants. The positive effects of abiotic stresses on increasing the amounts of phytochemical compounds such as phenolic acids, tannins and flavonoids in different tissues of horticultural plants have been reported in several studies [Aziz et al. 2008, Khorasaninejad et al. 2011, Roodbari et al. 2013, Li et al. 2014, Fathi et al. 2020]. Increasing secondary metabolites in response to environmental stresses is one of the plant's defensive mechanisms to alleviate the adverse effects of stresses [Hosseini et al. 2021]. Several studies have shown that the influence of stresses on the metabolites of medicinal plants is strongly correlated with stress severity. High levels of abiotic stresses increase secondary metabolite contents and can disrupt the pathways of metabolite biosynthesis by inducing negative effects on the plants' enzymatic and antioxidant activities and altering gene expression [Khorasaninejad et al. 2011, Li et al. 2014, Fathi et al. 2020].

Cultivation of peppermint under a deficit irrigation strategy (50% of field capacity) will cause a significant decrease in some important morphological characteristics such as plant height, leaf number and leaf area index, leading to lower photosynthetic rate, dry matter and accumulation rate of secondary metabolites in plant tissues. Total phenolic compounds and flavonoid contents of peppermint leaves increased by 34.8% and 43.6%, respectively, when irrigation was carried out with 70% of field capacity [Khorasaninejad et al. 2011]. Increasing the ROS accumulation rate in plant cells induced by drought stress leads to increasing phenolic compounds and flavonoids as antioxidant agents [Aziz et al. 2008, Khorasaninejad et al. 2011, Roodbari et al. 2013]. Phenolic compounds act as antioxidants by donating electrons to peroxidase enzymes and detoxifying hydrogen peroxide [Li et al. 2014]. Under moderate levels of drought stress, the highest increase rate in phenolic compounds and flavonoids was observed in rosmarinic acid and hesperidin. Drought stress also caused the production of phenolic and flavonoid compounds such as coumaric acid, luteolin, quercetin, naringenin, and vanillin in peppermint, which was not observed in plants grown under normal conditions [Charles et al. 1990, Khorasaninejad et al. 2011]. It seems that the induction of drought stress leads to the expression of some new genes that lead to the biosynthesis of new phenolic compounds and flavonoids in peppermint. Studies

have shown that drought stress is generally a negative factor for peppermint menthol content [Khorasaninejad et al. 2011, Abdi et al. 2018]. Based on reports, drought stress, even in low severities, reduces menthol content in different tissues of peppermint [Abdi et al. 2020]. Measurements have shown that this negative effect comes from the influence of drought stress on *mr* gene expression. Down-regulation of *mr* gene expression will occur in water deficit conditions, and there is a strong negative correlation between drought stress severity and expression level of *mr* gene [Croteau et al. 2005, Davis et al. 2005]. However, the results of some studies have shown that when drought stress, along with the other elicitors such as salt stress, nanoparticles and hormonal agents, is induced on peppermint, the influence of drought stress on essential oil content and components is generally determined through the interaction between the factors. Mostly low severities of drought stress in interaction with salt stress, nanoparticles, methyl jasmonate and salicylic acid can enhance peppermint essential oil content and quality [Alhaithloul et al. 2019, Abdi et al. 2018, Abdi et al. 2020].

Salt stress is one of the abiotic stresses which can affect secondary metabolite biosynthesis in peppermint [Aziz et al. 2008]. Different reports have been published about the effect of salinity on the secondary metabolites of peppermint [Roodbari et al. 2013, Li et al. 2014, Fathi et al. 2020, Hosseini et al. 2021]. Some studies have shown that inducing salinity stress during peppermint cultivation can increase the content of secondary metabolites in the aerial parts of the plant. However, other research demonstrated that salinity negatively impacts the biosynthesis of essential oil and other secondary metabolites in peppermint. According to Roodbari et al. [2013], growing peppermint under salinity conditions leads to more energy consumption by plant cells leading to lower carbon content that is available for the biosynthesis of secondary metabolites. Moreover, changing the hormonal ratios (abscisic acid to cytokinin) and lowering the amounts of cytokinin in plant aerial parts under salinity conditions are considered other important reasons for the lower biosynthesis rate of secondary metabolites. Studies on the influence of salinity on essential oil content and constituents demonstrated that doubling the salinity level led to a 48.28% decrease in essential oil content. A 55.18% decrease was also observed in essential oil yield when the salinity level increased by three times. Lower levels of salinity increased menthol content, but increased salinity levels to concentrations higher than 3 g L^{-1} NaCl drastically decreased menthol content in peppermint essential oil [Fathi et al. 2020]. Assessment of *mr* enzyme activity showed that its activity rate is strongly negatively correlated with the severity of salt stress. Pulegone, isomenthone, linalool and myrcene were the other essential oil components whose concentrations exhibited a negative correlation with salinity level [Hosseini et al. 2021]. Peppermint essential oil is mostly produced by epidermal oil glands, which are carbon heterotrophic. Therefore, a lower photosynthetic rate induced by higher levels of salinity leading to lower available carbon could be considered the main reason for lower essential oil content under salinity conditions [Roodbari et al. 2013, Li et al. 2014]. Similar to the other abiotic stresses, salinity generates high levels of excited energy and ROS, leading to damage to the cell membrane and a higher electrolyte leakage rate. Peppermint cells were found to produce increased levels of phenolic compounds and flavonoids in response to salinity, which acts as an abiotic stimulus. This response serves as a defensive mechanism to combat aggressive agents [Aziz et al. 2008]. Increased biosynthesis of polyphenols, especially in photosynthetic structures, and a rise in the amount and activity of glutathione s-transferase enzyme (this enzyme is involved in the transfer of flavonoids to vacuole) were recorded in peppermint grown under saline conditions [Li et al. 2014]. Moreover, studies have shown that induction of salinity stress at low levels increases the PAL enzyme activity in peppermint cells. However, increasing stress severity not only reduces PAL enzyme activity but also leads to a higher polymerization rate of soluble phenolic compounds, leading to a reduction in total phenol content [Roodbari et al. 2013, Li et al. 2014, Fathi et al. 2020]. Finally, the current study showed that the effect of salinity and drought stress on the biosynthesis of secondary metabolites in peppermint tissues drastically depends on the stress severity rate (Fig. 4).

Alhaithloul et al. [2019] demonstrated that cultivation of peppermint under heat stress conditions causes the accumulation of glycine betaine, inositol and mannitol in plant cells. The maximum accumulation

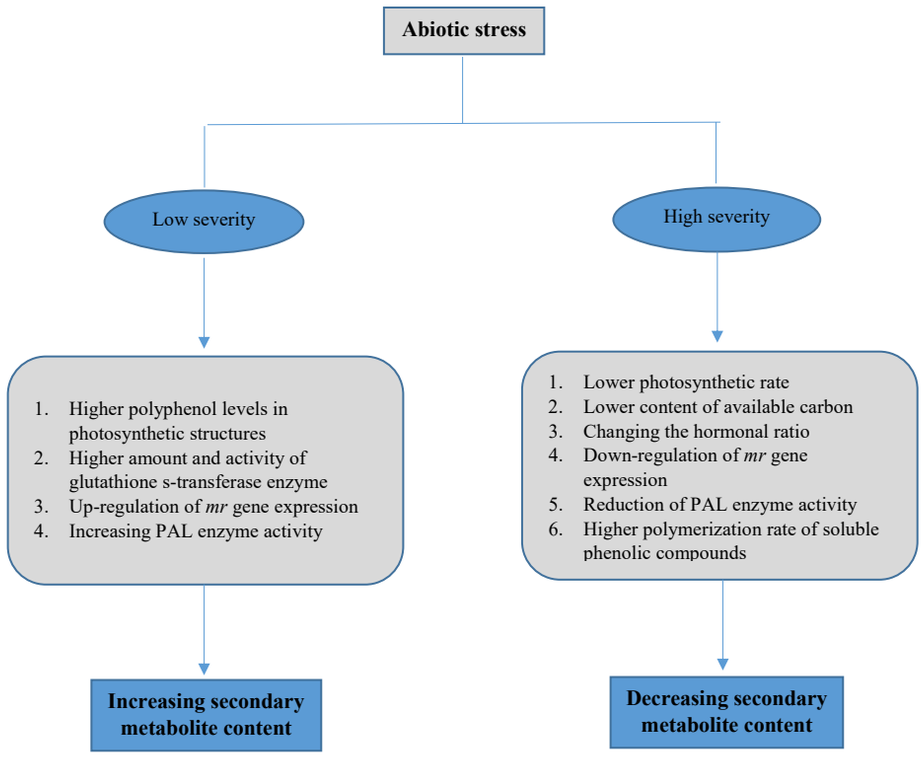

Fig. 4. The influence of abiotic stresses on the biosynthesis of secondary metabolites in peppermint is strongly corre-lated with the severity of stress

rates of these compounds were observed when the heat stress was inducted with low levels of drought stress. A molecular assessment also showed that the expression rate of genes involved in the shikimic acid pathway and biosynthesis of some alkaloids, such as anthocyanins and lignin, up-regulated by induction of heat stress during peppermint cultivation. The effect of heat stress on the *mr* gene is similar to that of drought stress, which indicates a negative correlation between heat stress severity and the *mr* gene expression rate. Studies have shown that cultivation of peppermint under temperatures of 23–25 °C leads to the highest *mr* enzyme activity rate and accumulation of menthol in peppermint aerial parts [Heydari et al. 2018, Alhaithloul et al. 2019].

Chemical elicitors. *Heavy metals.* Accumulation of heavy metals in agricultural soils through irrigation with contaminated wastewater not only causes soil pollution but also significantly reduces food quality and safety [Valko et al. 2005, Nazerieh et al. 2018, Nemati Lafmejani et al. 2018, Azimychetabi et al. 2021, Ahmad et al. 2022]. Among the heavy metals, Cd and Pb have been studied and investigated more than the others due to their high stability in the environment [Valko et al. 2005, Azimychetabi et al. 2021]. According to reports, plants need these elements in low concentrations, and accumulation of high amounts of heavy metals in soil can negatively affect seed germination, seedling growth and both the qualitative and quantitative characteristics of mature plants [Nemati Lafmejani et al. 2018, Azimychetabi et al. 2021, Ahmad et al. 2022]. According to Zheljazkov et al. [2006], the total yield of peppermint was not significantly changed when cultivated in soil enriched with heavy metals (Cd, Pb and Cu), although the menthol content was considerably decreased. In addition, the essential oil of peppermint plants did not contain any heavy metals. Menthol, as the most important sec-

ondary metabolite of peppermint, is bio-synthesized in eight steps, including the production of geranyl diphosphate from isopentenyl pyrophosphate, which is catalyzed by geranylgeranyl diphosphate synthase enzyme, the biosynthesis of limonene by the activity of limonene synthase enzyme, the biosynthesis of menthone from limonene through several oxidation, reduction, and polymerization reactions, and finally, the biosynthesis of menthol from the menthone by the activity of the menthone reductase enzyme [Croteau et al. 2005]. Thus, menthol is produced through a multi-enzyme pathway, and changes in the activity rate of each enzyme can affect the menthol biosynthesis rate in peppermint. Studies have shown that low concentrations (lower than 50 ppm) of some heavy metals such as Mn, Cd and Pb can up-regulate the expression of the limonene synthase gene, leading to more amounts of precursor for menthone and, subsequently, menthol biosynthesis. However, high concentrations (more than 100 ppm) of these heavy metals can reduce menthol biosynthesis rates by down-regulating the expression of the menthone reductase enzyme [Valko et al. 2005]. Nazerieh et al. [2018] reported that the influence of Se on secondary metabolites of peppermint is significantly related to the concentration of metals used in treatments. Based on their results, low concentrations of Se can increase total phenolic content and menthol in peppermint leaves by enhancing phenylalanine ammonia lyase (a key enzyme of phenylpropanoid metabolism) and menthone reductase enzyme activities. Cultivation of peppermint in soils with low concentrations of Cd and Pb did not affect the percentage of essential oil in the leaves. However, when the concentration of these heavy metals was increased to 100 ppm, both the quantity and quality of the leaf essential oil were reduced. This reduction may be linked to a decrease in the total dry matter of the leaves [Valko et al. 2005]. Since terpenoids produced by leaf epidermal cells are considered consumers of carbon produced through photosynthesis, the essential oil biosynthesis rate in peppermint leaves is remarkably correlated with the continuous production of photosynthetic carbon. High amounts of heavy metals can negatively affect essence production through photosynthesis rate reduction [Nazerieh et al. 2018, Lafmejani et al. 2018, Azimychetabi et al. 2021]. Studies on the influence of Cd on secondary metabolites of peppermint have shown that cultivating this crop in soils with high concentrations of Cd leads to decreasing menthol content and increasing menthofuran and pulegone contents. Increasing Cd levels in a substrate are followed by down-regulation of menthone reductase and pulegone reductase enzyme expression and up-regulation of menthofuran synthase enzyme expression [Azimychetabi et al. 2021]. Ahmad et al. [2022] reported that the reduction of secondary metabolites content in aerial parts of peppermint induced by cultivation in soils enriched with heavy metals is correlated with changes in activities of two vital regulatory enzymes, phenylalanine ammonia-lyase and deoxy-D-xylulose-5-phosphate reductoisomerase. These two enzymes are key factors in the shikimic aid pathway (an important pathway for phenolic compound biosynthesis) and the methylerythritol 4-phosphate pathway (an important pathway for terpenes biosynthesis); see Figure 5.

Nanoparticles. Nanoparticles of different elements have been used as chemical elicitors in different crops and cultivation processes to enhance qualitative and quantitative characteristics [Abdelsalam et al. 2023]. Nanoparticles can easily go through the pores of the cell wall and enter the plasma membrane due to their tiny size. Thus, their biological efficiency could be higher than elements of larger sizes [Ahmad et al. 2018]. Using these factors at different physiological steps, from seed germination to flowering and fruiting, has been reported in different crops such as wheat, rice, apple, basil, *etc*. [Ahmad et al. 2010, Ali et al. 2016, Jankovskis et al. 2022]. Considerable changes in enzymatic activity and gene expression leading to better growth, development and defensive responses were observed after applying these elicitors during crop cultivation [Nemati Lafmejani et al. 2018]. To date, the influence of iron, copper, titanium dioxide and iron oxide nanoparticles on secondary metabolites of peppermint has been investigated.

Using titanium dioxide nanoparticles at a concentration of 150 mg L^{-1} increased the essential oil content of peppermint by 39.4% compared to a control. This treatment also led to a higher menthol content (9.6% higher than the control) in treated plants. Treatment of peppermint with titanium dioxide nanoparticles leads to higher photosynthetic pigment content (chlorophyll a, chlorophyll b, total chlorophyll content, carotenoids

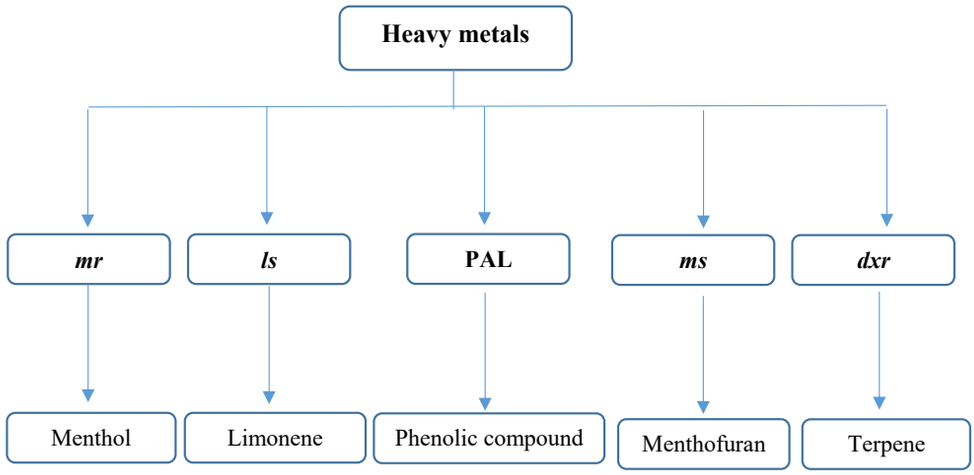

Fig. 5. The influence of heavy metals on the activity of enzymes involved in secondary metabolite biosynthesis in peppermint; *mr*: menthone reductase, *ls*: limonene synthase, PAL: phenylalanine ammonia-lyase, *ms*: menthofuran synthase and *dxr*: deoxy-D-xylulose-5-phosphate reductoisomerase

and anthocyanin) and subsequent photosynthetic rate. Both an increase in *mr* gene expression and a higher activity rate of menthone reductase enzyme were observed in treated plants with titanium dioxide nanoparticles, leading to higher menthol content compared to the control. Increased growth rate, photosynthesis rate, expression of terpene biosynthesis enzymes and density and diameter of oil glands were considered the most important reasons for the enhanced production of secondary metabolites in peppermint treated with titanium dioxide nanoparticles [Ahmad et al. 2018].

According to Nemati Lafmejani et al. [2018], there were no significant differences between the concentrations of 0.5, 1 and 1.5 g L^{-1} of iron nanoparticles in terms of their influence on monoterpene content in peppermint. These treatments increased the essential oil content of peppermint by 50–60%. The concentrations of menthone and menthol in treated plants also increased by more than 65% and 34%, respectively, compared to control. Iron nanoparticle foliar application at the flowering stage increased the menthofuran content in peppermint leaves more than twice.

Using iron oxide nanoparticles at 30 µm concentration during peppermint vegetative growth and flowering stage led to a higher essential oil content of treated plants (3.56%) compared to the control (2.19%). Studies have shown that iron oxide nanoparticles can influence the types and proportions of major components of peppermint essential oil. When the peppermint plants were sprayed with iron oxide nanoparticles at a concentration of 30 µm, menthone (49.67%), menthol (22.19%), 1,8-cineol (7.90%), pulegone (2.86%), menthofuran (2.84%), cis-sabinene hydrate (2.52%) and germacrene D (1.69%) were the major components of peppermint essential oil. Furthermore, the proportion of menthone increased and that of pulegone doubled. Increasing Fe concentration from 10 to 30 µm resulted in a higher total essential oil content, however, its quality decreased due to a reduction in menthol content and an increase in menthofuran content [Askary et al. 2016].

Copper, as an essential micronutrient, plays vital roles in different physiological processes such as cell wall metabolism, biosynthesis of regulatory proteins, mitochondrial respiration and biosynthesis of phytohormones. Copper acts as a cofactor for the activity of several enzymes involved in some of the most important plant physiological processes [Lafmejani et al. 2018]. The effect of copper nanoparticles on secondary metabolites of peppermint is correlated with the nutrient concentration used in the treatment. Studies have shown that using high concentrations of copper reduces the total secondary metabolite content of some crops, such as beans, wheat, and peppermint, because

of its toxicity effects and growth inhibition. According to reports, foliar application of copper nanoparticles at the concentration of 1 g L^{-1} during the flowering stage of peppermint could be a widely used technique to increase the essential oil content of peppermint. However, exceeding this concentration may reverse the increase in essential oil production [Nemati Lafmejani et al. 2018]. Nemati Lafmejani et al. [2018] reported that using copper nanoparticles during the reproductive growth of peppermint will increase the leaf menthol and phenolic compound contents by 15 and 18%, respectively.

Hormonal elicitors. Studies have shown that hormonal elicitors can affect gene expression, enzymatic pathways and biosynthesis of metabolites in plants. Hormonal elicitors are used during crop cultivation for different purposes, such as increasing productivity rate, enhancing tolerance rate against biotic and abiotic stresses or improving qualitative characteristics [Soleymani et al. 2015, Çoban et al. 2016, Soleymani et al. 2017, Abdi et al. 2018, Abu El-Leel et al. 2021]. Based on reports, methyl jasmonate, salicylic acid, gibberellic acid, and melatonin are some hormonal elicitors that can significantly affect the secondary metabolite content and essential oil quality of peppermint.

Methyl jasmonate. Foliar application of methyl jasmonate at 0.1% concentration at the vegetative stage can induce alteration of gene expression pattern in peppermint. Twenty-one out of 33 transcript-derived amplicons were the new transcripts. This change in gene expression pattern induced by methyl jasmonate application led to a significant increase in volatile oil percentage. The total concentration of monoterpene hydrocarbons increased by 6.85% in treated plants compared to control. The concentration of oxygenated monoterpenes also increased by 4.42% in treated plants compared to non-treated plants. Increasing menthol content and decreasing menthone content and menthofuran gene expression (which produces higher quality essential oil) were the other effects of methyl jasmonate application during the vegetative growth of peppermint [Abu El-Leel et al. 2021]. As mentioned, methyl jasmonate application can induce changes in gene expression patterns. Genes such as *pr*, *mfs* and *ls* were considered the most important genes involved in changes in the content of monoterpenes induced by methyl jasmonate application. Generally, *pr, mfs and ls* are key genes in the monoterpene biosynthesis pathway in peppermint [Krzyzanowska et al. 2012]. Previous studies have shown that up-regulation of the expression of these genes in response to methyl jasmonate application will result in higher menthol content [Abu El-Leel et al. 2021]. The expression of these genes will lead to higher amounts and activities of some key enzymes involved in menthol biosynthesis in peppermint. Moreover, increasing the pulegone and menthofuran biosynthesis was observed after the up-regulation of the expression of *pr* and *mfs* genes induced by methyl jasmonate application [Afkar et al. 2013, Soleymani et al. 2017]. The results have demonstrated that the response of *pr* and *ls* genes to methyl jasmonate application is faster than that of *mfs* gene, which means that the expression level of *mfs* gene might be related to the expression of two other genes. This indicates that the effect of methyl jasmonate application on monoterpenes of peppermint is not only induced by changes in transcript levels but also by varying the rates of expression among different genes. This has a significant impact on changes in secondary metabolites [Afkar et al. 2013].

Salicylic acid. Salicylic acid is a widely used chemical elicitor to improve the metabolite profile, nutritional value and antioxidant characteristics of peppermint [Abdi et al. 2020]. For instance, Figueroa-Pérez et al. [2015] reported that the application of salicylic acid at 1 mM concentration increased the total phenolic content of treated plants, and some secondary metabolites such as sinapic acid, rutein and naringin were detected only in salicylic acid-treated plants. Saharkhiz and Goudarzi [2014] reported that using salicylic acid at a concentration of 150 mg L^{-1} significantly increased the essential oil content compared to untreated plants. In particular, salicylic acid treatment mostly increased menthone (15.8–18.1%) and menthol (46.3–47.4%) content. The effect of salicylic acid on the essential oil of peppermint is correlated with the concentration of treatment. Previous studies have shown that using salicylic acid at a concentration of ≤2 mM led to increasing total essential oil content by 1–2%, but on the other hand, increasing the concentration of the treatment chemical up to 2 mM reduced the essential oil content and quality [Cappellari et al. 2019]. However, under water stress conditions, using salicylic acid

treatment at concentrations higher than 2 mM resulted in increased essential oil contents (more than 2-fold compared to control) [Abdi et al. 2020]. Increasing phenolic compounds, especially cinnamic acid and salvianolic acid, which occur through an increase in phenylalanine ammonia-lyase enzyme activity, as well as higher flavonoid levels, particularly eriocitrin and narirutin, induced by salicylic acid treatment, were also reported in various studies [Saharkhiz and Goudarzi 2014, Haydari et al. 2019, Abdi et al. 2020]. The influence of salicylic acid treatment on the expression of genes involved in monoterpenes biosynthesis in peppermint demonstrated that using treatments at a concentration below 1 mM had no significant effect on *ls* and *pr* gene expression, while increasing the treatment concentration led to up-regulating gene expressions and higher monoterpenes biosynthesis rate in peppermint [Cappellari et al. 2019]. Figueroa-Pérez et al. [2015] reported that treatment of peppermint plant with salicylic acid at a concentration of 0.5 mM can increase some triterpene and steroid saponins (such as phytolaccagenic acid, phytolaccagenic acid, hederagenin, serjanic acid, campesteryl, stigmastery and sitosteryl) and the concentration of some alkaloids (such as choline, trigonelline, nicotinic acid, sitsirikine, vinblastine, vindoline, catharanthine and vinleurosine). Based on the results of these authors, changes in the saponin and alkaloid profiles induced by salicylic acid treatment led to improving anti-diabetic effects of peppermint. To develop systemic acquired resistance (SAR), a plant must generate a signal in the pathogen-inoculated tissue that travels (presumably through the vasculature) to the uninoculated portions of the plant, in which it signals defense responses. Radio-tracer studies in tobacco and cucumber initially indicated that some of the salicylic acid (SA) in systemic leaves was synthesized in the inoculated leaf, raising the possibility that SA was the mobile signal [Mölders et al. 1996, Shulaev et al. 1995]. Consistent with this possibility, pathogen-induced SA was shown to move through the apoplast before being loaded to phloem in Arabidopsis [Lim et al. 2016], and SA was detected in phloem sap in pathogen-infected plants [Lim et al. 2016, Métraux et al. 1990, Mölders et al. 1996, Yalpani et al. 1991]. However, analyses of chimeric tobacco generated by grafting combinations of wild type (wt) or SA-deficient rootstocks and scions (the upper half of the plant) revealed that plants containing a wt scion developed SAR even if the rootstock was SA-deficient. By contrast, plants containing an SA--deficient scion failed to develop SAR, regardless of the rootstock [Pallas et al. 1996, Vernooij et al. 1994]. Overall, these studies suggest that SA accumulation is required in uninoculated tissues to signal SAR, but SA is not likely the critical mobile signal.

Over the years, efforts to identify the mobile SAR signal have identified several candidates. The first SAR signal to be identified was the SA derivative MeSA. This finding was rapidly followed by the discovery that other compounds, including a nine-carbon dicarboxylic acid azelaic acid (AzA), glycerol-3-phosphate (G3P) or a G3P-dependent factor, the abietane diterpenoid dehydroabietinal (DA), and the lysine (Lys) derivative pipecolic acid (Pip), also are mobile inducers of SAR. In addition, SAR signaling mediated by some of these small metabolites appears to depend on one or both the lipid transfer protein (LTP) defective in induced resistance 1 (DIR1) and the LTP-like protein azelaic acid-induced 1 (AZI1). Since these signals and the complex network through which they interact have been the subject of several reviews published to date [Dempsey and Klessig 2012, Shah and Zeier 2013, Shah et al. 2014, Singh et al. 2017], only some of the more recent findings will be summarized here.

Genetic, molecular, and biochemical analyses have led Pradeep Kachroo and colleagues to propose that SAR is activated by parallel pathways mediated by SA and AzA/G3P [Singh et al. 2017, Wendehenne et al. 2014]. In this model, pathogen infection leads to the accumulation of SA and nitric oxide (NO), which triggers the accumulation of ROS via an amplification loop (Fig. 3). ROS, in turn, generate AzA from precursor C18 unsaturated fatty acids (FAs). AzA then induces the synthesis and accumulation of G3P, which travels via the symplast to the phloem and subsequently induces SAR, in conjunction with SA, in the systemic tissue [Lim et al. 2016, Wang et al. 2014, Yu et al. 2013]. DIR1 and AZI1, which interact with each other as well as themselves, impact this pathway by forming a positive feedback loop with G3P [Yu et al. 2013]. Since pathogen-induced accumulation of AzA and G3P as well as SAR were compromised in Arabidopsis mutants that lack the ability to synthesize the galactolipids monogalactosyldiacylglycerol (MGDG)

or digalactosyldiacylglycerol (DGDG), it was further hypothesized that AzA is generated via oxidation of C18 unsaturated FAs on MGDG and DGDG lipids, rather than free FAs [Gao et al. 2014]. Interestingly, the DGDG-defective mutant dgd1 but not the MGDG-defective mutant mgd1 failed to accumulate NO or PR-1 transcripts after pathogen infection; dgd1 plants also displayed reduced accumulation of free SA and SAG in pathogen-inoculated leaves and reduced free SA accumulation in the systemic leaves. Thus, DGDG and MGDG appear to have additional, distinct functions that impact different steps of the SAR signaling pathway [Klessig et al. 2018].

Gibberellic acid. Gibberellic acid, as a phytohormone and plant growth regulator, is recognized as an important effective factor in eliciting the production of secondary metabolites in plant cells [Bose et al. 2013]. However, in contrast to this, previous studies have shown that exogenous application of gibberellic acid on peppermint plants led to lower menthol content in treated plants. The transcript levels of genes involved in the first steps of menthol biosynthesis, such as *gds*, *lh*, *ls* were not affected by gibberellic acid treatment, but the expression of genes involved in later stages, such as *neo-red* and *m-deh* was down-regulated by applying this treatment [Soleymani et al. 2015]. Gibberellic acid treatments increase the total phenolic compound of peppermint (especially caffeic acid) by enhancing PAL enzyme activity. Moreover, the positive effects of gibberellic acid treatments on the formation of trichomes and increasing their density and diameter, leading to higher essential oil content was reported in *Menta arvensis* plants [Bose et al. 2013], and similar effects could be found in peppermint through further research.

Melatonin. Melatonin is a brassinosteroid hormone that plays several important roles in regulating different physiological processes such as germination, rooting and defensive responses against plant stresses [Haydari et al. 2019]. According to previous studies, using melatonin as a plant growth regulator during peppermint cultivation can significantly increase its essential oil yield [Haydari et al. 2019]. Haydari et al. [2019] reported that treatment of peppermint with melatonin at a concentration of 10–30 M (40 d after seed sowing) increased total concentrations of oxygenated monoterpenes and oxygenated sesquiterpenes. The amount of monoterpene hydrocarbons was not affected by melatonin treatments, but the foliar application of melatonin at a dose of 30 M increased menthol content by 14.3%. In addition, the amount of menthone and pulegone in treated plants was higher than that of the control, and menthofuran decreased with increasing melatonin treatment concentration from 10 to 30 M. To date, the mechanisms of melatonin effect on changes in enzymatic activities, biological pathways and gene expression in peppermint have not been investigated.

CONCLUSION

Given the economic importance of plant secondary metabolites and the increase in their demand, researchers are looking for inexpensive and useful strategies to increase the content and quality of plant secondary metabolites. Using abiotic elicitors such as UV radiation, salt and drought stress, and phytohormones during different growth stages of peppermint could be introduced as an effective method to enhance the content and quality of desired compounds. The effect of abiotic elicitors on secondary metabolites of peppermint is often correlated with the dosage, concentration or severity of abiotic elicitors and choosing the appropriate dosage or intensity is remarkably important in achieving the desired results. Abiotic elicitors can affect the secondary metabolites profile of peppermint mostly through changes in gene expression and plant enzymatic activity. In recent decades, most of the conducted research has covered the effects of abiotic elicitors on essential oils and monoterpenes (especially menthol) in peppermint, and more research on other important secondary metabolites of peppermint is required.

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AUTHORS' CONTRIBUTION STATEMENT

ANK, GE and MER compiled the literature and wrote the manuscript. MG edited and reviewed the manuscript. All authors approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

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

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