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RESEARCH PAPER

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THE EFFECT OF BIOCHAR AND CONSORTIA OF BENEFICIAL BACTERIA ON THE OCCURRENCE OF ARBUSCULAR MYCORRHIZAL FUNGI IN CULTIVATION OF CUCUMBER, STRAWBERRY AND APPLE PLANTS

Edyta Derkowska[1], Beata Sumorok[2], Lidia Sas-Paszt[3], Krzysztof Górnik[4], Sławomir Głuszek[5]

ABSTRACT

The aim of the study was to evaluate the effect of newly developed microbial consortia on the degree of colonization of cucumber, strawberry, and apple roots by arbuscular mycorrhizal fungi and to determine the number of spores of these fungi in the rhizosphere soil. The following strains of beneficial bacteria were used in the 3 consortia: 1 – *Bacillus licheniformis* TES10B3, TES5B21, GOS10B9; *Streptomyces* sp. GOS5B1, 2 – *Pseudomonas* sp. Pi22B, Pi25C, *Klebsiella* sp. NAzot2, 3 – *Priestia* sp. TES5B10C, GOS5B22; *Bacillus licheniformis* GOS10B151. All consortia have been enriched with biochar. It was found that the use of Consortium 2 with biochar had asignificant effect on increasing the colonization of roots by arbuscular mycorrhizal fungi. Consortium 3 significantly increased the formation of mycorrhizal fungi spores in the rhizosphere soil in 2023, while in the following year the highest spore count was observed in the soil after the application of Consortium 2 with biochar. This beneficial effect makes both biochar and bacterial consortia recommended in the cultivation of horticultural plants to improve their growth and development and to improve the quality of soils, especially those that are poor in organic matter. Consortium 2 is recommended as a biostimulant for growing fruit and vegetable plants.

Keywords: root colonization, rhizosphere soil, spore count, biostimulant

INTRODUCTION

Feeding the ever-growing human population is one of the most significant challenges facing global agriculture. Environmental factors that limit food security and affect crop production are undoubtedly soil degradation [Lal 2020]. The decrease in the level of organic matter and nutrients in the soil has led producers to excessively use mineral fertilizers in order to maintain high-quality crops [Sas-Paszt et al. 2023]. However, this practice causes environmental pollution and greenhouse gas emissions, as well as a reduction in the population of beneficial microorganisms in the soil [Derkowska et al. 2024].

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Considering the above problems, biochar is becoming more widely used as a sustainable alternative in agriculture to improve soil quality and protect the natural environment [Jatuwong et al. 2024]. Used as a soil improver or as a component of biofertilizer to increase soil fertility and enhance its bio-physico-chemical properties, it can reduce the need for mineral fertilizers [Nepal et al. 2023, Kabir et al. 2023]. The components contained in biochar such as nitrogen (N), phosphorus (P), calcium (Ca), magnesium (Mg), potassium (K) and high organic carbon content are essential for plant growth, and high porosity, low density, high capacity and neutral to alkaline pH make it increasingly used in the cultivation of horticultural and agricultural plants [Kabir et al. 2023]. The addition of biochar to soil, whether in the form of biofertilizers or composts, can increase soil organic carbon content and porosity, stabilize pH, immobilize toxic heavy metal elements, and increase soil microbial activity, which affects the development of the root system of crops and improves soil nutrient uptake [Nepal et al. 2023, Kabir et al. 2023, Sifton et al. 2023]. Biochar also plays an important role in reducing global greenhouse gas emissions and reducing atmospheric CO_2 concentration [Jabborova et al. 2021]. As a carrier of organic matter, it has been extensively studied for its effects on plants, however the effects of biochar on microbiome structure and biodiversity in different soil conditions have not yet been fully understood [Xu et al. 2023a]. Assessing the impact of biochar on soil microbial diversity in horticultural crops is crucial for maintaining soil health, enhancing productivity, and mitigating the adverse effects of intensive crop cultivation and climate change [Xu et al. 2023b]. When assessing soil quality, we often pay attention to the roles of microorganisms in the soil environment, the size and structure of their populations, and species diversity [Ren et al. 2022]. Different plant species grown in the same soil can affect the development of different groups of microorganisms [Zhang et al. 2022].

Soil microorganisms, through the decomposition of organic matter in the soil, make nutrients available to plants and are also responsible for the mineralization, adsorption and binding of elements supplied to the soil with both mineral and organic fertilizers [Li et al. 2022, Fuke et al. 2021]. The microbiome, occurring in the rhizosphere soil, i.e. the one directly surrounding plant roots, contains characteristic species of microorganisms, attracted by root exudates released into the soil [Perreault and Laforest-Lapointe 2022]. Sometimes it has been called the second plant genome [Zhang et al. 2022]. Direct interactions between crop roots and soil microorganisms have a significant impact on the growth, health and adaptation of plants to unfavorable environmental conditions [Petipas et al. 2021]. Plant growth-promoting bacteria (PGPB) are an important group of microorganisms that primarily colonize the rhizosphere of plants. They can support plant growth and development by increasing the bioavailability and uptake of carbon, nitrogen, and essential minerals from the soil [Racioppo et al. 2023]. They also contribute to increased plant yield, reduced pathogen infestation, and alleviate biotic and abiotic stress [Sharma et al. 2025]. In the natural environment, arbuscular mycorrhizal fungi (AMF) participate in mutualistic symbiotic associations with various crop species, are an essential component of the rhizosphere microbiome and play an important role in nutrient uptake from the rhizosphere and improve plant nutrition [Fall et al. 2022]. The use of mycorrhizal fungi as components of biostimulants and biofertilizers is a significant and environmentally friendly approach to modern agriculture [Shi et al. 2022, Sun et al. 2023]. AMF are widely used in ecological and sustainable crop production because they provide a number of benefits, e.g. they affect plant nutrition by providing micro and macro elements, participate in nitrogen fixation and participate in soil carbon cycling through the decomposition of organic matter [Ebbisa 2022]. Mycelial hyphae can affect soil structure and prevent its erosion, participate in the remediation of soils contaminated with heavy metals, support biodiversity and regulate water uptake by plants [Ebbisa 2022]. Studies by many authors have shown that the species composition and abundance of mycorrhizal fungi are regulated by the plants with which these fungi live in symbiosis, but the presence of AMF can also affect the abundance and composition of other species of microorganisms found in the rhizosphere soil [Emmett et al. 2021, Wang et al. 2021]. However, the conditions and mechanisms responsible for this relationship between mycorrhizal fungi and other microorganisms remain unknown. Ultimately, the effectiveness of nutrient uptake from the rhizosphere soil depends on this cooperation, which in turn affects the growth and development of plants and the biodiversity of the soil environment.

In this work, we undertook research on the influence of specific soil bacterial consortia and biochar on the level of colonization of cucumber, strawberry and apple plant roots by arbuscular mycorrhizal fungi naturally occurring in soil and the number of spores in the rhizosphere soil under the influence of their use.

MATERIALS AND METHODS

The research included 3 series of field experiments carried out at the Experimental field of the National Institute of Horticultural Research (INHORT) in Skierniewice, Poland (cucumber, strawberry) and the Experimental Orchard of INHORT in Dąbrowice near Skierniewice (apple).

Characteristics of the applied materials

Biochar was produced by rapid pyrolysis (at 280°C for 5 minutes) from coniferous wood chips containing 80% of organic matter and 20% of organic carbon.

Bacterial strains from SYMBIO BANK by INHORT, which have a beneficial effect on plant growth and development, were used to create consortia of microorganisms [Derkowska et al. 2023]: Consortium 1 – strains: *Bacillus licheniformis* TES10B3, TES5B21, GOS10B9; *Streptomyces* sp. GOS5B1; Consortium 2 – strains: *Pseudomonas* sp. Pi22B, Pi25C, *Klebsiella* sp. NAzot2; Consortium 3 – strains: *Priestia* sp. TES5B10C, GOS5B22, *Bacillus licheniformis* GOS10B151.

The consortia were applied in the form of a liquid suspension with a concentration of microorganisms of 10^6 cfu/ml after 72 hours of cultivation, with the exception of the *Streptomyces* sp. GOS5B1 strain after 7 days of cultivation.

Description of experiments

Cucumber, strawberry and apple plants were planted in podzolic soil with pH 6.2, and a quality rating of 3B, which contained macro-elements at the level of: P – 7.5, K – 12.4, Mg – 5.8 mg/100 g and microelements at the level of: B – 2.4, Cu – 4.8, Fe – 862, Mn – 75.5, Na – 4.35, Zn – 3.7 mg/1000 g, and the humus content was 1.2%. The preceding crop was mustard plants, and the soil was cultivated before planting the plants in accordance with the recommendations for cucumber, strawberry and apple plantations. The experiment was established in a randomized block design in four replications for each plant species. Prior to setting up the experiment granulated poultry manure at a rate of 200 g per plot for cucumber and strawberry, and 300 g per tree for apple, was applied. Biochar was applied at a rate of 2L per plot, mixed with the topsoil layer. Microbial consortia were applied in the root system zone around the growing plants by soil watering, at the following doses: cucumber and strawberry – 40 ml per plant, apple – 400 ml per tree The application of microbial consortia, biochar and manure was carried out in the spring of each year of the experiment, at the beginning of the growing season.

Experiment I. Cucumber seeds Octopus F1 cv. (Syngenta, Netherlands) were sown into the soil in May 2023 and 2024, respectively. Each plot ($2m^2$) constituted an experimental unit, where 14 seeds were sown with a spacing of 0.3 m within the row and 1 m between rows. Standard care treatments were performed during the experiment.

Experiment II. Frigo A+ strawberry plants of the Lycia variety (produced by Vivai Mazzoni Societa Agricola, Italy) were planted in April 2022 at a spacing of 0.9 m × 0.2 m. Each experimental combination consisted of 15 plants per plot. Standard maintenance procedures were performed on the strawberry experimental plots before planting, and in each of the two years of the experiment.

Experiment III. One-year-old maiden apple trees of the Gold Milenium variety, grafted onto M. 9 rootstock (produced by the Center for Elite Nursery Material in Prusy, Poland), were planted in May 2018 at a spacing of 2 m × 4 m. Until the experiment was established, standard maintenance procedures for commercial orchards were performed. The study involved 4-year-old apple trees with an experimental plot area of 12 m^2 (3 trees per plot) and standard maintenance procedures were performed on them.

The experiment consisted of 8 treatments, namely: Control (only granulated poultry manure at the beginning of the growing season), Biochar, Consortium 1, Consortium 2, Consortium 3, Consortium 1 + Biochar, Consortium 2 + Biochar, and Consortium 3 + Biochar.

Determination of root colonization by arbuscular mycorrhizal fungi naturally occurring in soil

The roots of apple, strawberry and cucumber plants (10 g from each replication), collected in July 2023 and 2024, were stained according to the method developed in the Department of Microbiology and Rhizosphere of INHORT [Derkowska et al. 2015]. Next, microscopic specimens were prepared and examined with a Nikon Eclipse 50i microscope (objectives with magnifications of 20×, 40×, 60×, 100×) and photographic records of the observed mycorrhizal structures were produced. The assessment of the degree colonization of the roots by arbuscular mycorrhizal fungi was performed by the method of Trouvelot et al. [1986]. Based on the results, the frequency of mycorrhiza occurrence (F%) was calculated using the computer program MYCOCALC, available on the website: http://www2.dijon.inra.fr/mychintec/Mycocalc-prg/MYCOCALC.EXE (Tables 1–6).

Counting of the number of spores of mycorrhizal fungi naturally occurring in rhizosphere soil

Samples of rhizosphere soil collected from the plants of every experimental combination, collected in July 2023 and 2024, were used to weigh out 100 g portions for further analyses. These were then placed in bottle containers and made up to 1 litre with distilled water. The resulting suspensions were shaken for approx. 1 hour

and placed in a refrigerator for 24 h at 4°C. After that, the soil solutions were filtered through a column of sieves (0.5 mm, 0.125 mm, 0.0063 mm, and 0.0045 mm). The fractions of soil remaining on the successive sieves were washed away with distilled water into Petri dishes (120 mm), to which sucrose (5 g per dish) was added. The samples thus prepared were examined using a Nikon SMZ 800 stereoscopic microscope, fishing out and counting spores of mycorrhizal fungi found in them [Błaszkowski 2008, Tables 1–6].

Statistical analysis

The results were statistically analysed by one-way analysis of variance in a random block design. Multiple comparisons of means for the combinations were performed with Tukey's test at a significance level of $\alpha = 0.05$ using STATISTICA v.13.1 software (StatSoft Inc., 2011). Data not significantly different from each other are marked with the same letter.

RESULTS

Compared to the roots of control plants, the use of consortia of beneficial bacteria had a positive effect on the intensity and degree of root colonization by arbuscular mycorrhizal fungi present in the soil and the formation of their spores in the rhizosphere of cucumber, strawberry and apple plants.

Year 2023. Roots of cucumber plants after the application of Consortium 2 on its own, as well as in combination with biochar, were colonized to the greatest extent and most intensively by arbuscular mycorrhizal fungi. Similarly, applications of biochar and Consortium 1, as well as those applied in combination with biochar , also caused a significant increase in the colonization of roots by mycorrhizal fungi. Analyses of rhizosphere soil collected from experimental plots of cucumber plants showed that the combined application of Consortium 3 and biochar significantly increased the number of spores of arbuscular mycorrhizal fungi in the soil. On the other hand, the application of biochar and Consortium 2 had no significant effect on the number of spores in the rhizosphere soil of cucumber (Table 1).

The roots of strawberry plants after the combined application of Consortium 2 in combination with biochar were colonized to the greatest extent and most intensively by arbuscular mycorrhizal fungi. Similar results were obtained after the application of Consortium 2 on its own, while the application of biochar alone did not significantly affect the colonization of roots by AMF. Analysis of strawberry soil showed that the combined application of Consortium 3 and biochar significantly increased the number of spores of arbuscular mycorrhizal fungi in the soil. It is worth noting that a slightly lower number of these spores was found after the application of Consortium 2 in combination with biochar (Table 2).

The roots of apple trees after the application of Consortium 2 in combination with biochar and also after the application of Consortium 1 and 2 on their own were colonized to the greatest extent by arbuscular mycorrhizal fungi. In the roots of control trees, however, the colonization of roots was the lowest. Microscopic observations also allowed to state that the roots of apple trees were most intensively colonized by AMF after the application of Consortium 2 alone and in combination with biochar. Similarly to the soil from under cucumber and strawberry plants, also the soil from under apple trees after the combined application of Consortium 3 and biochar was characterized by the highest number of spores, and after the application of Consortium 2 and biochar alone, the analyzed soil contained the lowest number of spores (Table 3).

Year 2024. The combined use of Consortium 2 and biochar, and Consortium 2 applied alone significantly increased the colonization of cucumber roots by AMF. Only the roots of control plants were characterized by the lowest degree of root colonization by AMF. The highest relative intensity of mycorrhization was observed in the roots of cucumber plants after the application of Consortium 2, and Consortium 2 with biochar. Also the use of Consortium 2 with biochar significantly increased the number of spores of AMF in the rhizosphere soil of cucumber. Only in the control soil the number of spores was the lowest (Table 4).

In strawberry roots, it was found that the highest degree of root colonization was characteristic of plants after the combined application of Consortium 2 and biochar. The single application of Consortium 2 also significantly influenced the increase in root colonization by AMF, while the roots of control plants were colonized to the least extent. The combined application of Consortium 2 and biochar significantly influenced the intensity of colonization. Comparing the rhizosphere soil of strawberry, it was observed that the combined application of Consortium 2 and biochar increased the number of spores in the soil. Only the rhizosphere soil of control plants was characterized by the lowest number of spores (Table 5).

Table 1. Degree of cucumber root colonization by arbuscular mycorrhizal fungi and the number of spores in the rhizosphere soil after the application of beneficial bacteria and biochar in 2023 (INHORT Experimental Field in Skierniewice, 2023)

Treatment	F (%)	M (%)	m (%)	Spore count (per 100 g soil)
Control	15.56 a	1.02 a	5.90 a	9 a
Biochar	33.33 b-d	2.39 ab	7.10 a	14 a
Consortium 1	33.33 b-d	2.35 ab	6.97 a	32 b
Consortium 2	41.11 d	2.79 b	6.94 a	11 a
Consortium 3	22.22 ab	1.73 ab	7.89 a	28 b
Consortium 1 + Biochar	36.67 cd	2.75 b	7.51 a	29 b
Consortium 2 + Biochar	45.55 d	3.20 b	6.92 a	36 b
Consortium 3 + Biochar	25.56 a-c	2.18 ab	8.60 a	51 c

F – mycorrhizal frequency, M – relative mycorrhizal frequency (for the whole sample), m – absolute mycorrhizal frequency (for the segments in which there was some evidence of colonization by mycorrhizal fungi)

Table 2. Degree of strawberry root colonization by arbuscular mycorrhizal fungi and the number of spores in the rhizosphere soil after the application of beneficial bacteria and biochar in 2023 (INHORT Experimental Field in Skierniewice, 2023)

Treatment	F (%)	M (%)	m (%)	Spore count (per 100 g soil)
Control	12.22 a	0.90 a	6.94 a	12 a
Biochar	20.0 a	1.71 ab	8.56 a	22 b
Consortium 1	46.67 c	2.52 bc	5.36 a	42 cd
Consortium 2	51.11 cd	3.62 c	7.08 a	29 b
Consortium 3	34.44 b	1.53 ab	4.45 a	39 c
Consortium 1 + Biochar	35.56 b	2.69 bc	4.47 a	34 c
Consortium 2 + Biochar	58.89 d	3.70 c	6.26 a	51 d
Consortium 3 + Biochar	32.22 b	2.24 a-c	6.90 a	65 e

See note under table 1

Table 3. Degree of apple root colonization by arbuscular mycorrhizal fungi and the number of spores in the rhizosphere soil after the application of beneficial bacteria and biochar in 2023 (INHORT Experimental Orchard in Dąbrowice, 2023)

Treatment	F (%)	M (%)	m (%)	Spore count (per 100 g soil)
Control	12.22 a	0.99 a	8.02 a	24 a
Biochar	25.56 b	2.22 a-c	8.77 a	33 a
Consortium 1	42.22 c	2.89 bc	6.84 a	91 cd
Consortium 2	40.0 c	3.19 c	7.94 a	30 a
Consortium 3	27.78 b	1.88 a-c	6.81 a	65 b
Consortium 1 + Biochar	13.33 a	1.28 ab	8.78 a	74 bc
Consortium 2 + Biochar	40.0 c	2.78 bc	6.99 a	100 d
Consortium 3 + Biochar	27.78 b	2.20 a-c	7.90 a	128 e

See note under table 1

Table 4. Degree of cucumber root colonization by arbuscular mycorrhizal fungi and the number of spores in the rhizosphere soil after the application of beneficial bacteria and biochar in 2024 (INHORT Experimental Field in Skierniewice, 2024)

Treatment	F (%)	M (%)	m (%)	Spore count (per 100 g soil)
Control	25.56 a	2.18 a	8.60 b	13 a
Biochar	35.56 b	2.69 ab	4.47 a	24 ab
Consortium 1	46.67 c	3.21 ab	6.85 ab	34 bc
Consortium 2	58.89 d	3.70 b	6.26 ab	44 cd
Consortium 3	42.22 bc	3.08 ab	7.28 ab	38 c
Consortium 1 + Biochar	46.67 c	3.12 ab	6.67 ab	43 c
Consortium 2 + Biochar	58.89 d	3.24 ab	5.50 ab	56 d
Consortium 3 + Biochar	41.11 bc	2.70 ab	6.57 ab	36 c

See note under table 1

Table 5. Degree of strawberry root colonization by arbuscular mycorrhizal fungi and the number of spores in the rhizosphere soil after the application of beneficial bacteria and biochar in 2024 (INHORT Experimental Field in Skierniewice, 2024)

Treatment	F (%)	M (%)	m (%)	Spore count (per 100 g soil)
Control	22.22 a	1.78 a	7.96 b	14 a
Biochar	34.45 b	2.40 ab	6.88 ab	26 b
Consortium 1	50.0 cd	3.57 ab	6.12 ab	36 bc
Consortium 2	60.93 de	3.38 ab	5.70 ab	53 d
Consortium 3	44.44 bc	2.32 ab	5.21 a	44 cd
Consortium 1 + Biochar	44.45 bc	3.14 ab	7.06 ab	47 cd
Consortium 2 + Biochar	62.22 e	3.69 b	5.91 ab	66 e
Consortium 3 + Biochar	44.44 bc	2.50 ab	5.58 ab	38 c

See note under table 1

Table 6. Degree of apple root colonization by arbuscular mycorrhizal fungi and the number of spores in the rhizosphere soil after the application of beneficial bacteria and biochar in 2024 (INHORT Experimental Orchard in Dąbrowice, 2024)

Treatment	F (%)	M (%)	m (%)	Spore count (per 100 g soil)
Control	23.33 a	2.11 a	8.95 b	23 a
Biochar	33.33 ab	2.30 a	6.84 ab	34 a
Consortium 1	43.33 bc	2.81 ab	6.50 ab	35 a
Consortium 2	61.11 d	3.95 b	6.48 ab	67 bc
Consortium 3	46.67 c	3.12 ab	6.65 ab	56 b
Consortium 1 + Biochar	48.89 c	3.83 b	7.87 ab	81 c
Consortium 2 + Biochar	65.56 d	3.77 b	5.76 a	130 d
Consortium 3 + Biochar	48.89 c	3.14 ab	6.42 ab	64 b

See note under table 1

In the cultivation of apple trees, it was found that the combined application of Consortium 2 with biochar, and the application of Consortium 2 on its own significantly increased the colonization of roots by arbuscular mycorrhizal fungi. The application of Consortium 2 had the greatest effect on the intensity of mycorrhizal fungi colonization of apple tree roots. Soil analysis showed that under the influence of the combined application of Consortium 2 and biochar, the number of spores was the highest. However, the application of biochar and Consortium 1 did not significantly affect the number of spores in the rhizosphere soil of apple trees. The lowest number of spores was observed in the rhizosphere control soil (Table 6).

DISCUSSION

The results of our studies indicate the high effectiveness of using Consortium 2 and biochar in increasing the colonization of cucumber, strawberry and apple plant roots by arbuscular mycorrhizal fungi. Biochar applied together with Consortia 2 and 3 increased the formation of spores of these fungi in the rhizosphere soil of the studied plant species. These results are also confirmed by studies of other authors.

Due to the beneficial effect of biochar on the growth and yield of perennial plants, in recent years there has been an increased interest in its use in the field cultivation of horticultural plants, mainly fruit trees [Frąc et al. 2022]. Less is known, however, about the interaction of biochar with soil microorganisms and its impact on soil biodiversity, which is one of the factors influencing soil functioning and soil biodiversity [Chen et al. 2022].

Videgain-Marco et al. [2021] conducted a study to determine the effect of biochar derived from grapevine shoots on the activity of arbuscular mycorrhizal fungi and the effect of their multiplication in soil on plant resistance to water stress. The results showed an overall increase in AMF frequency and activity after the application of biochar produced at 400 °C in sandy-loam substrate. They also found that the addition of biochar increased AMF root colonization and spore count, and the developed inoculum containing arbuscular mycorrhizal fungi increased root colonization and spore count in the soil where lettuce plants were grown under limited irrigation conditions. Malik et al. [2019] assessed the effect of biochar application on the occurrence of arbuscular mycorrhizal fungi in the soil and roots of wheat and maize. The results showed that AMF root colonization in maize was significantly greater than in wheat, and biochar application significantly increased both the number of spores in the soil and the number of spores in the roots of wheat and maize plants, compared to the control plants and soil. The authors suggest that the use of biochar increased the number of native populations of arbuscular mycorrhizal fungi, occurring in wheat and maize crops growing in alkaline calcareous soil with low fertility, in both the soil and the roots of these plants. The results obtained in our study are consistent with those obtained by Videgain-Marco et al. [2021] and Malik et al. [2019].

Compared to the control plants, biochar application increased the degree of root colonization and the number of AMF spores in the rhizospheric soil of the tested cucumber, strawberry and apple plants. Studies by other authors have shown that the use of biochar increases soil microbial activity and improves its chemical and physical properties [Qayyum et al. 2020, El Nahhas et al. 2021]. Chen et al. [2025] studied the use of biochar to accelerate the rate of colonization of American ginseng (*Panax quinquefolius* L.) roots by arbuscular mycorrhizal fungi and to explore its potential mechanisms. The results of this study indicate that the use of biochar accelerates the colonization of AMF in roots. It was also found that biochar affected the presence of potentially beneficial microorganisms such as *Sphingobium*, *Sphingomonas*, and *Novosphingobium*, which accelerate the colonization of plant roots by arbuscular mycorrhizal fungi. This is confirmed by the results of our research, in which the use of beneficial microorganisms such as *Bacillus licheniformis*, *Pseudomonas* sp., *Klebsiella* sp., *Priestia* sp., which are components of Consortia 1 and 2, increased the degree of AMF colonization of cucumber, strawberry and apple roots and the number of spores in the rhizospheric soil.

Agricultural practice is moving towards the combined use of biochar and other organic and inorganic fertilizers [Bai et al. 2022] and beneficial soil microorganisms [Sas-Paszt et al. 2023]. The study conducted by Ning et al. [2019] aimed to determine the effect of mycorrhizal fungi and *Pseudomonas* sp. interactions on phosphorus (P) and nitrogen (N) uptake and on the root length, surface area and volume of celery plants after biochar application at low and high phosphorus fertilization. They observed that the rate of root colonization by AMF fungi increased as a result of the combined use of *Pseudomonas* sp. and biochar. Our results also indicate that the combined application of Consortium 2, which includes, among others, *Pseudomonas* sp. (Pi22B, Pi25C) bacteria and biochar had the greatest impact on the extent of root colonization by arbuscular mycorrhizal fungi of cucumber, strawberry and apple. In their study, Ashraf and Chen [2023] assessed the effect of biochar application on soil properties, the

growth of wild rye (*Elymus elymoides*) and diversity of mycorrhizal fungi. Soil samples were treated with different doses of biochar and the inoculated with arbuscular mycorrhizal fungi. The results confirmed their assumptions that the use of biochar had a significant effect on the growth and diversity of mycorrhizal fungi. The combined use of biochar and AMF significantly increased the number of mycorrhizal fungi such as: *Rhizophagus* to 62.67%, *Glomus* to 67.67%, *Clarediogous* to 38.33% and *Redochera* to 16.67%. Other results were obtained by Sun et al. [2022] who studied the effects of biochar and arbuscular mycorrhizal fungi on the growth of maize (*Zea mays* L.), mineral content and chlorophyll content of the plants. They observed that biochar application to soil significantly reduced mycorrhizal colonization by 40.58% in maize roots, accompanied by a significant decrease in mycorrhizal dependence from 80.57% to –28.67%. Zhao et al. [2024] evaluated the effects of biochar and arbuscular mycorrhizal fungi on the growth, physiological traits and genetic expression of rice plants subjected to Cd stress. The results showed that biochar significantly increased the rate of mycorrhizal colonization by 22.19%. Wen et al. [2024] conducted a study to determine the effects of biochar and the application of the arbuscular mycorrhizal fungus *Rhizophagus irregularis* on prairie grass growth and soil quality. They found that the combined application of biochar and *Rhizophagus irregularis* significantly changed the microbiological composition of the rhizosphere soil and its quality. It also significantly increased the number of beneficial *Bacillus* sp. bacteria. In our experiments, a different mechanism was used, in which the included strains of *Bacillus licheniformis* bacteria (Consortium 1 and 2) significantly increased the colonization of cucumber, strawberry and apple plant roots and the number of AMF spores in the rhizosphere soil.

CONCLUSIONS

The use of beneficial bacterial consortia positively affected the degree and intensity of root colonization by arbuscular mycorrhizal fungi in cucumber, strawberry, and apple plants compared to control plants.

In both study years (2023–2024), the use of Consortium 2, alone or in combination with biochar, was most effective in increasing the degree of AMF colonization of the roots of the studied plant species.

In 2023, the highest number of AMF spores in the rhizosphere soil was observed after the combined application of Consortium 3 and biochar, while in 2024, the combined application of Consortium 2 and biochar significantly increased both root colonization and the number of AMF spores in all studied plant species.

The results indicate that the selection of appropriate microorganisms and their combination into consortia, e.g. *Pseudomonas* sp. Pi22B, Pi25C, *Klebsiella* sp. NAzot2a (Consortium 2), can effectively stimulate the development of AMF both in roots and in soil, and their effect can be enhanced by the use of biochar.

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CONFLICT OF INTEREST

The authors declare that they do not have any known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHORS' CONTRIBUTIONS

All authors contributed to the study concept and design. L.S.-P. was responsible for the experimental design, methodology, supervision, and administration of the project. E.D. and B.S. conducted the study material preparation and laboratory evaluation. K.G. and S.G. performed data collection and statistical analysis, and together with E.D., they developed the tables. The first draft of the manuscript was written by E.D., and all authors commented on subsequent versions of the manuscript. K.G. provided linguistic proofreading. All authors read and approved the final version of the manuscript.

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RESEARCH PAPER

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EFFECT OF CYCLIC 3-HYDROXYMELATONIN (3-OHM) ON PEPPER SEEDLING EMERGENCE UNDER DROUGHT CONDITIONS

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ABSTRACT

Cyclic 3-hydroxymelatonin (3-OHM), a significant metabolite derived from melatonin (MEL) through its interaction with oxygen-containing compounds, is believed to play a crucial role in enhancing plant resistance to various abiotic stresses. Despite its importance, research on 3-OHM remains limited. Therefore, this study aimed to investigate the effects of exogenous 3-OHM treatments on the drought stress tolerance of pepper seedlings during the emergence phase. The application of 3-OHM to seeds at various concentrations (0, 10, 50, and 100 µM) notably improved seedling emergence performance under drought conditions compared to untreated controls. Furthermore, 3-OHM treatments significantly reduced oxidative stress markers such as hydrogen peroxide (H_2O_2) and thiobarbituric acid reactive substances (TBARS), while simultaneously enhancing the activities of key antioxidant enzymes including peroxidase (POD), superoxide dismutase (SOD) and catalase (CAT). Additionally, seedling proline and total chlorophyll contents increased significantly by 3-OHM application. Among the concentrations tested, 50 µM 3-OHM consistently showed the most pronounced beneficial effects across multiple parameters. These results underscore the potential utility of 3-OHM as a natural bioactive compound to mitigate the detrimental impacts of abiotic stress in agricultural crops. To further elucidate the physiological mechanisms and confirm the efficacy of 3-OHM, future studies should focus on comparative analyses with MEL, employing the optimal 3-OHM concentration identified herein.

Keywords: melatonin metabolite, abiotic stress, water stress, seedling growth, antioxidant activity

INTRODUCTION

Drought is a significant constraint on plant growth, development, and productivity, making the study of plant responses to drought stress an important focus in environmental research [Ahmad et al. 2009]. By decreasing imbibition and increasing the osmotic potential of the growth medium, drought stress leads to reduced germination rates and seedling vigor [Anjum et al. 2017]. Polyethylene glycol (PEG), synthetic, water-soluble, and non-ionic polymer with a wide range of molecular weight [Chazen et al. 1995], is frequently used to induce drought stress in experiments involving higher plants [Hajihashemi and Sofo 2018]. The use of PEG-6000 effectively elevates the osmotic potential of the growth medium, consequently limiting the imbibition capacity of seeds. Under drought conditions, plants experience an overproduction of reactive oxygen species (ROS), which can lead to the oxidation of vital cellular macromolecules, disrupt physiological and metabolic processes, and, in severe cases, result in plant death [Bewley 1997]. To counteract the damaging effects of oxidative stress, plants have developed a sophisti-

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cated antioxidant defense system that plays a crucial role in scavenging excess ROS and maintaining intracellular redox equilibrium [Ahmad et al. 2009]. This defense mechanism comprises both enzymatic and non-enzymatic antioxidants. The enzymatic component includes a range of enzymes such as peroxidase (POD), superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT), which collectively function to neutralize ROS and preserve redox stability within the cell. For example, SOD facilitates the conversion of superoxide radicals into hydrogen peroxide (H_2O_2) and molecular oxygen, after which CAT and APX further detoxify H_2O_2 to prevent cellular damage [Anjum et al. 2017].

In plants, MEL (N-acetyl-5-methoxytryptamine) plays a vital role in defending against oxidative stress by mitigating the damaging effects of ROS. Beyond its antioxidant properties, MEL also functions as a biostimulant, contributing to enhanced plant productivity through its involvement in cellular signaling pathways [Rehaman et al. 2021]. MEL biosynthesis in plants follows a metabolic pathway: tryptophan → tryptamine → serotonin → N-acetylserotonin → MEL. This process occurs under normal physiological conditions, particularly in actively growing tissues, and involves four key enzymatic steps. The enzymes responsible for each step include TDC (tryptophan decarboxylase), T5H (tryptophan 5-hydroxylase), SNAT (serotonin N-acetyltransferase), ASMT (N-acetylserotonin methyltransferase), COMT (caffeic acid O-methyltransferase), respectively [Back 2021].

An important branch of plant MEL research focuses on its metabolites. Metabolites resulting from MEL degradation—such as N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK), N-acetyl-5-methoxykynuramine (AMK), and the hydroxylated derivatives 2-OHM (2-hydroxymelatonin), 3-OHM (3-hydroxymelatonin), 4-OHM (4-hydroxymelatonin), and 6-OHM (6-hydroxymelatonin)-originally identified in animals, have also been detected in plant systems [Tan and Reiter 2020, Hardeland 2015]. These metabolites possess potent antioxidant properties and contribute substantially to MEL's broader antioxidant network [Reiter et al. 2016]. Notably, in terms of endogenous content, MEL metabolites represent the predominant forms of MEL present in plant tissues [Lee et al. 2016]. Among MEL derivatives in plants, 2-OHM and 3-OHM are recognized as the principal metabolites. These metabolites are generated through hydroxylation processes catalyzed by melatonin 2-hydroxylase (M2H) and melatonin 3-hydroxylase (M3H), respectively [Byeon and Back 2015, Lee et al. 2016]. Both enzymes are classified as members of the 2-oxoglutarate-dependent dioxygenase (2-ODD) protein family [Bugg 2003]. Interestingly, M2H and M3H are absent in animals, suggesting that the corresponding hydroxylated metabolites are specific to terrestrial plants [Lee and Back 2016]. The detection of MEL and its metabolites across plant species has spurred extensive research into their physiological functions and regulatory mechanisms. 2-OHM, in particular, has been shown to accumulate at higher levels than MEL itself in plant tissues [Byeon et al. 2015]. It is recognized not only for its antioxidant capacity but also for its pro-oxidant function, capable of inducing ROS production through respiratory burst oxidase homolog-mediated pathways. Korkmaz et al. [2023] reported that exogenous application of 2-OHM enhances seed germination and seedling emergence in pepper under abiotic stress. This dual role may be attributed to its ability to stimulate both enzymatic and non-enzymatic antioxidant systems [Shah et al. 2020a], thereby conferring broad protective effects against oxidative stress and environmental challenges [Shah et al. 2020b]. In addition, The cytochrome P450 monooxygenase enzyme CYP1A1, which participates in MEL metabolism, catalyzes the biosynthesis of 6-OHM. When the mouse-derived *MmCYP1A*1 gene was ectopically expressed in transgenic *Arabidopsis* and apple calli, both MEL and 6-OHM levels increased. This enhancement was associated with greater ROS-scavenging capacity and improved abiotic stress tolerance, suggesting a synergistic role of MEL and its hydroxylated metabolites in plant defense responses [Wang et al. 2023]. Álvarez-Diduk et al. [2016] reported that certain MEL derivatives, such as N-acetylserotonin (N-Ser) and 6-OHM, exhibit stronger peroxyl radical scavenging capacity than MEL itself. In a related study, Lee and Back [2019] showed that foliar application of 2-OHM significantly improved tolerance to cold and drought stress in cucumber and tomato seedlings, whether the stresses were applied individually or simultaneously. Moreover, 2-OHM treatment in rice seedlings under combined stress conditions led to increased proline accumulation, which contributed to enhanced stress resilience [Lee and Back 2016]. According to Korkmaz et al. [2023], application of 2-OHM under various abiotic stresses reduced the levels of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA), while elevating the concentrations of proline and antioxidant enzymes. Interestingly, the enzymatic activity responsible for MEL degradation-yielding metabolites such as 2-OHM and 3-OHM has been reported to be greater than that of enzymes involved in MEL biosynthesis. As a result, MEL and its metabolites often co-occur in plant tissues [Back 2021]. This co-existence has led to the hypothesis that MEL's protective functions under stress are not solely attributed to the parent molecule, but are also significantly influenced by its downstream metabolites, particularly 2-OHM. In support of this, Byeon and Back [2015]

successfully cloned M2H genes from rice, which encode 2-oxoglutarate-dependent dioxygenases that catalyze the conversion of MEL to 2-OHM. High levels of 2-OHM were observed in the roots of MEL-treated plants. Choi and Back [2019a, 2019b] further reported that both 2-OHM and 3-OHM, similar to MEL, follow a diurnal rhythm, with elevated concentrations detected at night. In addition, 3-OHM was found to significantly enhance tiller formation in rice by upregulating the expression of the *MOC1* gene. Lee and Back [2016] also noted that 2-OHM and 3-OHM improved plant tolerance to multiple abiotic stresses, such as drought and cold, through the upregulation of stress-responsive genes. Lee and Back [2022] reported that exogenous application of 3-hydroxymelatonin in *Arabidopsis thaliana* enhanced both vegetative growth and the initiation of flowering. Similarly, 4-OHM, another melatonin-derived metabolite, has been identified in plants alongside 2-OHM and 3-OHM [Hardeland 2016], and is thought to play a crucial role in modulating ROS levels under stress conditions [Pérez-González et al. 2017]. In this context, Shah et al. [2021] demonstrated that 4-OHM alleviated the adverse effects of nickel toxicity on the root and shoot systems of eggplant seedlings, thereby enhancing their stress resilience.

Although research on MEL metabolites remains limited, existing evidence suggests that these compounds may exert biological functions similar to MEL, particularly in plants exposed to environmental stresses. Their potential contribution to abiotic stress tolerance has drawn increasing attention, underscoring the importance of investigating their physiological roles and underlying mechanisms. Gaining a clearer understanding of these metabolites is also essential for elucidating the broader functional dynamics of their precursor, MEL. Especially, few studies have revealed the physiological functions of this molecule in plants 3-OHM, a MEL metabolite. Pepper is one of the most important vegetable crops worldwide, and its production is significantly affected by environmental stresses such as drought, with seedling emergence representing one of the most sensitive developmental stages. Accordingly, the present study was designed to examine the effects of 3-OHM, a key MEL-derived metabolite, on pepper (*Capsicum annuum* L.) seedling emergence in drought-stressed environments.

MATERIALS AND METHODS

Seed sterilization and treatments. Seed-borne microorganisms on pepper seeds from a uniform seed lot were eliminated through a 15 min treatment with sodium hypochlorite solution (1%). Sterilized pepper seeds were placed between two layers of blotting paper moistened with 3-OHM solutions at concentrations of 0, 10, 50, and 100 µM. After that, the seeds were incubated at 20 °C in darkness for 24 hours, followed by a 1 min rinse under running tap water. 24-hour incubation period was sufficient for compound absorption, and washing step was performed only to remove residual solution from the seed surface. After treatment, seeds were air-dried on paper towels for one day before drought stress emergence testing.

Emergence test. Following the application of 3-OHM, the experiment was conducted using a completely randomized design with four replications, each consisting of 40 seeds. In total, 1280 seeds were used in the experiment (4 concentrations of 3-OHM × 2 PEG levels × 4 replications × 40 seeds). The seeds were seeded in plastic containers, filled with a growth medium composed of perlite and peat in a 1:3 ratio. After sowing, each container received 200 mL of either distilled water (0 g L^{-1} PEG 6000, non-stress control) or a polyethylene glycol (PEG 6000) solution at 75 g L^{-1} (–0.41 MPa) to simulate drought stress. The containers were kept in a climate-controlled chamber (Memmert IPP110) set to 25 °C. The chamber was illuminated with white LED lights providing a photoperiod of 16 hours per day at an intensity of 225 µmol m^{-2} s^{-1}. During the emergence period, supplementary irrigation with respective PEG solutions was provided as needed to prevent the drying of the substrate surface. Seedling emergence was monitored daily until stabilization of the emergence count. A seedling was considered emerged upon the visible formation of a hypocotyl arc above the substrate. The emergence test was carried out over a 30-day period under drought stress conditions. Emergence parameters including FEP (final emergence percentage), E_{10-90} (emergence uniformity), and MET (mean emergence time) were determined using on the total number of emerged seedlings. Upon completion of the experiment, seedlings were harvested and their SFW (shoot fresh weight) was recorded. Immediately after, biochemical analyses were performed.

Determining chlorophyll, thiobarbituric acid reactive substances (TBARS), hydrogen peroxide (H_2O_2), and proline contents. Chlorophyll content was quantified using the method reported by Gunes et al. [2007]. For this, leaf samples randomly collected from each replicate were ground in 80% acetone. After filtration, chlorophyll concentrations were determined by measuring absorbance at 645 and 663 nm with a spectrophotometer

(Shimadzu UV-1800, Kyoto, Japan). The concentration of TBARS in pepper seedlings was quantified based on the approach by Posmyk et al. [2009] with minor alterations. Samples were ground in a 10% TCA (trichloroacetic acid) solution, and the clear extract was mixed with 0.6% TBA (thiobarbituric acid). The mixture was then incubated in a water bath for 20 min, and absorbance values were measured using a UV-Vis spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) at wavelengths of 532 nm, 600 nm, and 450 nm. H_2O_2 levels were determined by adapting the method from Ozden et al. [2009]. Shoots were fragmented in 0.1% (w/v) TCA, followed by mixing with 10 mM potassium phosphate buffer (pH 7.0) and 1 M potassium iodide. Subsequently, the absorbance was measured at 390 nm to determine the hydrogen peroxide content. Proline levels in the tissue were determined using the approach by Bates et al. [1973]. Fresh shoot samples were homogenized in 3% sulfosalicylic acid and filtered. An aliquot of 0.5 mL filtrate was reacted with glacial acetic acid and acid ninhydrin in test tubes, then incubated for one hour in a water bath. Once at room temperature, the reaction mixture was extracted with toluene and absorbance was measured at 520 nm.

Extraction and analysis of antioxidant enzymes. The activities of essential antioxidant enzymes-CAT (EC 1.11.1.6), POD (EC 1.11.1.7), and SOD (EC 1.15.1.1) were determined using the extraction methods outlined by Ozden et al. [2009]. Protein concentration in the extracts was determined via the Bradford assay [Bradford 1976]. Enzyme activities were subsequently quantified based on established methods reported by Gong et al. [2001] for CAT, Dolatabadian et al. [2008] for POD, and Agarwal and Pandey [2004] for SOD, and expressed as units per milligram of protein (unit mg^{-1} protein).

Statistical analysis. The trials was conducted using a completely randomized design with four replicates per treatment. Data processing and calculation of means were performed using Microsoft Excel (Office XP). Statistical analyses were carried out with SPSS version 20.0. To assess the significance of differences among treatment means, two-way analysis of variance (ANOVA) was applied, followed by Duncan's multiple range test at a 5% significance level.

RESULTS

Effect of 3-OHM application on drought stress. The data presented in Table 1 indicate that 3-OHM treatments influenced MET, FEP, $E_{10\text{-}90}$, and SFW of seedlings under both optimal and drought stress conditions. Drought stress significantly reduced seedling emergence, with FEP decreasing from 92.3% under optimal conditions to 81.1% under stress. Seeds treated with 3-OHM at all concentrations showed higher emergence rates compared to the untreated control. Under drought conditions, the highest emergence percentage (85.6%) was observed in seeds treated with 50 µM 3-OHM. While drought stress delayed emergence, increasing MET from 9.38 to 10.30 days, 3-OHM application improved emergence speed, lowering MET from 10.15 days in untreated seeds to 9.50 days at 50 µM treatment. Emergence uniformity ($E_{10\text{-}90}$) also increased from 3.60 days under optimal conditions to 5.15 days under drought stress. Additionally, drought stress significantly reduced seedling fresh weight, from 14.54 g to 6.13 g on average. However, seeds treated with 50 µM and 100 µM 3-OHM yielded seedlings with greater fresh weights (10.93 g and 10.68 g, respectively) compared to the untreated group (9.56 g).

Table 2 presents proline, H_2O_2, TBARS, and chlorophyll levels in pepper seedlings after 3-OHM seed treatments under drought and optimal conditions. Chlorophyll content increased from 59.7 mg g^{-1} under optimal conditions to 70.3 mg g^{-1} under drought stress. Seedlings derived from seeds treated with 3-OHM exhibited chlorophyll levels that were consistently higher, though comparable, to those of the untreated control. TBARS content rose from 0.35 nmol g^{-1} in optimal conditions to 0.77 nmol g^{-1} under drought stress. However, 3-OHM treatments at 10 µM, 50 µM, and 100 µM significantly reduced TBARS levels compared to the control. The interaction analysis between drought stress and 3-OHM treatments showed that the lowest TBARS content was detected in seedlings grown under optimal conditions with 3-OHM application, indicating reduced lipid peroxidation. In contrast, the highest TBARS accumulation was noted in seedlings that were exposed to drought stress without 3-OHM, suggesting that the absence of treatment exacerbated oxidative damage under stress conditions. Similarly, H_2O_2 content increased from 0.29 nmol g^{-1} under optimal conditions to 0.34 nmol g^{-1} under stress, but 3-OHM treatments at all tested concentrations significantly decreased H_2O_2 accumulation. Proline content was markedly elevated from 117.6 nmol g^{-1} in optimal conditions to 473.1 nmol g^{-1} under drought stress. Notably, all 3-OHM treatments enhanced proline accumulation compared to the untreated seeds (201.8 nmol g^{-1}), with the highest proline level observed in seedlings from seeds treated with 10 µM 3-OHM (344.6 nmol g^{-1}).

Table 3 summarizes the activities of antioxidant enzymes CAT, POD, and SOD in pepper seedlings following treatment with 3-OHM. Under optimal conditions, CAT activity was measured at 0.93 U mg^{-1} protein and increased to 1.88 U mg^{-1} protein when plants were exposed to drought stress. While 3-OHM treatment resulted in an increase of CAT activity to approximately 1.50 U mg^{-1} protein, this change did not reach statistical significance. POD activity showed a modest rise from 0.009 U mg^{-1} protein in optimal conditions to 0.01 U mg^{-1} protein under drought. Importantly, POD activity was significantly enhanced by 3-OHM application, with the 50 µM and 100 µM treatments yielding higher enzyme activity (0.011 U mg^{-1} protein) compared to the untreated group. Similarly, SOD activity increased from 2.01 U mg^{-1} protein in well-watered plants to 2.55 U mg^{-1} protein under drought conditions. Notably, seedlings treated with 50 µM 3-OHM showed a marked increase in SOD activity, reaching 4.29 U mg^{-1} protein, compared to 1.12 U mg^{-1} protein in untreated controls.

Table 1. Effect of pre-sowing seed treatments with 3-OHM on pepper seed final emergence percentage (FEP), mean emergence time (MET), emergence uniformity (E_{10-90}) and seedling fresh weight (SFW) under optimum and drought stress conditions. Values are means (n = 4)

Treatments	Doses	FEP (%)	MET (day)	E_{10-90} (day)	SFW (g $plant^{-1}$)
PEG					
0 gL^{-1}	–	92.3 ±3.35a	9.38 ±0.26b	3.60 ±0.35b	14.54 ±0.65a
75 gL^{-1}	–	81.1 ±4.06b	10.30 ±0.22a	5.15 ± 0.41a	6.13 ±0.44b
3-OHM (µM)					
0	–	80.9 ±5.94c	10.15 ±0.39a	4.81 ±0.68	9.56 ±4.09c
10	–	88.1 ±4.38b	9.89 ±0.43ab	4.46 ±0.83	10.17 ±3.99b
50	–	90.6 ±5.00a	9.50 ±0.44b	3.97 ±0.48	10.93 ±4.24a
100	–	87.2 ±7.19b	9.83 ±0.58ab	4.26 ±1.09	10.68 ±4.48a
PEG × 3-OHM					
0 gL^{-1}	0 µM	86.9 ±1.08c	9.77 ±0.44	4.12 ±0.95	13.65 ±0.12
	10 µM	92.5 ±0.00b	9.46 ±0.18	3.63 ±0.29	14.16 ±0.49
	50 µM	95.6 ±1.08a	9.06 ±0.50	3.48 ±0.79	15.17 ±0.11
	100 µM	94.4 ±1.07ab	9.26 ±0.16	3.17 ±0.12	15.16 ±0.30
75 gL^{-1}	0 µM	75.0 ±1.77f	10.54 ±0.17	5.49 ±1.00	5.46 ±0.24
	10 µM	83.8 ±2.80 d	10.32 ±0.35	5.30 ±0.84	6.19 ±0.61
	50 µM	85.6 ±2.07cd	9.94 ±0.38	4.45 ±1.02	6.69 ±0.36
	100 µM	80.0 ±1.76e	10.41 ±0.24	5.35 ±1.35	6.20 ±0.30
Significance					
PEG	–	***	***	***	***
3-OHM (µM)	–	***	*	N.S.	***
PEG × 3-OHM	–	*	N.S.	N.S.	N.S.

NS – not significant; *, **, *** significant at P < 0.05, 0.01 or 0.001, respectively

Table 2. Effect of pre-sowing seed treatments with 3-OHM on chlorophyll, TBARS, H_2O_2, and proline contents of pepper seedlings under optimum and drought stress conditions. Values are means (n = 4)

Treatments	Doses	Chlorophyll (mg g^{-1} FW)	TBARS (nmol g^{-1} FW)	H_2O_2 (nmol g^{-1} FW)	Proline (nmol g^{-1} FW)
PEG					
0 gL^{-1}	–	59.7 ±2.95b	0.35 ±0.09b	0.29 ±0.08b	117.6 ±22.1b
75 gL^{-1}	–	70.3 ±0.39a	0.77 ±0.13a	0.34 ±0.07a	473.1 ±100a
3-OHM (µM)					
0	–	66.8 ±3.61a	0.75 ±0.24a	0.42 ±0.01a	201.8 ±110c
10	–	63.4 ±7.34c	0.48 ±0.18b	0.26 ±0.03c	344.6 ±243a
50	–	65.7 ±3.93b	0.49 ±0.19b	0.24 ±0.03c	322.0 ±175b
100	–	64.0 ±6.46c	0.51 ±0.23b	0.36 ±0.01b	313.0 ±181b
PEG × 3-OHM					
0 gL^{-1}	0 µM	63.2 ±0.68b	0.51 ±0.02d	0.40 ±0.02	91.2 ±3.0e
	10 µM	56.0 ±0.73d	0.30 ±0.03e	0.23 ±0.02	101.6 ±2.0e
	50 µM	61.8 ±0.86b	0.30 ±0.03e	0.21 ±0.01	146.3 ±1.9d
	100 µM	57.6 ±0.68c	0.28 ±0.02e	0.35 ±0.02	131.5 ±1.2d
75 gL^{-1}	0 µM	70.4 ±1.02a	0.99 ±0.07a	0.44 ±0.04	312.3 ±22.2c
	10 µM	70.7 ±0.96a	0.66 ±0.04c	0.30 ±0.03	587.7 ±14.9a
	50 µM	69.7 ±0.79a	0.68 ±0.02c	0.27 ±0.04	497.8 ±8.7b
	100 µM	70.5 ±0.92a	0.74 ±0.02b	0.37 ±0.02	494.5 ±9.8b
Significance					
PEG	–	***	***	***	***
3-OHM (µM)	–	***	***	***	***
PEG × 3-OHM	–	***	**	N.S.	***

NS – not significant; *, **, *** significant at $P < 0.05$, 0.01 or 0.001, respectively

Table 3. Effect of pre-sowing seed treatments with 3-OHM on catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) enzyme activities of pepper seedlings under optimum and drought stress conditions. Values are means (n = 4)

Treatments		CAT (U mg^{-1} protein)	POD (U mg^{-1} protein)	SOD (U mg^{-1} protein)
PEG				
0 gL^{-1}	–	0.93 ±0.10b	0.009 ±0.0b	2.01 ±1.42b
75 gL^{-1}	–	1.88 ±0.07a	0.010 ±0.0a	2.55 ±1.14a
3-OHM (µM)				
0	–	1.33 ±0.47	0.009 ±0.0b	1.12 ±0.39c
10	–	1.50 ±0.39	0.009 ±0.0b	2.51 ±0.33b
50	–	1.44 ±0.56	0.011 ±0.0a	4.29 ±0.02a
100	–	1.35 ±0.48	0.011 ±0.0a	1.20 ±0.36c

Treatments		CAT (U mg^{-1} protein)	POD (U mg^{-1} protein)	SOD (U mg^{-1} protein)
PEG × 3-OHM				
0 gL^{-1}	0 µM	0.86 ±0.03	0.009 ±0.0	0.74 ±0.10
	10 µM	1.11 ±0.15	0.009 ±0.0	2.18 ±0.24
	50 µM	0.88 ±0.06	0.010 ±0.0	4.27 ±0.62
	100 µM	0.87 ±0.03	0.010 ±0.0	0.84 ±0.08
75 gL^{-1}	0 µM	1.81 ±0.18	0.009 ±0.0	1.51 ±0.11
	10 µM	1.88 ±0.07	0.009 ±0.0	2.84 ±0.50
	50 µM	1.99 ±0.06	0.012 ±0.0	4.31 ±4.30
	100 µM	1.83 ±0.63	0.011 ±0.0	1.55 ±0.17
Significance				
PEG	–	***	*	***
3-OHM (µM)	–	N.S.	***	***
PEG × 3-OHM	–	N.S.	N.S.	N.S.

NS – not significant; *, **, *** significant at P < 0.05, 0.01 or 0.001, respectively. SD values for POD enzyme activity were rounded to one decimal place and presented as 0.0

DISCUSSION

MEL, known as both a circadian rhythm regulator and a potent antioxidant, has prompted extensive research into its metabolites. In terrestrial plants, the primary MEL metabolites are 2-OHM, 3-OHM, and 4-OHM [Byeon et al. 2015]. MEL and its metabolites possess MEL-like functions and play an active role in improving stress tolerance by acting as antioxidants [Lee and Back 2019]. Studies have demonstrated that 2-OHM application reduces cadmium uptake and polyamine oxidase activity in pepper seedlings. Furthermore, 2-OHM has been reported to alleviate cadmium toxicity in cucumber seedlings, enabling cultivation in cadmium-contaminated soils [Shah et al. 2020a, b]. In the work of Lee and Back [2016] high proline accumulation was observed in rice seedlings treated with 100 µM 2-OHM under combined cold and drought stress, which contributed to increased capacity to withstand stress. Additionally, treatment with 50 µM 2-OHM positively influenced germination and seedling emergence in pepper seeds exposed to different types of stress, effects attributed to the stimulation of the antioxidant system [Korkmaz et al. 2023]. The metabolite 3-OHM has been documented to counteract oxidative stress under adverse environmental conditions by increasing antioxidant activity to mitigate stress-induced damage [Reiter et al. 2016, Choi and Back 2019a, 2019b]. In rice plants, increased expression of M3H genes, which facilitate the conversion of MEL into 3-OHM, was detected in MEL-treated leaves, accompanied by significant accumulation of 3-OHM. These findings were supported by cloning studies of 2-oxoglutarate-dependent dioxygenase (2-ODD). Collectively, these results indicate that MEL is metabolized to 3-OHM via M3H genes in plants; however, the precise physiological role of 3-OHM remains to be fully elucidated [Lee et al. 2016].

The findings of this research clearly demonstrate that 3-OHM has a mitigating effect on the adverse influence of drought stress during the emergence stage of pepper seedlings. Pre-sowing application of 3-OHM notably enhanced both emergence rate and final emergence percentage under water-deficit conditions (Table 1). Following 3-OHM application, stressed seedlings exhibited marked reductions in H_2O_2 and TBARS levels, while proline content, total chlorophyll concentration (Table 2), and activities of antioxidant enzymes (Table 3) were significantly enhanced. Comparable findings have been reported in rice seedlings [Lee and Back 2016] and *Arabidopsis* subjected to chilling stress, where 3-OHM treatment promoted proline biosynthesis and upregulated genes such as *Myb4* and *AP37*, which are crucial for abiotic stress tolerance. Additionally, Lee and Back [2022] demonstrated that 3-OHM stimulated growth and development in *Arabidopsis* and exhibited antioxidant activity approximately 15 times greater than MEL itself. In rice, 3-OHM levels displayed a circadian rhythm similar to MEL and notably increased tiller numbers via upregulation of the *MOC1* gene [Choi and Back 2019a]. Furthermore, under cadmium exposure, 3-OHM content decreased while 2-OHM remained stable in rice plants, implicating 3-OHM in the oxidative stress response [Choi and Back, 2019b]. These observations align with previous reports on the crucial role of 3-OHM in MEL-related physiological effects [Galano et al. 2014] and support its potential as an antioxidant com-

parable in efficacy to MEL [Tan et al. 2014]. Furthermore, it is suggested that 3-OHM also has MEL-like functions and plays an active role in seedling emergence under stress factors. Nonetheless, further research is warranted to elucidate the precise mechanisms by which 3-OHM exerts its physiological functions in plants and to confirm these preliminary findings.

CONCLUSIONS

Seed treatment with 50 µM 3-OHM significantly enhanced pepper seedling emergence under drought stress. This metabolite reduced oxidative damage by lowering H_2O_2 and TBARS levels while increasing proline content and antioxidant enzyme activities, thereby stabilizing cell membranes. These findings suggest that 3-OHM functions as an effective antioxidant, improving drought tolerance similarly to MEL. However, further comparative studies with MEL at this concentration are necessary to elucidate the precise physiological mechanisms. Continued research on MEL metabolites will advance understanding of their role in stress mitigation and clarify MEL's overall function in plants.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHORS' CONTRIBUTIONS

A. Karaca: investigation, conceptualization, data curation, funding acquisition, formal analysis, writing-review and editing. G. Yakupoğlu: investigation, conceptualization, data curation, funding acquisition, formal analysis, writing-review and editing. A. Korkmaz: writing-review and editing.

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RESEARCH PAPER

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INFLUENCE OF MYCORRHIZAL FUNGI ON PLANT GROWTH AND RHIZOSPHERE SOIL MICROBIOME OF TOMATO (*Solanum lycopersicum* L.)

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ABSTRACT

Mycorrhizal fungi increase plant resistance to stress factors such as drought, high or low temperatures, acidification, soil contamination with heavy metals, and the presence of soil pathogens. The aim of the study was to determine the effect of mycorrhizal inoculum on plant growth and the microbiome of the rhizosphere soil of tomato. The experiment was established in the greenhouse of the Felin Experimental Farm at the University of Life Sciences in Lublin. The research material consisted of tomato plants (*Solanum lycopersicum* L.) Lubań cv. The mycorrhizal inoculum Endo-VAM from Mykoflor (Końskowola, Poland) was used for mycorrhization of tomato seedlings. It contains spores and live mycelium of the following species: *Glomus intraradices*, *G. mosseae*, *G. claroideum*, *G. etunicatum*, *Gigaspora margerita*, and *Entrophospora* spp. Non-mycorrhized tomato seedlings served as a control. Six weeks after the application of the mycorrhizal inoculum, mycological analysis of tomato roots and microbiological analysis of rhizosphere soil were performed, and plant height, stem base diameter, fresh and dry root weight were determined. The roots of mycorrhized plants were found to be more abundantly colonized by fungi, including antagonistic species, than the roots of non-mycorrhized plants. The total number of bacteria, including *Pseudomonas* and *Bacillus* genera, and the total number of fungi isolated from the rhizosphere of mycorrhized plants were significantly lower than in the rhizosphere of control plants. Analysis of morphological parameters of tomato plants demonstrated a beneficial effect of the mycorrhizal inoculum on the growth of fresh and dry root weight, but did not demonstrate a significant effect of mycorrhiza on plant height or tomato stem base diameter.

Keywords: tomato, mycorrhiza, rhizosphere microbiota, SPAD, total chlorophyll

INTRODUCTION

Tomato is one of the most important vegetable crops worldwide, not only because of its economic importance but also due to the high nutritional value of its fruits [Yang et al. 2023]. Universally consumed tomato fruits provide numerous health benefits, which contribute to a healthy, balanced diet. Tomato fruits contain vitamins A, B, and C, as well as high levels of lycopene and carotenoids, which help protect against various lifestyle-related diseases [Kaboré et al. 2022, Lee et al. 2023, Li et al. 2024]. Tomato fruits are also used in the prevention of neurodegenerative diseases, reducing the incidence of certain chronic conditions. Tomatoes, as an important source of potassium, phosphorus, magnesium, and iron, are essential for proper nerve and muscle function [Raiola et al. 2014, Shah et al. 2021]. They are rich in minerals, essential amino acids, sugars, and dietary fiber. In addition to these

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nutrients, they also contain β-carotene and lycopene pigments [Natali et al. 2025]. Tomato cultivation is exposed to many adverse environmental factors, including pathogens. Many agrochemicals, such as mineral fertilizers and pesticides, are still used in tomato cultivation.

Although chemical horticulture is effective in crop protection, it also contributes to environmental pollution. The need to reduce synthetic fertilization and protect the environment has led to the development of formulations, whose main active components are beneficial microorganisms. The living organisms in biopreparations utilize various mechanisms of action and can affect the soil, plants, or both concurrently. The purpose of such formulas is to improve plant nutrient uptake, reduce the negative effects of abiotic environmental factors, combat phytopathogens, and promote plant growth and development. The main components of such preparations are bacteria and fungi, often isolated from the natural environment, frequently directly from the soil. Examples of such microorganisms are mycorrhizal fungi, which due to their multi-level interactions with plants are increasingly used as potential biocontrol agents [Głuszek et al. 2008]. Mycorrhiza is a strong symbiotic association between soil fungi and the fine plant roots. Structures formed through mycorrhiza benefit both partners, as the fungus receives organic compounds produced by the plant during photosynthesis, while the plant gains organic compounds and water. Mycorrhizal fungi are rarely specific to a single host, meaning that one species can colonize multiple plant species. Arbuscular mycorrhizal fungi (AMF), primarily from the phylum *Glomeromycota*, can protect plants from the harmful effects of abiotic stressors such as drought and heavy metals. The drought tolerance of mycorrhizal plants depends on the AMF species colonizing their roots. Mycorrhizal fungi are believed to modify the growth of aerial plant parts under drought stress by affecting complex plant responses to water deficiency, including phosphorus and potassium uptake, root respiration, photosynthesis, transpiration, and leaf osmotic potential [Głuszek et al. 2008, Jamiołkowska et al. 2018, 2020a]. Mitigation of heavy metal toxicity may result from reduced uptake by plants, either through accumulation in the extraradical mycelium or through changes in metal solubility. Mycorrhizal fungi have also developed various mechanisms to protect plant roots from pathogens. They colonize the root surface and compete with pathogenic microorganisms for nutrients produced by the plant. They stimulate the plant to produce and secrete defense-related compounds [Ruiz-Lozano et al. 2016]. In addition to the benefits conferred to plants, mycorrhizal fungi also contribute to the improvement of soil structure. These fungi form soil aggregates, which positively affect soil quality and plant health. Apart from these benefits, mycorrhiza increases plant resistance to diseases [Pozo and Azcón-Aguilar 2007]. Mycorrhizal fungi share certain traits with biotrophic pathogens and can trigger plant defense responses during the initial stages of symbiosis [Jung et al. 2012, Song et al. 2015]. For colonization to be successful, the fungus must manage these responses and actively modulate plant reactions – a phenomenon known as "priming". Stimulating plant defenses plays a central role in mycorrhiza-induced resistance [Fiorilli et al. 2024]. Gallou et al. [2011] discovered that mycorrhizal colonization increases tomato resistance to late blight by inducing a systemic defense response, and the jasmonic acid (JA) signaling pathway is essential for mycorrhiza-induced disease resistance. Drought tolerance in mycorrhizal plants depends on the species of arbuscular mycorrhizal fungi (AMF) colonizing their roots. These fungi are believed to modify the growth of aerial plant parts under drought stress by influencing complex plant responses to water deficit, including phosphorus and potassium uptake, root respiration, photosynthesis, transpiration, and leaf osmotic potential.

The aim of this study was to determine the effect of a mycorrhizal inoculant on selected plant morpho-physiological parameters, the rhizosphere soil microbiome, and the diversity of fungi colonizing tomato roots.

MATERIALS AND METHODS

Pot experiment

The experiment was conducted in the greenhouse of the Felin Experimental Farm at the University of Life Sciences in Lublin. The plant material consisted of tomato (*Solanum lycopersicum* L.) plants of the cultivar Lubań (PlantiCo – Hodowla i Nasiennictwo Ogrodnicze Zielonki sp. z o.o., Poland). Tomato seedlings were prepared following the standard procedures commonly used for this vegetable (Wysocka-Owczarek 2010). The three-week-old tomato seedlings were transplanted in the first ten days of May into 1-liter plastic pots filled with non-strilized Sterlux horticultural substrate (Agaris, Poland). The substrate used for experiment was based on high-moor peat and enriched with a multi-component NPK + Mg fertiliser (14–16–18) at a rate of 0.6 kg m^{-3}, with a pH of 5.5–6.5.

One plant was transplanted into each pot. To each pot partially filled with horticultural substrate 0.5 g of MF inoculum mixed with 30 ml of semi-liquid potato dextrose agar (PDA, Difco, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was added. Then, tomato seedlings were planted and supplemented with soil substrate. The

tomato seedlings were mycorrhized using the Endo-VAM mycorrhizal inoculum (Mykoflor, Końskowola, Poland) containing oospores and live mycelium of *Glomus intraradices*, *G. mosseae*, *G. claroideum*, *G. etunicatum*, *Gigaspora margerita*, and *Entrophospora* sp. The control group consisted of non-mycorrhized plants. For each experimental treatment, 25 plants were prepared (5 plants in 5 replicates). Plants were grown in pots, on cultivation tables over a capillary mat watered once a day for 2 minutes. The average temperature in the facility during the experiment was 23°C, with air humidity ranging from 30 to 35%. The facility was ventilated automatically. The pot experiment was carried out for 6 weeks.

Assessment of root colonization by mycorrhizal fungi. To assess the degree of root colonization by mycorrhizal fungi, 10 root hairs were collected from 10 randomly selected plants (100 root fragments per experimental treatment), at the end of the pot experiment (i.e. 6 weeks after inoculation of the rhizosphere soil with the mycorrhizal inoculum). The collected root fragments were stained according to the method developed at the Department of Microbiology and Rhizosphere of the Institute of Horticulture in Skierniewice. Microscopic slides were prepared from these roots by selecting 50 one-cm-long fragments from each experimental combination. The fragments were arranged in parallel on a glycerol-coated slide and gently pressed with a cover slip. The histological preparations were examined using a Nikon 50i microscope (40× objective magnification), and photographic documentation of mycorrhizal structures was made. The degree of tomato root colonization by arbuscular mycorrhizal fungi was assessed using the Trouvelot (1986) method. Based on the obtained results, mycorrhizal frequency (F%) and root colonization intensity (M%, m%) were calculated using the MYCOCALC software, available online at: https://www2.dijon.inrae.fr/mychintec/Mycocalc-prg/download.html.

Microbiological analysis of rhizosphere soil. The experiment determined the total number of fungi and bacteria, as well as the bacterial count of genera *Pseudomonas* and *Bacillus* in the rhizosphere soil of tomato. Rhizosphere soil was collected six weeks after inoculating the plants with the mycorrhizal fungi to obtain a representative composite sample for each experimental combination. 5 g of rhizosphere soil was collected from each pot and then mixed (pooled) within the experimental combination. Then 1 g of soil was taken and dried from each pooled sample per combination. After drying, 0.7 g of soil was obtained for the control (C) and 0.8 g for the mycorrhizal treatment (MF). Microbiological analysis was performed on 10 g of rhizosphere soil from each experimental combination. Each soil sample was mixed with 9 ml of sterile distilled water. Subsequently, serial dilutions of the soil solution were prepared. Appropriate dilutions of the soil solutions were plated onto culture media in Petri dishes. For each experimental combination and microbial group, 9 replicates (9 Petri dishes) were prepared with the culture media described above, onto which dilutions of the soil suspension were plated according to the method of Patkowska and Konopiński [2014]. For the isolation of total fungi, Martin's medium was used, containing 20 g of agar, 5 g of peptone, 10 g of dextrose, 1 g of KH_2PO_4, and 0.5 g of $MgSO_4 \cdot 7H_2O$, supplemented with streptomycin and Bengal rose. Streptomycin and Bengal rose were mixed with Martin's liquid medium after sterilization in an autoclave, before pouring the medium into Petri dishes. For the isolation of total bacteria, nutrient agar supplemented with glucose was used (Difco, Becton, Dickinson and Company, Sparks, MD, USA). For the isolation of bacteria from the genus *Pseudomonas*, Pseudomonas Agar F (Difco, Becton, Dickinson and Company, Sparks, MD, USA) supplemented with penicillin and nystatin was applied. Penicillin and nystatin were mixed with liquid medium Pseudomonas Agar F medium after sterilization in an autoclave, before pouring the medium into Petri dishes. Triptic Soy Agar (Difco, Becton, Dickinson and Company, Sparks, MD, USA) was used to isolate bacteria from the genus *Bacillus* [Jamiołkowska et al. 2020b].

Root mycological analysis. Mycological analysis of tomato roots was conducted six weeks after mycorrhizal inoculation (beginning of flowering, BBCH 62), following the method described by Jamiołkowska [2007]. For the analysis, roots were collected from 10 plants per experimental combination. The roots were cleaned of substrate residues and rinsed under running water for 20 minutes. They were then surface-sterilized in a 10% sodium hypochlorite solution. The roots were cut into 0.5-cm fragments, and 10 fragments were placed on each Petri dish. Glucose-potato agar (PDA, Difco, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was used in the study. Twenty dishes were prepared for each experimental combination. The dishes with root fragments were incubated at 24°C for 7 days. Subsequently, the resulting fungal colonies were transferred onto potato-dextrose agar (PDA, Difco, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and identified to the genus level based on microscopic characteristics and available mycological monographs.

Assessment of root fresh and dry weight. The analysis was conducted on 15 roots from plants in each experimental combination. The fresh root mass was expressed as g FW^{-1}. The roots were then dried for 7 days in a ventilated room at 23–25°C and weighed, and the results were expressed as g DW^{-1}.

Plant height and stem base thickness measurements. The assessment of plant height and stem base diameter was performed 6 weeks after mycorrhizal inoculation of the plants (beginning of the flowering stage – BBCH 62). The height of the aerial plant part (cm) was measured from the soil surface to the plant apex for each plant in the combination. Stem thickness (mm) was measured at the base of each plant in the experimental combination using a caliper (Limit CDN-NT IP67).

Leaf greenness (SPAD) and total chlorophyll measurements. Leaf greenness of tomato plants was measured during the experiment using a SPAD-502 Plus chlorophyll meter (Konica Minolta). Non-invasive measurements were taken at three time points, at 7-day intervals, starting 10 days after mycorrhizal inoculation (15 May 2024, 22 May 2024, and 29 May 2024). The measurements were conducted on the third fully developed leaf from the apical meristem. Based on the measurements, SPAD values were converted to total chlorophyll content using the formula provided by Shenker et al. [2004] for tomato plants:

$$\text{Chlorophyll} = \frac{\text{SPAD} - 6.6}{27.3} \ (\text{mg g}^{-1}\,\text{FW})$$

Statistical analysis

The collected data were analyzed using Statistica software version 13.3 (1984–2017 TIBCO Software INC, Palo Alto, CA, USA). The normality of data distribution was tested using the Shapiro-Wilk test. A one-way analysis of variance (ANOVA) was conducted, and the significance of differences was assessed using the Tukey post hoc test and Kruskal-Wallis test at a significance level of $p = 0.05$.

RESULTS

Evaluation of root colonization by mycorrhizal fungi. Laboratory assessment of tomato roots showed that roots of mycorrhized plants (MF) were colonized by mycorrhizal fungal structures at 30%, whereas mycorrhizal frequency in control roots (C) was 18.89% (Table 1, Figure 1). Relative mycorrhizal intensity (M) was higher in the mycorrhizal treatment (MF – 2.59%) than in the control (C – 1.7%). No arbuscules (A) were observed in the roots of either mycorrhized or control plants. No significant differences were observed in absolute mycorrhizal intensity between the experimental combinations (m) – as shown in Table 1.

Table 1. Colonization of tomato roots by mycorrhizal fungi

Experimental treatment	F (%)	M (%)	m (%)	A (%)
MF	30.0[b]	2.59[a]	8.68[a]	0
C	18.89[a]	1.70[a]	8.92[a]	0

MF – mycorrhized plants, C – control plants, F – mycorrhiza frequency, M – relative mycorrhiza intensity, m – absolute mycorrhiza intensity (refers to fragments where any mycorrhizal colonization was observed), A – absolute arbuscule abundance (refers to fragments where arbuscules were detected); [a, b] – values in the same column marked with the same letter do not differ significantly at $p \leq 0.05$

Microbiological analysis of rhizosphere soil. The laboratory analysis revealed variation in the quantitative and qualitative composition of rhizosphere microorganisms. Mycorrhization of the plants significantly reduced the total number of bacteria and fungi in the tomato rhizosphere. The bacterial population in the rhizosphere of mycorrhized plants was 32.2×10^6 CFU g^{-1} DW soil, whereas it was twice as high in the control (72.4×10^6 CFU g^{-1} DW) soil (Table 2). The total number of fungi in the rhizosphere of mycorrhized plants was also significantly lower (3.1×10^5 CFU g^{-1} DW soil) than in the control (8.8×10^5 CFU g^{-1} DW soil) – as shown in Table 2. The mycorrhizal inoculum inhibited the growth of *Bacillus* sp. and *Pseudomonas* sp. in the tomato rhizosphere soil. The population of *Pseudomonas* sp. in the control was nearly twice as high (12.7×10^6 CFU g^{-1} DW soil) as in the rhizosphere soil of mycorrhized tomato plants (6.6×10^6 CFU g^{-1} DW soil). A lower number of bacteria from the genus *Bacillus* was also detected in the rhizosphere soil, but this difference was not statistically significant (Table 2).

Figure 1. Mycelium (m) and vesicles (vs) of mycorrhizal fungi in tomato roots; MF – mycorrhized plants, C – control plants; microscope objective magnification: 40×

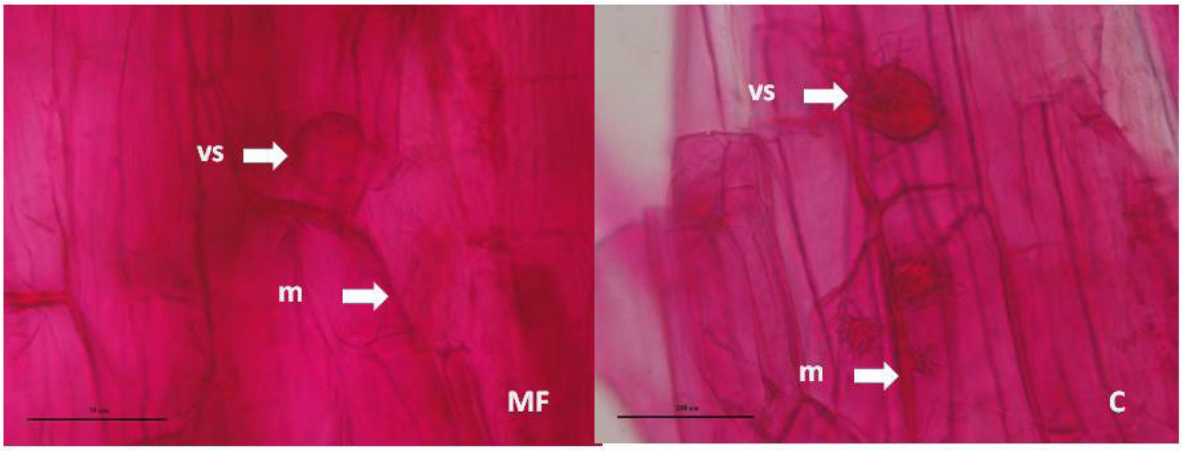


Table 2. Number of bacteria and fungi isolated from tomato rhizosphere soil

Experimental treatment	Total bacterial count(10^6 CFU g^{-1} DW soil) ±SD	*Bacillus* sp. count (10^6 CFU g^{-1} DW soil) ±SD	*Pseudomonas* sp. count (10^6 CFU g^{-1} DW soil) ±SD	Total fungal count (10^5 CFU g^{-1} DW soil) ±SD
MF	32.2 ±2.75[b]	1.5 ±0.21[a]	6.6 ±0.96[b]	6.6 ±1.60[b]
C	72.4 ±18.74[a]	2.37 ±0.77[a]	12.7 ±2.40[a]	8.8 ±1.08[a]

MF – mycorrhized plants, C – control plants, [a, b] – values in the same column marked with the same letter do not differ significantly at $p \leq 0.05$

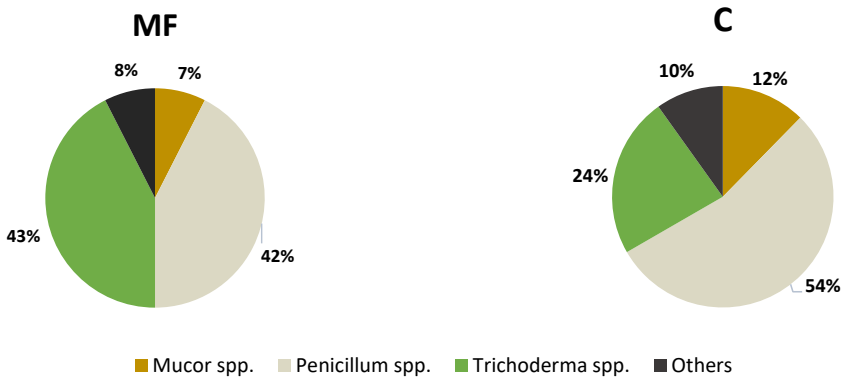
Fungal diversity colonizing tomato roots. The mycological analysis of tomato roots identified a total of 160 fungal colonies belonging to 10 genera (Table 3). The most abundant genera were *Penicillium* spp., *Trichoderma* spp., and *Mucor* spp. *Trichoderma* (33.1%), *Penicillium* (48.8%), and *Mucor* (10.0%) accounted for the largest proportions of the overall fungal composition (Table 3). *Penicillium* spp. were the most frequently isolated fungi in the control (C; 54.0%) – as shown in Figure 2. Meanwhile, the genus *Trichoderma* was dominant (43.0%) in the mycorrhizal treatment (MF) – as shown in Table 3 and Figure 2. The presence of mycorrhizal fungi led to a reduction in the abundance of other genera (including *Penicillium* spp. and *Mucor* spp.) in favor of the development of antagonistic *Trichoderma* spp. fungi (Figure 2).

Table 3. Diversity of fungi colonizing tomato roots

Fungal genus	Colony number		Total	Percentage (%)
	MF	C		
Alternaria spp.	1	1	2	1.3
Aspergillus sp.	–	1	1	0.6
Cladosporium sp.	1	1	2	1.3
Colletotrichum sp.	1	–	1	0.6
Humicola spp.	1	1	2	1.3
Mucor spp.	6	10	16	10.0
Penicillium spp.	34	44	78	48.8
Rhizopus sp.	–	1	1	0.6
Trichoderma spp.	34	19	53	33.1
Zygorhynchus sp.	1	3	4	2.4
Total	79	81	160	100

MF – mycorrhized plants, C – control plants

Figure 2. Percentage of selected fungal genera colonizing tomato roots; MF – mycorrhized plants, C – control plants



Plant height and stem base thickness. The study did not reveal any significant differences between the experimental combinations. Control plants were taller (101.14 cm) than mycorrhized plants (96.62 cm), but differences were not statistically significant. Similar trends were observed for stem base thickness, with control plants having thicker stems than mycorrhized tomatoes (Table 4).

Table 4. Mean plant height (cm) and stem base diameter of tomato plants (mm)

Experimental treatment	Plant height (cm) ±SD	Stem base diameter (mm) ±SD	Fresh root weight (g) ±SD	Dry root weight (g) ±SD
MF	96.62 ±6.48[a]	9.15 ±0.27[a]	41.67 ±8.28[a]	5.9 ±1.65[a]
C	101.14 ±8.80[a]	9.25 ±0.89[a]	35.77 ±4.71[a]	5.01 ±0.64[a]

MF – mycorrhized plants, C – control plants, [a, b] – values in the same column marked with the same letter do not differ significantly at $p \leq 0.05$

Fresh and dry root weight. The highest fresh root weight was observed in mycorrhized plants, with an increase of 16.5% compared to the control. Similarly, the highest dry root weight was recorded in mycorrhized plants, showing a 17.7% increase compared to the control. The recorded increase in fresh and dry weight was not statistically significant (Table 4). The results, however, indicated an increasing trend in this trait following mycorrhizal inoculation (Table 4).

Leaf greenness index and total chlorophyll content. The tomato leaf greenness index showed an increasing trend during plant development (Table 5). The highest SPAD value was recorded at the third measurement for mycorrhized tomato leaves (40.9), but it was not significantly higher compared to the control plants. Similar increasing trends in SPAD values were observed at the other time points, with mycorrhized plants showing higher SPAD values than the control plants. These differences, however, were not statistically significant (Table 5). Tomato leaves also showed variation in total chlorophyll content depending on the experimental combination, with the highest values in plants with mycorrhizal inoculum, although the difference from the control was not significant (Table 5).

Table 5. Leaf greenness index (SPAD) and total chlorophyll content in tomato leaves (mg g^{-1} FW)

Measurement	I		II		III	
Experimental treatment	C	MF	C	MF	C	MF
SPAD ±SD	34.21 ±3.20[a]	36.43 ±4.69[a]	35.41 ±3.72[a]	36.69 ±3.55[a]	40.18 ±3.43[a]	40.9 ±2.60[a]
Chlorophyll (mg g^{-1} FW) ±SD	1.01 ±0.12[a]	1.09 ±0.17[a]	1.06 ±0.14[a]	1.10 ±0.13[a]	1.23 ±0.13[a]	1.26 ±0.10[a]

C – control plants, MF – mycorrhizal plants; [a, b] – values within rows for each measurement date followed by the same letter do not differ significantly at $p \leq 0.05$

DISCUSSION

Tomato is a species with a high capacity for forming mycorrhizal symbiosis [Pokluda et al. 2021]. Many authors have confirmed the positive effect of mycorrhizal fungi on tomato plants [Leventis 2021, Felföldi et al. 2022, Singh et al. 2024, Zhang et al. 2024]. Plants that interact with mycorrhizal fungi exhibit increased resistance to adverse environmental conditions [Delaeter et al. 2024]. It has been shown that arbuscular mycorrhizal fungi (AMF) protect plants against the negative effects of biotic and abiotic factors, including drought. Through the symbiosis with plants, they increase gas exchange, leaf water content, stomatal conductance, and plant transpiration rate [Morte et al. 2000, Mena-Violante et al. 2006]. AMF maintain cell membrane integrity, improve nutrient and water uptake, and protect the photosynthetic apparatus from drought-induced oxidative stress. They enhance photosynthetic efficiency, enable the accumulation of hormones and phenols, and reduce the buildup of reactive oxygen species (ROS) by increasing antioxidant activity and gene expression [Tang et al. 2022].

Singh et al. [2024] demonstrated the beneficial effects of mycorrhizal fungi on tomato growth parameters, including plant height, stem diameter, leaf number and area, aerial organ weight, as well as root weight and volume, compared with non-inoculated plants. Our study confirmed the positive effect of a multi-component mycorrhizal inoculum on the fresh and dry root weight of tomatoes, as well as on total chlorophyll content in the leaves of mycorrhized plants. Felföldi et al. [2022] demonstrated that mycorrhizal inoculation improved tomato plant growth, yield, and fruit quality parameters, highlighting the beneficial effect of mycorrhiza on water and mineral uptake from the soil. In the present study, however, mycorrhization had no significant effect on plant height or stem base thickness. This can be explained by the fact that measurements were taken six weeks after plant inoculation, when the effect of mycorrhization was not yet apparent in morphological traits. Hart and Reader [2002] reported that the average time for root colonization, i.e., from inoculation to initial colonization, ranged from 1 to 3 weeks for *Glomus* spp., and from 6 to 8 weeks for *Gigaspora* spp. Smith and Smith [2011] have emphasized that the use of arbuscular mycorrhizal fungi does not always result in a significant increase in plant vegetative biomass. Jamiołkowska et al. [2020b] demonstrated that mycorrhization of tomato roots with *Claroideoglomus etunicatum* and *Rhizophagus intraradices* improved plant uptake of certain macro- and micronutrients (Ca, K), and the root length of tomato plants, particularly those treated with *Claroideoglomus etunicatum*, was significantly greater compared to the control. The authors found out that inoculation of tomato roots with both mycorrhizal fungal strains significantly increased the number of leaves and improved plant health. Tomato yield was not significantly affected by mycorrhization; however, *Claroideoglomus etunicatum* reduced the number of diseased fruits more effectively than *Rhizophagus intraradices* compared to the control [Jamiołkowska et al. 2020b].

The current study showed that inoculation with a multi-component mycorrhizal inoculum modulated the microbiota of tomato rhizosphere soil. Mycorrhizal fungi reduced the total number of bacteria and fungi in the tomato rhizosphere, including antagonistic bacteria from the genera *Bacillus* and *Pseudomonas*. Similar results were obtained by Jamiołkowska et al. [2020b], who reported a reduction in the total number of bacteria, including *Bacillus* sp. and *Pseudomonas* sp., as well as a decrease in the total number of fungi in the rhizosphere soil of sweet pepper inoculated with mycorrhizal inoculum. In our experiment, a higher number of *Trichoderma* spp. fungi was found on the roots of plants in combination with MF. This phenomenon can be explained by the changing composition of root exudates of mycorrhizal plants. Because Mycorrhizal fungi influence root exudates, modify the environmental conditions for microbial growth in the rhizosphere, and thereby alter the composition of soil microbiota [Amer and Abou-el-Seoud 2008, Bücking et al. 2008, Al-Askar and Rashad 2010, Zarea et al. 2011]. Root border cells (RBCs) also play an active role in this process by secreting metabolites into the environment, shaping the populations of microorganisms colonizing the root zone, and participating in interactions between plants and soil microorganisms [Jaroszuk-Ściseł et al. 2009]. This phenomenon is particularly important for stimulating the activity of microorganisms antagonistic to soil pathogens, promoting the growth of microorganisms such as: *Mortierella*, *Pseudogymnoascus*, *Mucor*, *Trichoderma*, *Conocybe*, *Pseudaleuria*, and *Acremonium* [Jamiołkowska et al. 2020b].

Mycological analysis of tomato roots showed that the number of antagonistic fungi of the genus *Trichoderma* spp. was higher in the roots of mycorrhized plants than in those of control plants. Fungi of the genus *Trichoderma* benefit plants by suppressing pathogenic agents. They are strong competitors and exhibit both antibiotic and parasitic activity against phytopathogens. They grow rapidly in the soil and suppress other microorganisms [Błaszczyk et al. 2014]. Enzymes produced by *Trichoderma* spp. (including cellulases, hemicellulases, chitinases, and glucanases) facilitate the degradation of pathogen cell walls [Chen et al. 2025]. It can be assumed that myc-

orrhizal fungi also have a significant impact on the fungal mycobiota of tomato roots inoculated with mycorrhizal fungi. Al-Hmoud and Al-Momany [2015] demonstrated that mycorrhizal fungi reduce the number of pathogens through competition for nutrients, and the application of the mycorrhizal fungus *Glomus intraradices* significantly decreased *Fusarium* spp. infections in the studied plants by promoting plant growth and increasing root volume. The present study confirmed these findings, showing a significantly higher abundance of *Trichoderma* fungi on the roots of mycorrhized plants. Mycorrhizal fungi also improve plant resistance to diseases, in part by regulating changes in root anatomical structure [Jamiołkowska et al. 2020a, Weng et al. 2022]. The most important elements of this phenomenon include accelerated lignification of the cell wall and an increased number of cell layers, formation of hyphal networks and a pectic nodule structure in the root epidermis, callose deposition, and production of hydroxyproline-rich glycoproteins (structural components of the cell wall) [Scervino et al. 2007, Balestrini and Bonfante 2014, Basyal and Emery 2021]. Fujita et al. [2022], using molecular analyses, demonstrated the efficacy of a mycorrhizal inoculum based on *Gigaspora margarita* in protecting tomato plants against *Botrytis cinerea* and *Pseudomonas syringae* pv. tomato. Defense-related genes associated with salicylic acid (SA) and jasmonic acid (JA) were expressed more rapidly and strongly in tomato plants colonized by *G. margarita* compared to control plants, as the plant's immune system was stimulated by mycorrhizal colonization. Resistance induced by this mechanism was effective against both fungal and bacterial pathogens.

The effectiveness of root colonization by mycorrhizal fungi depends on multiple factors, including the type of mycorrhizal inoculum and environmental conditions such as light availability, pH, soil fertility, and moisture [Jamiołkowska et al. 2018, Derkowska et al. 2024]. Literature indicates significant differences in the colonization rate between different species of arbuscular mycorrhizal fungi, highlighting consistent trends characteristic of their taxonomic family [Hart and Reader 2002]. Fungi of the genus *Gigaspora* (family: *Gigasporaceae*), despite their low colonization rate and limited internal root colonization, tend to form extensive, dense, and robust hyphal networks on the root surface [Caccia et al. 2024]. In contrast, fungi of the genus *Glomus* (family: *Glomaceae*) are characterized by a rapid colonization rate, but they produce a more dispersed mycelium with finer hyphae [Säle et al. 2021]. Simultaneously, previous studies have emphasized the need for the coexistence of these mycorrhizal genera to achieve improved effects [Hart and Reader 2002]. Subramanian et al. [2006] demonstrated that the inoculation of tomato seedlings with *Rhizophagus intraradices* increased root colonization by up to 48%. The present study indicates that the applied mycorrhizal inoculum resulted in a colonization frequency of 30%, over one-third higher than in the control combination. The mycorrhizal fungal species and the colonization time also play a significant role in the process of plant root colonization [Hart and Reader 2002].

The study also demonstrated the effect of plant mycorrhization on chlorophyll content in tomato leaves, a important indicator of plant physiological status [Caradonia et al. 2019]. Literature confirms a direct relationship between the chlorophyll content in the plant and the SPAD parameter values defined as a measure of "leaf greenness" [Shenker et al. 2004]. The results showed that leaves of mycorrhized tomato plants had slightly higher chlorophyll content than the control leaves. Although these differences were not statistically significant, it can be assumed that mycorrhization had a positive effect on photosynthesis. Similar experiments were conducted by Fracasso et al. [2020], who confirmed a positive correlation between SPAD values and photosynthesis rate. Similarly, Majkowska-Gadomska et al. [2016] showed that the mycorrhizal inoculum improved the nutritional status of tomato plants determined based on the leaf greenness index (SPAD). The higher photosynthetic efficiency in mycorrhized plants results from an increased density of stomata in the leaves and elevated levels of photosynthetic pigments (increased concentrations of chlorophyll a; b; a + b; and carotenoids) [Głuszek et al. 2008, Chitarra et al. 2016].

The application of mycorrhizal fungi in the form of a multi-component inoculum is highly promising and should be recommended in integrated tomato production systems as an element supporting natural plant physiology, immunity, and protection against phytopathogens. The effectiveness of commercial mycorrhizal fungal inoculants naturally depends on the quality and quantity of the inoculum, compatibility with cultivated plant species, native microbial communities, environmental factors, and soil management practices [Basirus et al. 2021]. The pot experiments confirmed the multidirectional impact of the mycorrhizal inoculum as a factor improving photosynthesis, contributing to increased root weight, and promoting a higher abundance of antagonistic fungi (*Trichoderma* spp.) in plant roots. The present findings indicate that plant mycorrhization can reduce the use of certain agrochemicals (mineral fertilizers and fungicides) and improve the safety of the harvested crops.

CONCLUSIONS

1. Plant mycorrhization reduced the total number of bacteria and fungi in the tomato rhizosphere.

2. The mycorrhizal inoculum increased the abundance of antagonistic fungi of the genus *Trichoderma* in tomato roots.

3. Plant mycorrhization increased the fresh and dry weight of tomato roots, although the increase was not statistically significant.

4. Leaves of mycorrhized tomato plants showed higher SPAD values and total chlorophyll content, although these differences were not statistically significant compared to control plants.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHORS' CONTRIBUTIONS

A.J.: working on a conception and assumptions. A.J., W.K.: developing the methods. A.J., W.K., E.P.: conducting research. A.J., W.K., E.P.: writing a manuscript, review and editing.

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RESEARCH PAPER

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PHYSIOLOGICAL RESPONSES OF GREEN BEANS TO POSTHARVEST SALICYLIC ACID TREATMENTS UNDER COLD STORAGE CONDITIONS

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ABSTRACT

This study evaluated the effects of different salicylic acid (SA) concentrations (0.5, 1, and 2 mM) on the postharvest quality of green beans during cold storage (4 ±1 °C). Physiological parameters (respiration rate, electrolyte leakage, weight loss, and cutting resistance), biochemical traits (total soluble solids, TSS), and colorimetric properties (L*, h°, C*, and browning index) were assessed to determine treatment efficacy. Multivariate analyses, including principal component analysis (PCA), correlation analysis, and hierarchical heatmap clustering, were applied to characterize multidimensional treatment responses. Salicylic acid exhibited dose-dependent and parameter-specific effects. The 0.5 mM SA treatment improved visual quality by maintaining higher chroma values and a more stable hue angle, while also enhancing cutting resistance. The 1 mM dose increased respiration rate and electrolyte leakage, indicating elevated metabolic activity and membrane stress. In contrast, 2 mM SA provided a more balanced preservation of overall quality by reducing weight loss and maintaining moderate color stability. Regardless of treatment, storage duration significantly affected color parameters (L*, h°, and browning index), reflecting natural chlorophyll degradation and senescence processes. PCA revealed that PC1 (43.0% of total variance) was primarily associated with visual quality attributes (L*, C*, TSS, and browning index), PC2 (19.9%) with stress-related variables (respiration rate and electrolyte leakage), and PC3 (12.1%) with cutting resistance. Heatmap clustering and correlation analysis supported these relationships by grouping color and biochemical attributes separately from stress indicators. Overall, the results demonstrate that SA dose optimization is critical for maintaining postharvest quality of green beans under cold storage conditions. These findings highlight the usefulness of multivariate approaches in integrating physiological and visual quality attributes and support the practical application of optimized salicylic acid doses for extending the postharvest life of green beans.

Keywords: *Phaseolus vulgaris* L., postharvest quality, low-temperature storage, color attributes, electrolyte leakage, dose optimization

INTRODUCTION

Green bean (*Phaseolus vulgaris* L.) is an economically and nutritionally important vegetable crop belonging to the Fabaceae family and is widely consumed as immature pods in many regions of the world. The genus *Phaseolus* comprises more than 50 species, among which *P. vulgaris*, *P. lunatus*, *P. coccineus*, *P. acutifolius*, and *P. polyanthus*

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are domesticated [USDA 2025]. Green beans are valued for their high dietary fiber and protein content, as well as essential micronutrients such as vitamins A, C, and K, and minerals including calcium, potassium, iron, and magnesium [Brigide et al. 2014]. Owing to these nutritional attributes, green beans contribute significantly to balanced diets and food security. Globally, green bean production reached 1.36 million tons in 2023 [FAO 2023]. The United States, Morocco, the Philippines, and Mexico are among the leading producers, while Türkiye ranks fifth with an annual production of 70,021 tons [TÜİK 2023]. Although production levels fluctuate annually, Türkiye has shown nearly a 15% increase over the past three decades, highlighting the growing agricultural and economic importance of this crop. Despite its importance, green beans are highly perishable due to its high moisture content (≈90%) and intense metabolic activity after harvest. Because pods are harvested at an immature stage, they exhibit a high respiration rate and are therefore prone to rapid senescence and quality deterioration [Ogumo et al. 2018]. Postharvest losses are commonly associated with water loss, textural softening, browning, decay, and reduced visual and nutritional quality [El-Mogy and Kitinoja 2019]. Consequently, reducing respiration rate and maintaining membrane integrity are critical for preserving postharvest quality. Cold storage is the most widely used method to slow metabolic activity and extend the shelf life of green beans. Storage at 5–7.5 °C and 95–100% relative humidity allows green beans to maintain acceptable quality for 8–12 days, and up to 21 days under optimal handling conditions [Gross et al. 2016, El-Mogy and Kitinoja 2019]. However, exposure to low temperatures may cause physiological stress on the pods, potentially leading to quality deterioration and reduced marketability if not properly managed [Zong et al. 1992, Lv et al. 2024]. Therefore, complementary postharvest treatments are often required to enhance physiological tolerance and maintain quality during cold storage. Various physical and chemical approaches have been investigated to delay senescence and preserve quality in perishable vegetables, including gibberellic acid [Ding et al. 2015], intermittent warming [Liu et al. 2015], biodegradable coatings [Germano et al. 2019], 1-methylcyclopropene, methyl jasmonate [Rehman et al. 2021], nitric oxide [Wang et al. 2015], chitosan-based coatings [Elbagoury et al. 2022], and salicylate-related compounds [Zhang et al. 2015]. Among these treatments, salicylic acid (SA) has received increasing attention due to its role as an endogenous signaling molecule involved in plant stress responses. Salicylic acid is an aromatic phenolic compound known to delay postharvest senescence by enhancing membrane stability, stimulating antioxidant defense systems, and modulating ethylene biosynthesis through the inhibition of ACC synthesis and conversion [Leslie and Romani 1988, Özeker 2005, Erbaş and Koyuncu 2019]. Previous studies have demonstrated that SA application can improve postharvest performance and quality retention of several horticultural commodities under cold storage conditions. Nevertheless, information on the dose-dependent effects of SA on the physiological performance and quality attributes of green beans during low-temperature storage remains limited. Therefore, the objective of this study was to evaluate the effects of postharvest salicylic acid treatments at different concentrations (0, 0.5, 1, and 2 mM) on the physiological responses and major quality attributes of green beans (*Phaseolus vulgaris* L.) during cold storage. This study aims to provide insight into the potential of SA as a practical and safe postharvest treatment for maintaining green bean quality under low-temperature storage conditions.

MATERIALS AND METHODS

Plant material. Green bean pods (*Phaseolus vulgaris* L., cv. Ayşe Kadın) were obtained from a commercial grower in Antalya, Türkiye, during the 2024 spring growing season. The pods were transported to the Postharvest Physiology Laboratory (Department of Horticulture, Faculty of Agriculture) under cooled conditions. Upon arrival, visibly defective pods (broken, bruised, diseased, or mechanically damaged) were removed. Marketable pods were washed under running tap water to eliminate dust and field residues, then air-dried prior to treatment.

Salicylic acid treatments, packaging and storage. The green beans were subjected to four postharvest treatments. In the control treatment, the pods were immersed in tap water. In the 0.5 mM SA treatment, the beans were immersed in a 0.5 mM salicylic acid solution. For the 1 mM SA treatment, the pods were immersed in a 1 mM salicylic acid solution, and in the 2 mM SA treatment, they were immersed in a 2 mM salicylic acid solution. All immersions were carried out for 5 minutes, after which the pods were surface-dried prior to storage. After drying, pods were packed into lidded PET containers (10 pods per container) for storage and subsequent analyses. Packaged pods were stored for 18 days at 4 ±1 °C and 85–90% RH. Measurements were conducted on days 0, 3, 6, 9, 12, 15, and 18.

Respiration rate. The respiration rate (RR) was determined using an O_2/CO_2 gas analyzer (Systech Illinois Gaspace 6000). Three PET packages per treatment were used. CO_2 accumulation inside each container was recorded, and RR was calculated using following formula, correcting for daily CO_2 leakage from the PET material.

$$\mathrm{RR} = \frac{(C_t - C_0) \times V}{w \times t}$$

where:
RR = respiration rate (mL CO_2 kg^{-1} h^{-1}),
C_t = CO_2 concentration at time t,
C_0 = initial CO_2 concentration,
V = package volume (L),
w = sample weight (kg),
t = elapsed time (h).

Electrolyte leakage (EL). Electrolyte leakage was quantified following Kasım and Kasım [2015], with minor modifications. Three pods per replicate were cut into ~1 cm pieces, rinsed twice in distilled water, then immersed in 50 mL distilled water for 2 h at room temperature. Initial conductivity (EC_1) was measured. Samples were then frozen (–18 °C), thawed, and final conductivity (EC_2) was recorded. EL was calculated using:

$$\mathrm{EL}\ (\%) = \frac{\mathrm{EC}_1}{\mathrm{EC}_2} \times 100$$

Weight loss. Weight loss was determined gravimetrically using the same PET containers throughout storage. Weight loss (%) was calculated using following formula:

$$\text{Weight loss}\ (\%) = \frac{W_0 - W_t}{W_0} \times 100$$

where:
W_0 = initial weight (g),
W_t = weight (g) at each sampling day.

Cutting resistance. Cutting resistance was measured using a Shimadzu EZ-LX texture analyzer fitted with a Warner–Bratzler shear blade. Three pods per replicate were evaluated. Results were expressed in Newtons (N).

Total soluble solids. TSS (%) was measured using a digital refractometer (Atago Co. Ltd., Japan). Juice extracted from pods was filtered and analyzed directly. Calibration was performed using distilled water.

Color measurement. Color values (L*, *a**, *b**) were recorded using a Minolta CR-400 colorimeter. Five pods per replicate were randomly selected, and readings were taken from the midsection of each pod. Calibration was performed using a standard white tile (L = 97.52, a = –5.06, b = 3.57). The following indices were calculated:

$$\mathrm{Hue} = \tan^{-1}\left(\frac{b^*}{a^*}\right), \mathrm{BI} = \frac{100(x-0.31)}{0.17}, \mathrm{C} = \sqrt{a^2 + b^2}$$

Statistical analysis. The experiment was arranged in a completely randomized design (CRD) with three biological replications, each replication consisting of 10 pods. The study followed a factorial structure with two fixed factors: salicylic acid (SA) concentration (0, 0.5, 1, and 2 mM) and storage duration (0, 3, 6, 9, 12, 15, and 18 days). All statistical analyses were performed using replicate-level data rather than averaged values. Data were subjected to two-way analysis of variance (ANOVA) to evaluate the main effects of SA concentration, storage duration, and their interaction. When significant differences were detected, mean separations were performed using Duncan's Multiple Range Test (DMRT) at a significance level of $p < 0.05$. Results are presented as mean ± standard error (SE) based on three replications (n = 3). Multivariate analyses, including principal component analysis (PCA), Pearson correlation matrix, and hierarchical clustering heatmap, were conducted to explore relationships among quality parameters and to visualize treatment-dependent patterns. These multivariate analyses were performed using the Python programming language, employing standard scientific libraries for data analysis and visualization.

RESULTS AND DISCUSSION

Analysis of variance (ANOVA)

The analysis of variance (ANOVA) revealed that salicylic acid (SA) treatments significantly affected several postharvest quality attributes of green beans (*Phaseolus vulgaris* L.) during cold storage (Table 1). Specifically, respiration rate, electrolyte leakage, weight loss, cutting resistance, hue angle, chroma, and browning index were all influenced by the treatment factor ($p < 0.05$). In contrast, SA treatments had no significant effect on soluble solids content (SSC) or L* values ($p > 0.05$). Storage duration exhibited a strong and highly significant effect ($p < 0.001$) on all measured parameters, confirming that cold storage time is a major determinant of physicochemical and physiological changes in green beans. The interaction between treatment and storage duration was not significant for most variables, with the exception of cutting resistance, indicating that texture responded differently to SA doses across storage intervals.

Table 1. Analysis of variance (*F* and *p* values) for the effects of salicylic acid treatments and storage duration on the quality parameters of green beans (*Phaseolus vulgaris* L.)

Parameter	Treatment	Storage duration	Treatment × Storage duration
Respiration rate	2.214*	483.881***	0.363 ns
Electrolyte leakage	2.657*	115.085***	1.827 ns
Weight loss (%)	15.399***	461.048***	0.617 ns
Cutting resistance (CR, N)	9.240**	7.787***	3.686***
Soluble solids content (SSC)	0.464 ns	29.030***	0.554 ns
L* value	0.408 ns	9.869***	0.553 ns
Hue angle (h°)	5.523*	52.265***	0.792 ns
Chroma (C)	2.601*	8.818***	1.304 ns
Browning index (BI)	1.703*	9.541***	0.818 ns

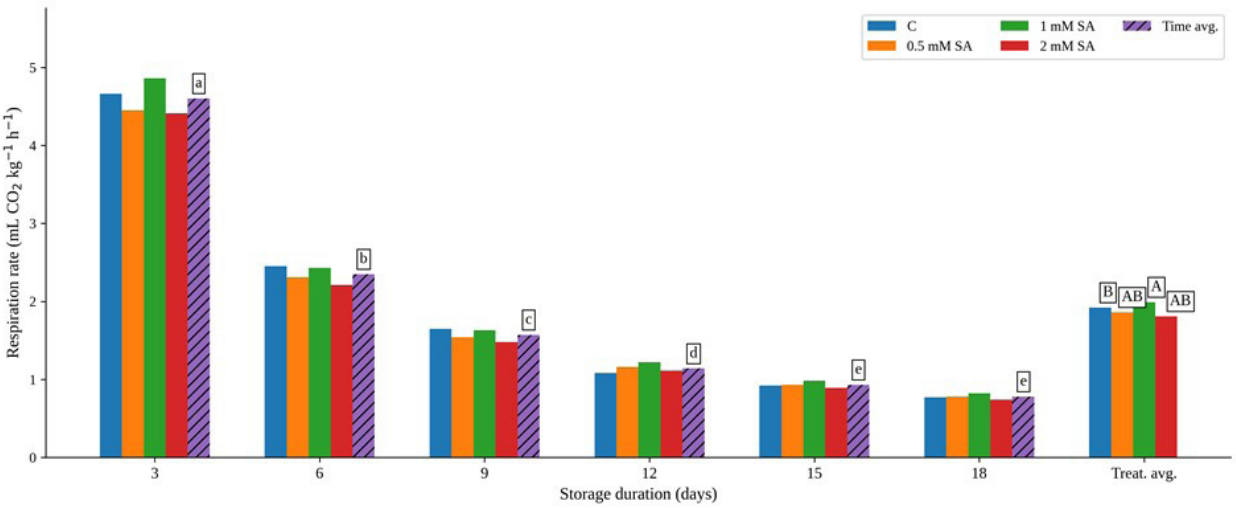
* Significance levels: ***$p < 0.001$, **$p < 0.01$, $p < 0.05$, ns = not significant. Treatments: 0 (Control), 0.5, 1, and 2 mM SA. Sampling days: 0, 3, 6, 9, 12, 15, and 18

Respiration rate

Figure 1 shows the changes in respiration rate (RR, mL CO_2 kg^{-1} h^{-1}) of green beans during cold storage. A gradual and significant decrease in RR was observed as storage progressed ($p < 0.001$). The highest respiration rate occurred on day 3 (4.60 mL CO_2 kg^{-1} h^{-1}), indicating high initial metabolic activity, whereas the lowest value was recorded on day 18 (0.78 mL CO_2 kg^{-1} h^{-1}). This trend reflects a natural decline in metabolic intensity under prolonged low-temperature conditions. Similar reductions in RR during cold storage have been reported for other legumes; for example, Kaur et al. [2022] demonstrated that respiration significantly decreased in cold-stored green pea pods, consistent with the present findings.

Salicylic acid (SA) treatments also significantly affected respiration rate ($p < 0.05$). The 1 mM SA treatment resulted in the highest RR among all treatments, whereas 2 mM SA resulted in a noticeable reduction compared with the control. The difference between these two doses was statistically significant, indicating a dose-dependent, bidirectional response. The stimulatory effect of 1 mM SA suggests that this concentration may enhance respiratory enzyme activity or energy metabolism, thereby increasing CO_2 production. In contrast, 2 mM SA appears to exert a suppressive influence, potentially moderating metabolic activity under cold stress. These observations align with mechanistic studies showing that the effect of SA on respiration is strongly dependent on concentration and tissue type. Shugaev et al. [2014] reported that SA up to 1 mM acted as a mitochondrial uncoupler in sugar beet roots, stimulating oxygen uptake in the absence of ADP but exhibiting little effect when ADP was present. The authors also noted that in *Lupinus albus* cotyledons, oxygen uptake increased only slightly in response to SA, suggesting that plant mitochondrial responses vary with both dose and exposure duration. Together, these findings support the present observation that moderate SA concentrations (1 mM) may transiently activate metabolic pathways, whereas higher concentrations (2 mM) may limit respiration through inhibitory or protective mechanisms.

Figure 1. Changes in respiration rate of green beans during cold storage as affected by salicylic acid (SA) treatments



Respiration rate (mL CO_2 kg^{-1} h^{-1}) of green beans treated with different SA concentrations (0, 0.5, 1, and 2 mM) over 18 days of storage at 4 ±1 °C. Different lowercase letters (a–e) indicate significant differences among storage days, while uppercase letters (A–B) indicate significant differences among treatments according to Duncan's multiple range test ($p < 0.05$). Bars represent mean ± SE (n = 3)

Table 2. Electrolyte leakage (%) of green beans treated with different concentrations of salicylic acid (SA) during cold storage

Parameter	Storage duration (days)							Treatment average
	0	3	6	9	12	15	18	
Control	7.2 $^{\pm 0.6}$ ns	18.8 $^{\pm 1.6}$ ns	7.4$^{\pm 1.8}$ ns	12.5 $^{\pm 1.6}$ ns	20.7 $^{\pm 1.8}$ ab	13 $\pm^{0.7\ ab}$	12.9 $^{\pm 0.8}$ ns	13.2 B
SA 0.5 mM	7.2 $^{\pm 0.6}$ ns	18.7 $^{\pm 1.8}$ ns	8.6$^{\pm 0.6}$ ns	12.6 $^{\pm 0.8}$ ns	18.7 $^{\pm 0.5}$ b	15.3 $^{\pm 1.3}$ a	15.4 $^{\pm 2.3}$ ns	13.8 AB
SA 1 mM	7.2 $^{\pm 0.6}$ ns	18.4 $^{\pm 1.3}$ ns	9.3$^{\pm 0.2}$ ns	13.6 $^{\pm 2.0}$ ns	23.5 $^{\pm 3.2}$ a	11.9 $^{\pm 2.4}$ b	15.6 $^{\pm 3.0}$ ns	14.2 A
SA 2 mM	7.2 $^{\pm 0.6}$ ns	17.3 $^{\pm 0.7}$ ns	8.8±0.6 ns	12.7 $^{\pm 2.1}$ ns	18.4 $^{\pm 0.9}$ b	13.1 $^{\pm 1.4}$ ab	13.9 $^{\pm 1.2}$ ns	13.1 B
Time average	7.2 f	18.3 b	12.8 e	12.8 d	20.3 a	13.3 cd	14.5 c	–

Mean electrolyte leakage (EL) values (%) of green beans subjected to salicylic acid treatments (0, 0.5, 1, and 2 mM) across storage days (0–18). Values represent means ± SE (n = 3). Different lowercase letters indicate significant differences among storage days, and uppercase letters indicate differences among SA treatments according to Duncan's multiple range test ($p < 0.05$)

Electrolyte leakage

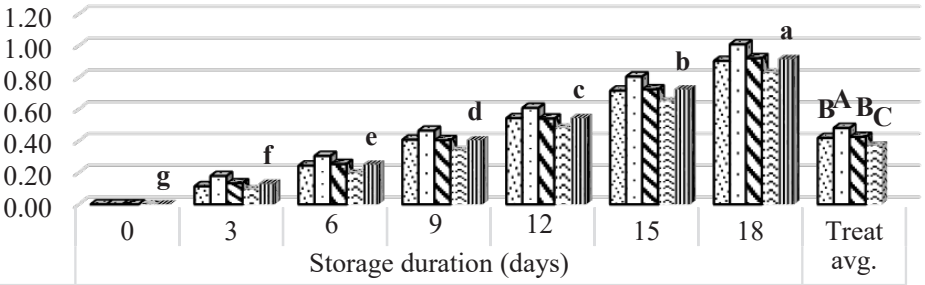
Electrolyte leakage (EL) is a key physiological indicator reflecting the integrity of cellular membranes, and therefore provides a reliable measure of membrane stability and sensitivity to chilling stress. In this study, EL values increased progressively across all treatments as storage time advanced (Table 2), demonstrating the cumulative impact of cold stress on membrane functionality. Analysis of treatment means showed that the lowest EL value occurred in the 2 mM SA treatment (13.1%), whereas the highest EL value was recorded in the 1 mM SA treatment (14.2%). These results indicate that 1 mM SA did not confer membrane protection in green beans; rather, under cold-storage conditions, this concentration may have induced a mild stress stimulus that enhanced membrane permeability. By contrast, 0.5 mM SA and 2 mM SA treatments fell within the same statistical group as the control, suggesting that these concentrations maintained membrane stability at levels comparable to untreated pods. The dose-dependent nature of SA responses observed here aligns with previous reports highlighting the dual, sometimes contrasting, roles of SA depending on concentration, tissue type, and environmental conditions. The general rise in EL during storage is characteristic of chilling-induced physiological disruption, including membrane lipid phase transitions, reactive oxygen species (ROS) accumulation, and lipid peroxidation – processes that collectively weaken membrane barrier properties [Wang et al. 2018]. The elevated EL values under the 1 mM SA treatment suggest that this dose may have failed to adequately stimulate antioxidant defenses or may have transiently activated oxidative pathways, thereby increasing membrane leakage. Previous studies have similarly noted variable EL responses depending on SA concentration. For example, Zhang et al. [2015] reported that a chitosan

(0.5%) + SA (0.1%) coating effectively reduced EL in cucumber, whereas Khademi et al. [2019] demonstrated that 1 mM SA decreased EL in bananas by strengthening membrane structure during cold storage. In contrast, the present findings show that 2 mM SA reduced EL more effectively than the lower doses. This suggests that higher SA concentrations may have more strongly activated antioxidant enzyme systems, improving membrane resistance to chilling-related oxidative damage. Overall, the EL results confirm that SA exhibits a concentration-dependent effect on membrane stability in green beans. While 0.5 mM and 2 mM SA provided partial membrane protection, 1 mM SA unexpectedly increased membrane permeability, indicating overstimulation of stress-related metabolic processes rather than mitigation.

Weight loss

Weight loss is a major quality determinant in green vegetables, primarily resulting from moisture loss through transpiration as well as respiratory mass loss. In the present study, green bean pods exhibited a significant increase in weight loss ($p < 0.001$) as storage progressed. This progressive rise can be attributed to the inherently high moisture content and broad surface area of green bean pods, which render them highly prone to dehydration even under optimal low-temperature storage conditions. Supporting this observation, Manzoor et al. [2019] reported that green beans, with moisture levels approaching 90% and ongoing metabolic activity at harvest, deteriorate rapidly and maintain relatively high respiration rates despite cold storage. Among the salicylic acid treatments, 2 mM SA demonstrated the greatest effectiveness in minimizing weight loss, displaying significantly lower values than all other treatments ($p < 0.001$). Although statistical differences among treatments were evident, overall weight loss remained low, with final values ranging between 0.83% and 1.01%, indicating that dehydration did not reach levels that would negatively affect visual quality. The dose-dependent trend observed in this study highlights the superior ability of 2 mM SA to limit water loss, likely through enhanced stomatal regulation, reduced epidermal transpiration, and improved membrane stability. Similar mechanisms were previously. Jahan [2019]

Figure 2. Weight loss (%) of green beans subjected to different salicylic acid (SA) treatments during cold storage. Lowercase letters (a–g) indicate significant differences among storage days, while uppercase letters (A–C) show significant differences among SA treatments according to Duncan's multiple range test ($p < 0.05$)



	0	3	6	9	12	15	18	Treat avg.
C	0.00	0.11	0.24	0.41	0.54	0.72	0.90	0.42
SA 0.5 mM	0.00	0.18	0.30	0.46	0.61	0.81	1.01	0.48
SA 1 mM	0.00	0.13	0.25	0.40	0.54	0.72	0.92	0.43
SA 2 mM	0.00	0.09	0.20	0.34	0.48	0.65	0.83	0.37
Time avg.	0.00	0.13	0.25	0.40	0.54	0.72	0.92	–

reported that SA mitigated oxidative damage in heat-stressed tomatoes by reducing reactive oxygen species and preserving membrane integrity, while El-Beltagi et al. [2025] showed that SA strengthened antioxidant defenses and improved anatomical structures associated with water retention. In contrast, the 0.5 mM SA treatment resulted in higher weight loss, suggesting that lower concentrations may be insufficient to activate protective pathways, while the 1 mM SA treatment behaved similarly to the control, indicating minimal regulatory influence. Collectively, these findings align with the hormetic dose–response behavior of salicylic acid, wherein low doses are ineffective, moderate doses stimulate stress responses, and higher doses exert protective effects depending on plant species and tissue type [Miura and Tada 2014, Brito et al. 2018, Li et al. 2022].

Cutting resistance

Cutting resistance (CR) values of green beans exhibited clear variations throughout storage, and both storage duration ($p < 0.001$) and SA treatments ($p < 0.01$) significantly influenced this parameter (Table 3). As one of the primary indicators of textural quality, CR reflects changes in tissue firmness, elasticity, and turgor pressure during postharvest storage. At the beginning of storage (day 0), beans exhibited a CR value of 49.9 N, which increased sharply to 57.4 N by day 3. This peak likely represents an early-stage physiological adjustment to cold conditions, during which transient moisture redistribution or slight epidermal hardening may temporarily enhance tissue firmness. Thereafter, CR values gradually declined and reached 50.3 N on day 18, indicating progressive softening of the pod tissues as senescence advanced – a typical pattern reported for cold-stored legumes and other non-climacteric vegetables. Based on treatment means, the 0.5 mM SA treatment resulted in the highest CR value (55.1 N), significantly exceeding all other treatments. This suggests that low SA concentration was insufficient to stabilize membranes or mitigate cold-induced stress, potentially allowing passive water loss and leading to increased tissue rigidity. Similar observations have been reported in other horticultural commodities, where suboptimal SA doses induced mild oxidative stress that promoted cell wall stiffening rather than protection [Liu et al. 2022]. Conversely, the lowest CR values were recorded in the 1 mM SA treatment, although this group did not differ statistically from the control or 2 mM SA treatments. The reduced CR in 1 mM SA–treated pods indicates a crisper and more hydrated tissue structure, suggesting that this concentration helped maintain membrane stability and regulate oxidative stress, thereby preserving turgor pressure. These findings are consistent with the dose-dependent nature of SA responses, in which moderate concentrations often exhibit the most beneficial effects on tissue quality. Supporting evidence from García-García et al. [2022] confirms that CR measurements reliably detect texture alterations in bean pods during storage, while Wang et al. [2022] demonstrated that SA can enhance firmness and mitigate water loss, although its efficacy varies with concentration. Collectively, the results indicate that SA exerts a biphasic, dose-dependent effect on textural stability. The 0.5 mM SA dose promoted tissue stiffening – likely through insufficient stress mitigation – whereas the 1 mM dose maintained crispness by better preserving cellular integrity. The 2 mM SA dose showed intermediate behavior, providing moderate stabilization without marked textural enhancement. These findings highlight that optimizing SA concentration is crucial for maintaining desirable texture attributes in green beans during cold storage.

Table 3. Effect of different salicylic acid (SA) treatments on cutting resistance (N) of green green bean pods during cold storage (4 ±1 °C)

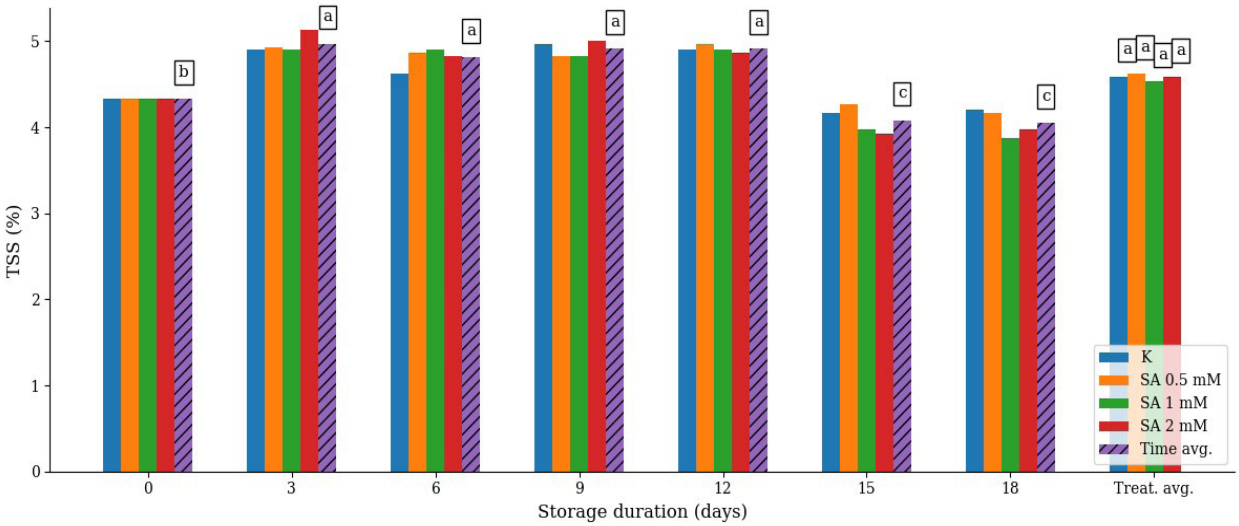
Parameter	Storage duration (days)							Treatment average
	0	3	6	9	12	15	18	
Control	49.7 $^{\pm 0.4}$ ns	58.7 $^{\pm 0.7}$ a	54.3 $^{\pm 4.8}$ ab	55.6 $^{\pm 1.0}$ ns	45.7 $^{\pm 1.0}$ b	53.7 $^{\pm 1.3}$ a	47.1 $^{\pm 2.4}$ b	52.1 B
SA 0.5 mM	50.4 $^{\pm 0.4}$ ns	60.5 $^{\pm 0.6}$ a	56.6 $^{\pm 5.9}$ a	52.3 $^{\pm 3.4}$ ns	55.8 $^{\pm 4.9}$ a	53.1 $^{\pm 1.5}$ a	57.4 $^{\pm 2.2}$ a	55.1 A
SA 1 mM	49.7 $^{\pm 0.5}$ ns	54.0 $^{\pm 1.3}$ c	53.4 $^{\pm 2.8}$ ab	51.4 $^{\pm 1.2}$ ns	47.5 $^{\pm 1.5}$ b	48.6 $^{\pm 0.1}$ b	47.7 $^{\pm 1.0}$ b	50.3 B
SA 2 mM	49.7 $^{\pm 1.0}$ ns	56.3 $^{\pm 1.6}$ b	46.6 $^{\pm 1.8}$ b	50.8 $^{\pm 9.6}$ ns	58.2 $^{\pm 4.8}$ a	51.9 $^{\pm 3.1}$ ab	49.1 $^{\pm 1.8}$ b	51.8 B
Time average	49.9 b	57.4 a	52.7 b	52.5 ab	51.8 ab	51.8 ab	50.3 ab	–

Values represent mean ± standard deviation (n = 3). Different lowercase letters within each row indicate significant differences among storage days according to Duncan's multiple range test ($p < 0.05$). Different uppercase letters in the "Treatment average" column indicate significant differences among treatments. "ns" denotes non-significant differences. Cutting resistance is expressed in Newtons (N); higher values indicate firmer and more elastic pods, whereas lower values represent crisper and less rigid tissue with higher moisture content

Total soluble solids

The effect of salicylic acid (SA) treatments on the total soluble solids (TSS) content of green beans was statistically non-significant ($p > 0.05$), indicating that none of the applied SA concentrations produced a measurable change in soluble solids during storage (Figure 3). In contrast, storage duration exerted a highly significant influence on TSS ($p < 0.001$). The fluctuations observed in TSS throughout the storage period were independent of treatment effects and are likely attributable to physiological processes such as carbohydrate utilization during respiration, concentration changes associated with moisture loss, progressive textural modifications, and minor developmental differences among pods despite simultaneous harvest.

Figure 3. Effects of different salicylic acid (SA) treatments on the total soluble solids (TSS, %) content of green beans during cold storage



Values represent the mean of three replicates (n = 3). Different lowercase letters (a–c) denote significant differences among storage days according to Duncan's multiple range test ($p < 0.05$). Treatment effects were not significant (ns)

The similarity among treatment means demonstrates that SA at 0.5, 1, or 2 mM does not markedly affect sugar metabolism or the accumulation of soluble solids in green beans. This outcome is consistent with previous research indicating that SA primarily influences stress-related physiological responses rather than carbohydrate metabolism. For example, El-Beltagi et al. [2022] reported that SA did not significantly alter TSS in broccoli florets, although it improved tissue firmness and pigment stability. Similarly, Hanaei et al. [2022] observed no substantial changes in TSS in SA-treated bell peppers; however, SA effectively enhanced stress tolerance-related parameters such as reduced weight loss and suppressed respiration. Collectively, these findings suggest that the postharvest role of SA is more closely associated with maintaining membrane integrity, modulating oxidative responses, and stabilizing tissue structure rather than altering soluble solids content, which is largely governed by inherent physiological and biochemical processes during storage.

Color values (L, h°, C, BI)

Table 4 presents the effects of different salicylic acid (SA) treatments on the L*, hue (h°), chroma (C*), and browning index (BI) values of green beans during storage. According to the ANOVA results (Table 1), SA treatments had no significant effect on L* ($p > 0.05$), whereas hue, chroma, and BI were significantly affected by the treatment factor ($p < 0.05$). Storage duration was highly significant for all color parameters, including BI ($p < 0.001$), indicating substantial color evolution during the storage period.

L* Values. SA treatments caused no meaningful changes in L*, and the mean values (65.3–65.8) remained statistically similar. L* increased slightly during early storage (days 9–12), indicating a transition toward lighter green tones. This trend is consistent with previous findings reporting increased L* values during cold storage as a result of cellular and structural changes affecting light reflectance [Yılmaz 2022]. By the end of storage, L* values slightly declined, reflecting the onset of tissue senescence. Differences reported between intact and green-cut beans in earlier studies [Kasım and Kasım 2015] highlight the influence of tissue integrity on L* evolution.

Hue (h°). Hue values decreased progressively throughout storage, demonstrating a gradual shift from vivid green toward more yellowish coloration. SA treatments modulated hue values to a limited extent: the lowest hue was observed in the 2 mM SA treatment (≈115.1°), suggesting slightly more advanced chlorophyll degradation compared with the control and 0.5 mM SA treatments. This decline reflects the well-documented degradation of chlorophyll and the increasing prominence of carotenoids during postharvest senescence [El-Mogy and Kitinoja 2019, Zhang et al. 2021].

Chroma (C*). Chroma values, which indicate color saturation, were significantly influenced by SA dose. Notably, 0.5 mM SA treatment resulted in higher chroma values than the control, suggesting better preservation of color vividness. This observation agrees with previous reports showing that SA-based treatments can delay yel-

lowing and help maintain color intensity by reducing oxidative stress and slowing pigment degradation [El-Beltagi et al. 2022, Kibar et al. 2024]. Chroma increased during mid-storage (days 9–12), reflecting a brighter color appearance, but declined toward the end of storage as senescence progressed.

Table 4. Effects of salicylic acid (SA) treatments on the color attributes (L, hue, chroma) and browning index (BI) of green beans during cold storage

Parameter		Storage duration (days)							Treatment average
		0	3	6	9	12	15	18	
L values	Control	64.49 ±2.6	61.71 ±1.1	65.12 ±1.4	66.52 ±1.0	68.33 ±1.2	65.99 ±0.9	66.81 ±0.6	65.57 ns
	SA 0.5 mM	64.49 ±2.6	61.61 ±1.6	64.84 ±0.8	67.23 ±1.2	66.65 ±1.8	67.05 ±1.1	68.99 ±0.3	65.84 ns
	SA 1 mM	64.49 ±2.6	62.56 ±0.9	65.62 ±0.8	64.47 ±0.1	67.16 ±1.9	65.84 ±1.2	66.69 ±1.7	65.26 ns
	SA 2 mM	64.49 ±2.6	63.34 ±0.7	65.57 ±0.5	66.72 ±1.2	67.73 ±1.1	66.96 ±0.7	66.07 ±1.2	65.84 ns
	Time average	64.49 c	62.3 d	65.29 bc	66.23 ab	67.47 a	66.46 ab	67.14 a	–
Hue	Control	117.0 ±1.0	117 ±0.3	116.9 ±0.2	116.4 ±0.2	115.7 ±0.4	114.5 ±0.5	113.4 ±1.0	115.9 a
	SA 0.5 mM	117.0 ±1.0	117.9 ±0.4	117.2 ±0.5	116.3 ±0.4	115.7 ±0.8	114.0 ±0.3	113.7 ±1.0	116.0 a
	SA 1 mM	117.0 ±1.0	116.8 ±0.6	116.6 ±0.4	116.3 ±0.8	115.6 ±0.7	113.4 ±0.8	112.8 ±0.2	115.5 ab
	SA 2 mM	117.0 ±1.0	116.1 ±0.7	116.5 ±0.6	115.9 ±0.9	114.6 ±1.2	112.3 ±1.6	113.1 ±0.7	115.1 b
	Time average	117.0 a	116.9 a	116.8 ab	116.2 b	115.4 c	113.6 d	113.2 d	–
Chroma	Control	32.86 ±1.0	31.67 ±1.37	33.17 ±0.28	34.98 ±0.38	33.23 ±3.93	34.71 ±0.42	35.38 ±0.40	33.71 b
	SA 0.5 mM	32.86 ±1.0	33.93 ±0.52	34.24 ±0.66	35.38 ±0.23	35.61 ±0.36	34.80 ±1.07	34.32 ±0.44	34.53 a
	SA 1 mM	32.86 ±1.0	33.64 ±0.26	33.72 ±0.62	35.55 ±0.46	35.34 ±0.13	34.91 ±0.57	34.2 ±0.48	34.32 ab
	SA 2 mM	32.86 ±1.0	33.79 ±0.71	33.58 ±0.47	35.3 ±0.45	34.24 ±1.14	33.29 ±1.24	33.67 ±0.81	33.96 ab
	Time average	32.86 d	33.26 d	33.68 bc	35.30 a	34.85 a	34.43 ab	34.54 ab	–
BI	Control	38.51 ±2.23	39.07 ±3.70	38.51 ±0.3	41.07 ±0.57	38.17 ±2.60	43.73 ±2.22	45.76 ±1.08	40.69 b
	SA 0.5 mM	38.51 ±2.23	41.57 ±1.27	40.15 ±0.69	41.24 ±0.21	43.00 ±1.30	42.69 ±2.87	42.54 ±1.47	41.53 ab
	SA 1 mM	38.51 ±2.23	41.82 ±1.41	39.45 ±1.75	44.06 ±0.77	42.25 ±2.78	45.78 ±1.31	44.59 ±1.07	42.35 a
	SA 2 mM	38.51 ±2.23	42.35 ±2.33	39.35 ±0.64	42.08 ±0.84	42.92 ±1.52	43.35 ±4.80	43.67 ±1.32	41.75 ab
	Time average	38.51 c	41.20 b	39.37 c	42.11 b	41.58 b	44.13 a	44.14 a	–

Values represent mean ± standard error (n = 3). Different lowercase letters within rows indicate significant differences among storage days ($p < 0.05$), while different uppercase letters within columns indicate significant differences among SA treatments ($p < 0.05$) according to Duncan's multiple range test. "ns" denotes non-significant differences ($p > 0.05$). SA: salicylic acid; BI: browning index

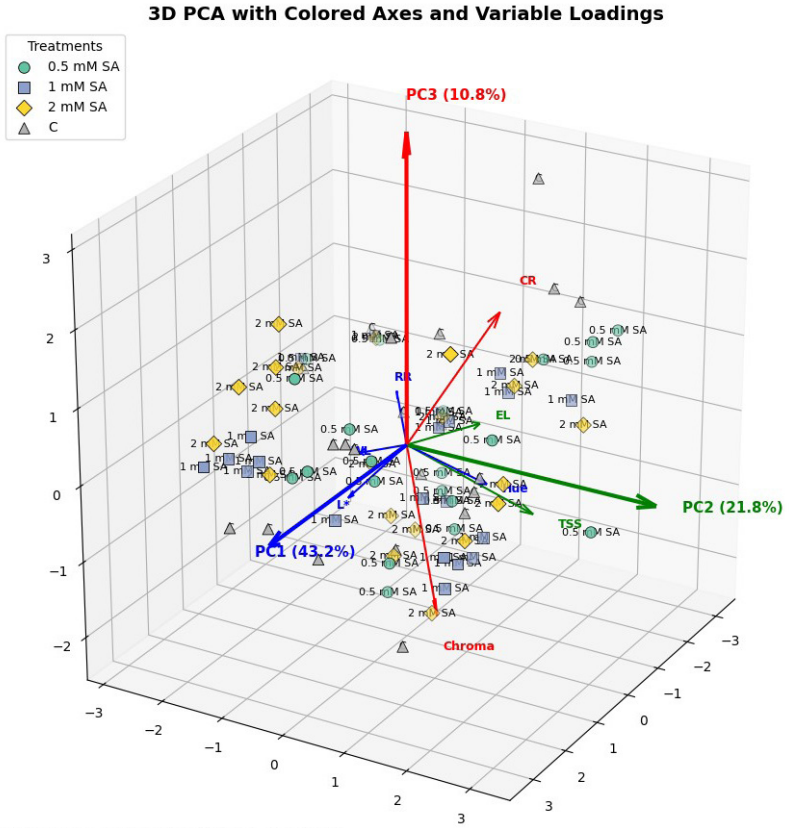
Browning index (BI). BI values provide an integrative measure of enzymatic and non-enzymatic browning, reflecting phenolic oxidation and pigment degradation. SA treatments significantly affected BI means ($p < 0.05$). The highest BI was observed in the 1 mM SA group (42.35), whereas the lowest BI belonged to 0.5 mM SA (41.53), followed closely by 2 mM SA and the control. These results indicate that 0.5 mM SA most effectively mitigated browning, whereas 1 mM SA slightly promoted it, possibly due to dose-dependent shifts between antioxidant activation and pro-oxidative stimulation – an effect previously described for SA in postharvest tissues. Storage duration strongly increased BI values, rising from approximately 38.5 at day 0 to 44.1–44.7 at day 18, reflecting progressive browning during senescence. This increase can be attributed to chlorophyll loss, phenolic oxidation, and membrane degradation – processes commonly associated with postharvest tissue aging. Similar browning progression has been documented in green beans and other vegetables during cold storage [El-Mogy and Kitinoja 2019, Zhang et al. 2021]. The multivariate PCA analysis (Figure 4) further supports these findings. The positive loading of BI on PC1 – together with L*, chroma, and TSS, suggests that browning progression is closely aligned with general color and compositional changes. The separation of the 0.5 mM SA treatment from higher BI regions indicates its stronger ability to preserve visual quality, whereas clustering of the 1 mM SA treatment near higher BI loadings, aligns with the observed increase in browning and decreased membrane stability. These multivariate relationships confirm that SA exhibits dose-dependent and parameter-specific effects, with 0.5 mM SA providing the most balanced preservation of appearance, color saturation, and browning resistance. Overall, storage duration was the dominant factor shaping color transitions, including lightness, hue, saturation, and browning. SA treatments exerted moderate but dose-dependent effects: 0.5 mM SA best preserved chroma and minimized browning, 2 mM SA slightly accelerated hue shift, 1 mM SA increased browning and was associated with less fa-

vorable color stability. Collectively, these findings indicate that SA modulates color attributes mainly through its influence on stress physiology and oxidative balance rather than direct effects on pigments, and that 0.5 mM SA emerges as the most effective dose for retaining visual quality in green beans during cold storage.

PCA, correlation matrix and heatmap findings

The multivariate evaluation obtained through the three-dimensional PCA, correlation matrix analysis, and hierarchical cluster-based heatmap (Figure 4) provided an integrated interpretation of how salicylic acid (SA) doses influenced the quality attributes of green beans. Overall, the treatments exhibited dose-dependent and parameter-specific effects.

Figure 4. 3D principal component analysis (PCA) of quality attributes in green beans treated with salicylic acid



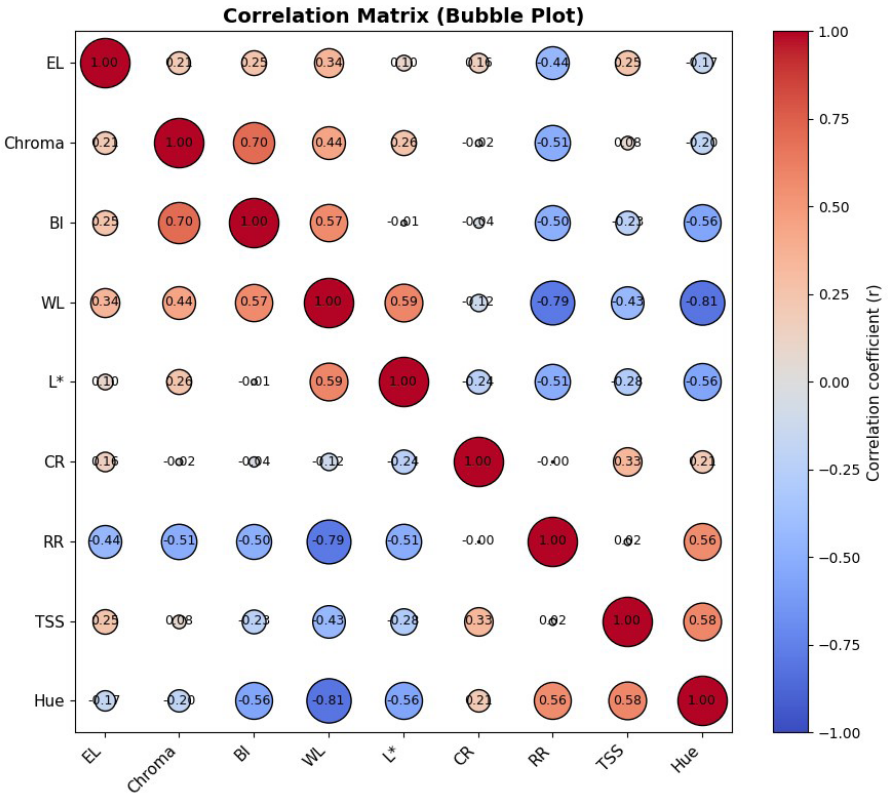
Total explained variance: 75.87% (PC1 = 43.2%, PC2 = 21.8%, PC3 = 10.8%)

PCA. According to the PCA results, the first three principal components explained 75.04% of the total variance, distributed as PC1 (43.0%), PC2 (19.9%), and PC3 (12.1%). PC1 was primarily associated with L*, chroma, hue, TSS, and to some extent, BI, indicating that this axis represented the overall appearance quality, including color brightness and saturation. The close alignment of the L* and chroma vectors suggested that pods with brighter and more saturated color tended to exhibit slightly lower BI values. The clustering of the 0.5 mM SA treatment along PC1 demonstrated that this dose supported higher color brightness, greater color saturation, and relatively elevated TSS values, consistent with Table 4 showing increased chroma under 0.5 mM SA. Therefore, this dose appeared to best preserve visual quality. PC2 was mainly associated with respiration rate (RR) and electrolyte leakage (EL), both indicators of stress physiology. The positive relationship between RR and EL on this axis indicated that higher respiration was accompanied by increased membrane permeability, reflecting reduced membrane stability. The distinct separation of the 1 mM SA treatment along PC2 confirmed that this dose enhanced metabolic activity and accelerated membrane deterioration, consistent with previously observed increases in RR and EL. PC3 was dominated by cutting resistance (CR), indicating that textural firmness represented an independent determinant

of quality. The positioning of the 0.5 mM SA treatment distant from other treatments along PC3 confirmed that this dose produced the highest cutting resistance, consistent with Table 3.

The correlation matrix further supported the multivariate structure revealed by the PCA analysis (Figure 5). A moderate positive correlation was observed between L* and hue ($r \approx 0.56$), indicating that brighter pods tended to retain greener color tones during storage. This relationship reflects the coordinated progression of color lightness and chlorophyll preservation. In addition, chroma exhibited a strong positive correlation with the browning index (BI) ($r \approx 0.70$), suggesting that increased color saturation was accompanied by intensified browning, likely due to parallel biochemical changes associated with senescence and pigment transformation. Respiration rate (RR) showed a strong negative correlation with weight loss – WL ($r \approx -0.79$), indicating that higher respiratory activity was not directly associated with greater water loss. This suggests that WL is more strongly governed by physical properties of the cuticle and epidermal structure rather than by metabolic intensity alone. Moreover, the positive correlation between RR and electrolyte leakage – EL ($r \approx 0.44$) confirmed that elevated metabolic activity was closely linked to membrane destabilization and increased cellular permeability. A moderate positive association between total soluble solids (TSS) and hue ($r \approx 0.58$) further indicated that pods with higher soluble solids tended to maintain greener coloration.

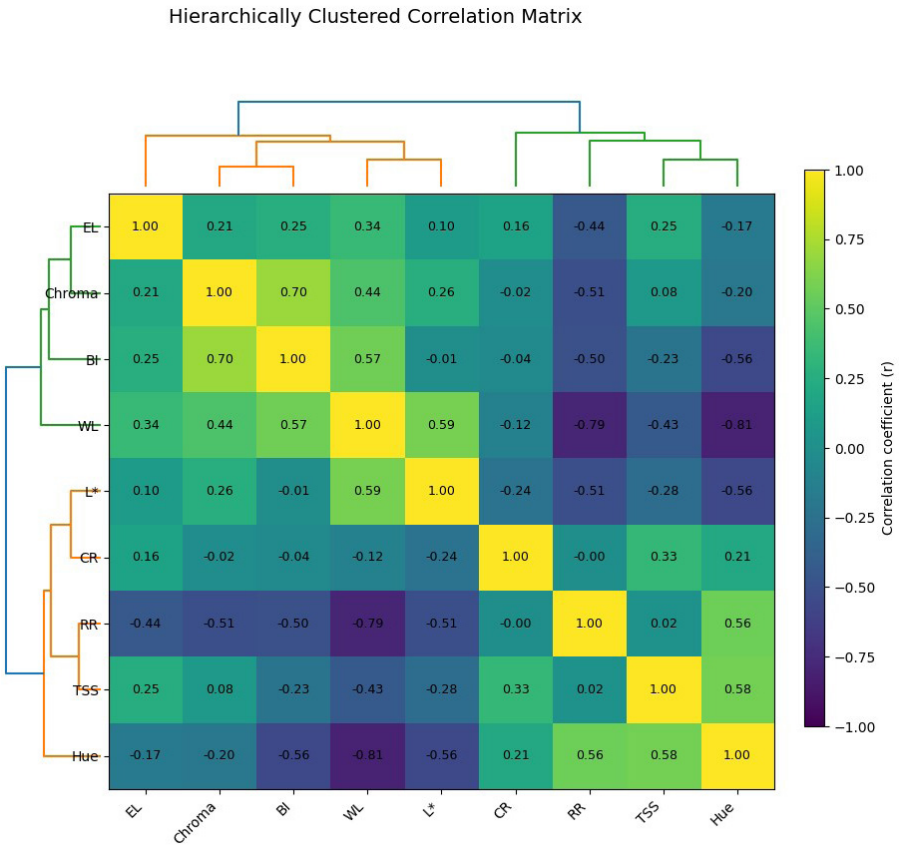
Figure 5. Correlation matrix of quality parameters in green beans treated with salicylic acid



Red circles indicate positive correlations, blue circles negative correlations, and circle size reflects the strength of each correlation

Heatmap and hierarchical clustering. The hierarchically clustered heatmap provided a clear visualization of these relationships and revealed three distinct parameter clusters (Figure 6). The first cluster consisted of chroma, BI, WL, and L*, representing visual and color-related quality attributes. This cluster closely corresponded to PC1 in the PCA, confirming that these parameters co-varied and jointly described overall visual quality changes. The second cluster included RR and EL, reflecting stress-related physiological responses; their close association indicates that increased respiration accelerated membrane degradation, in agreement with the stress axis represented by PC2. The third cluster comprised TSS and Hue, indicating a distinct subgroup related to compositional and pigment-related attributes, partially overlapping with visual quality but exhibiting independent behavior.

Figure 6. Hierarchically clustered correlation heatmap of quality attributes in green beans



The heatmap displays pairwise Pearson correlations among quality variables (WL, L*, EL, chroma, BI, RR, hue, CR and TSS) under different SA treatments. Positive correlations are shown in warm colors and negative correlations in cool colors, with dendrograms illustrating variable clustering patterns

Collectively, the PCA, correlation matrix, and heatmap analyses consistently demonstrated that salicylic acid (SA) applications exerted multidimensional and dose-dependent effects on postharvest quality of green beans. The 0.5 mM SA treatment emerged as the most advantageous dose by enhancing visual quality attributes (brightness and saturation) and supporting tissue firmness. In contrast, 1 mM SA intensified metabolic stress, as reflected by elevated respiration rate and electrolyte leakage, thereby limiting overall quality. The 2 mM SA treatment provided a more balanced and stable quality profile, without pronounced positive or negative deviations. These integrated findings emphasize that optimizing SA concentration is critical for preserving both physiological integrity and visual quality of green beans, and highlight the value of multivariate analytical approaches for interpreting complex postharvest responses.

CONCLUSION

This study demonstrates that salicylic acid exerts a clear dose-dependent influence on the postharvest physiology and visual quality of green beans. Among the tested concentrations, 0.5 mM SA was the most effective in maintaining color vibrancy, delaying browning, and enhancing tissue firmness – attributes strongly linked to consumer preference and market value. In contrast, 1 mM SA treatment induced higher respiration and electrolyte leakage, indicating elevated metabolic stress and reduced membrane stability. The 2 mM dose provided relatively stable quality preservation, particularly through reduced weight loss, but did not match the visual quality improvements observed at 0.5 mM. Multivariate analyses (PCA, correlation matrix, heatmap) clearly distinguished treatment responses and confirmed that color-related parameters co-varied closely with biochemical traits, whereas stress indicators (RR, EL) formed a separate cluster. PCA further revealed that quality variation was primarily explained by

visual attributes (PC1), followed by stress physiology (PC2) and texture firmness (PC3). Overall, the integrated findings indicate that 0.5 mM SA represents an optimal concentration for preserving postharvest quality by balancing physiological stability with attractive visual characteristics. These results highlight the importance of SA dose optimization for extending shelf life, delaying senescence, and improving the postharvest performance of green beans. Future research should explore combined treatments (e.g., SA + $CaCl_2$, SA + MAP) and molecular-level approaches to further elucidate underlying mechanisms. The multivariate analyses strengthened these conclusions by clearly separating visual quality attributes from stress-related physiological parameters, thereby confirming the dose-dependent responses observed in the univariate analyses. This integrated analytical approach provides a comprehensive understanding of postharvest quality dynamics and supports the selection of optimal salicylic acid concentrations for commercial cold storage applications.

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AUTHORS' CONTRIBUTIONS

R.K.: conceptualization, methodology, software, validation, formal analysis, investigation, data curation, writing – original draft, writing – review & editing, visualization. M.U.K.: conceptualization, methodology, software, writing – review & editing, visualization. S.G, M.D. and YG: formal analysis, investigation, visualization.

DATA AVAILABILITY

Data will be made available on request.

CONFLICT OF INTERESTS

The authors declare that they have no competing financial or non-financial interests that could have influenced the research, authorship, or publication of this article.

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RESEARCH PAPER

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EVALUATION OF THE EFFECT OF SODIUM ALGINATE COMBINED WITH THYME ESSENTIAL OIL ON THE POSTHARVEST SHELF LIFE OF WASHINGTON NAVEL ORANGE (*Citrus sinensis* cv. Washington Navel)

Hesamoddin Hamzeh[1], Rasool Etamadipoor[2], Mehrdad Babarabie[3]

ABSTRACT

Citrus fruits, belonging to the Rutaceae family, are among the most widely cultivated fruits worldwide. However, oranges are highly susceptible to postharvest quality deterioration during storage. Weight loss, firmness reduction, and biochemical changes are among the major factors limiting the shelf life of citrus fruits. This study aimed to evaluate the effects of sodium alginate edible coatings enriched with thyme essential oil on the postharvest quality of Washington navel oranges. Fruits were treated with sodium alginate alone or in combination with thyme essential oil and stored at 20 ±2°C and 65 ±5% relative humidity for 60 days. The results demonstrated that coated fruits exhibited significantly lower weight loss and better firmness retention compared with untreated fruits. In addition, the coatings maintained higher levels of titratable acidity, total soluble solids, total phenolic content, flavonoids, and ascorbic acid throughout storage, leading to enhanced antioxidant capacity. Furthermore, the combined application of sodium alginate and thyme essential oil effectively preserved cellular membrane integrity, as indicated by reduced ion leakage. Overall, sodium alginate coatings enriched with thyme essential oil can be considered a safe and effective strategy to delay physiological senescence and improve the postharvest quality of Washington navel oranges.

Keywords: alginate, thyme, preservation, antioxidants, oranges

INTRODUCTION

Citrus fruits (*Citrus* spp.), belonging to the Rutaceae family, are among the most widely cultivated fruits worldwide [Suri et al. 2022]. They are rich in various bioactive compounds, including flavonoids, essential oils, carotenoids, limonoids, and synephrine, which provide protection against various diseases such as cancer, inflammatory conditions, digestive disorders, and cardiovascular diseases [Lu et al. 2023]. Orange has gained significant importance due to its wide range of applications, including juice production, jam, confectionery, and extracts. Its consumption provides numerous nutritional benefits, including flavonoids, vitamin C, potassium, beta-carotene, and dietary fiber [Saini et al. 2022]. Washington navel orange (*Citrus sinensis* cv. Washington Navel) is one of the most important sweet orange varieties and is highly valued for its delicious taste, nutritional properties, and seedless nature [El-Khalifa et al. 2022]. This cultivar originates from the Bahia orange (Bahia), which was selected and cultivated in Brazil. Washington navel oranges are large, weighing between 200 and 500 grams, and have a thick peel

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[El-Gioushy and Eissa 2019]. Despite the high production volume of oranges, significant postharvest losses occur due to improper handling and storage methods [Barsha et al. 2021]. Preserving the quality and quantity of fruits and vegetables after harvest is crucial for economic sustainability.

A recent approach to extending shelf life, maintaining nutritional benefits, and preventing physical and textural deterioration of fruits involves the application of edible coatings [Ali et al. 2025]. These coatings form a thin, consumable layer on the fruit's surface, creating a barrier against moisture loss, oxygen exchange, and nutrient depletion [Wang et al. 2020, Kaur et al. 2024]. Edible coatings are an environmentally friendly innovation designed to maximize fruit quality and longevity [Prakash et al. 2020, Kaur et al. 2024].

Sodium alginate-based edible coatings have demonstrated significant potential in fruit preservation [Wang et al. 2019]. Sodium alginate and its derivatives exhibit several biological activities, including antioxidants, coagulating, antimicrobial, biocompatibility, wound healing, low toxicity, and tissue engineering effects [Nkede et al. 2024]. Additionally, sodium alginate is considered an ideal edible coating due to its biocompatibility, biodegradability, non-toxicity, physicochemical properties, rheological behavior, and film-forming ability. Dulta et al. [2022] examined the effect of sodium alginate (1%) and chitosan (0.5%) coatings enriched with nano-zinc oxide (0.5 g/L) on orange quality and found that the coatings significantly improved quality parameters compared to uncoated oranges [Dulta et al. 2022]. The coated samples exhibited lower rates of pH change, total soluble solids variation, and titratable acidity reduction. In another study, cherries coated with 3% sodium alginate demonstrated delayed weight loss, acidity reduction, softening, and color change [Chiabrando and Giacalone 2015]. Similarly, an experiment on strawberries revealed that a sodium alginate-calcium chloride edible coating effectively reduced respiration rate and transpiration while delaying pH increase and soluble solids accumulation. Additionally, the coating preserved the sensory properties of sliced strawberries, such as color and texture [Alharaty and Ramaswamy 2020].

Garden thyme (*Thymus vulgaris*), belonging to the Lamiaceae family, is widely recognized in traditional medicine for its expectorant, antitussive, bronchodilatory, antispasmodic, carminative, and diuretic properties [Borugă et al. 2014]. Thyme essential oil contains over 40% phenolic compounds, primarily thymol and carvacrol, which possess strong antimicrobial properties. Key components of thyme essential oil include thymol, carvacrol, linalool, γ-terpinene, p-cymene, β-myrcene, and terpinen-4-ol [Nadi et al. 2023]. In a study examining the effects of a sodium alginate (SA) and thyme essential oil (TEO) coating on fresh pistachios, treated samples exhibited lower weight loss and superior quality indices compared to untreated samples, with the highest sensory scores observed in the SA-TEO coated pistachios [Shakerardekani et al. 2021]. Fatemi et al. [2011] investigated the impact of natural essential oils, including thyme and peppermint, on controlling green mold and improving postharvest orange quality. Their results indicated that thyme essential oil exhibited strong antifungal properties against citrus fungal diseases. Similarly, Akbari et al. [2024] evaluated the effects of thyme essential oil on the microbial and physiological quality of green bell peppers, finding that it minimized peroxidase activity and respiration rate without adversely affecting texture, total phenolic content, antioxidant activity, pH, or soluble solids. This treatment contributed to the extended shelf life and improved postharvest quality of bell peppers.

Despite extensive research on sodium alginate and thyme essential oil-based edible coatings in postharvest fruit preservation, no study has specifically investigated the combined effect of sodium alginate and thyme essential oil coatings on the postharvest shelf life of Washington navel oranges. Therefore, this study aims to evaluate the application of a sodium alginate-thyme essential oil edible coating on the postharvest quality and shelf life of Washington navel oranges.

MATERIALS AND METHODS

Experimental design. This study was conducted as a factorial experiment based on a completely randomized design during the 2023–2024 period in the Plant Physiology Laboratory of the Department of Agriculture, Hormozgan University. The experimental treatments consisted of sodium alginate at two concentrations (1% and 2%) and thyme essential oil at two concentrations (150 and 300 mg L^{-1}), applied individually and in combination, along with an uncoated control. Each treatment was performed in three biological replicates, with three fruits per replicate. Measurements were carried out at three storage times (0, 30, and 60 days).

Fruit material and maturity. Washington navel oranges (*Citrus sinensis* L.) were harvested at commercial maturity from an orchard located in Kerman province, Iran. Fruits were selected based on uniform peel color, similar size and weight, and absence of visible defects or mechanical damage. To ensure homogeneity of fruit maturity, initial total soluble solids (TSS) and titratable acidity (TA) were measured at harvest (day 0), and only fruits with comparable initial quality attributes were used in the experiment.

Preparation and application of edible coatings. Sodium alginate (chemical formula: $C_6H_9NaO_7$) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Thyme essential oil (*Thymus vulgaris* L.) was obtained from Ayat Essences Company (Tehran, Iran). Edible coating solutions were prepared by dissolving sodium alginate in distilled water at concentrations of 1% and 2% (w/v) with continuous stirring until complete dissolution. Thyme essential oil was added to the alginate solutions at concentrations of 150 and 300 mg L^{-1} and homogenized thoroughly.

Prior to coating, fruits were washed with distilled water and air-dried at room temperature. Fruits were then immersed in the coating solutions for 2 min, allowed to drain, and dried at room temperature before storage.

Storage conditions. Coated and uncoated fruits were stored at 20 ±2 °C and 65 ±5% relative humidity for 60 days. Analyses were performed at the beginning of storage (day 0) and after 30 and 60 days of storage.

Measured traits. At the beginning of the experiment (T0), the following parameters were measured: fruit firmness, total soluble solids (TSS), ascorbic acid, titratable acidity (TA), total phenolic content, flavonoids, antioxidant capacity, and ion leakage. After 30 (T1) and 60 (T2) days, in addition to the mentioned traits, fruit decay and weight loss percentage were also evaluated.

Weight loss percentage. To determine the percentage of weight loss, the weight of all fruits in each container (three fruits together) was measured at the beginning of the experiment and again at 30 and 60 days using a digital scale with an accuracy of 0.01 g. Finally, weight loss percentage was calculated using Equation (1).

Equation 1:

$$\text{Weight loss (\%)} = [(\text{initial weight} - \text{final weight}) / \text{initial weight}] \times 100$$

Firmness. Fruit firmness was measured using a Lutron FS-1001 penetrometer equipped with an 8 mm cylindrical probe (Lutron Electronic Enterprise Co., Ltd., Taipei, Taiwan). Measurements were conducted on unpeeled fruits at two opposite equatorial positions, and the average value was recorded for each fruit. Firmness values were expressed in Newtons (N).

Total soluble solids (TSS). TSS content was determined using a digital refractometer (Prismatech PTRP100, Iran). A single drop of orange juice was placed on the device's prism, and the TSS value was recorded in Brix degrees [Ayala-Zavala et al. 2007].

Titratable acidity (TA). Titratable acidity was determined using the Ayala-Zavala method with slight modifications, and its value was calculated using Equation 2 [Ayala-Zavala et al. 2007].

Equation 2:

$$\text{TA} = \left(\frac{\text{volume of NaOH consumed} \times \text{normality of acid equivalent}}{\text{sample volume}}\right) \times 100$$

Ascorbic acid. Ascorbic acid (Vitamin C) was determined using the Etemadipoor method. A 100 µL sample of orange juice was added to a test tube containing 10 mL of 1% metaphosphoric acid and vortexed for 10 seconds. Then, 1000 µL (1 mL) of this mixture was transferred to 9 mL of indophenol solution and vortexed again for 10 seconds. The absorbance of the samples was read at 515 nm using an ELISA reader (BioTek Epoch 2TC Take3, BioTek Instruments, Inc., Winooski, VT, USA). In this experiment, 1% metaphosphoric acid was used as a blank [Etemadipoor et al. 2019].

Antioxidant capacity. To prepare the methanolic extract of the juice, a 1 : 3 ratio of orange juice to 85% methanol was used. Antioxidant capacity was measured using the DPPH free radical scavenging assay. In this experiment, 85% methanol was used as a blank, and the DPPH solution served as the control [Sheng et al. 2018]. The percentage of free radical inhibition was calculated using Equation (3).

Equation 3:

$$\text{Antioxidant capacity} = [(\text{AC} - \text{OD}) / \text{AC}] \times 100$$

where AC represents the absorbance of the control and OD represents the absorbance of the samples.

Total phenols. Total phenolic content was determined using the Folin-Ciocalteu reagent. A 150 µL sample of methanolic extract was transferred to a 2 mL microtube, and 750 µL of 10% Folin-Ciocalteu reagent (Merck KGaA, Darmstadt, Germany) was added. After 5 minutes, 600 µL of 7% sodium carbonate was added. The samples were incubated for 90 minutes in the dark on a shaker (SHAKER-M model, Noorsanat Azma Ferdows Co., Iran). The total

phenolic content was then measured at 760 nm using an ELISA reader (BioTek Epoch 2TC Take3, BioTek Instruments, Inc., Winooski, VT, USA) [Singleton and Rossi 1965].

Flavonoids. Flavonoid content was measured using the aluminum chloride ($AlCl_3$) colorimetric method. A 100 µL methanolic extract was placed in a 2 mL microtube, and 300 µL of 85% methanol was added. Then, 20 µL of 10% aluminum chloride and 20 µL of 1 M potassium acetate were added. Finally, 560 µL of distilled water was added, and the mixture was shaken for 30 minutes in the dark. The absorbance was then measured at 415 nm using an ELISA reader [Chang et al. 2002].

Ion leakage. Ion leakage was measured using the method described by Masoumi et al. [2010]. A 0.5 g sample of orange peel was placed in a 50 mL Falcon tube with 20 mL of distilled water and left for 24 hours. After 24 hours, the initial electrical conductivity (EC1) was measured using an EC meter (AD3000 model, ADWA Instruments, Szeged, Hungary). The samples were then incubated in a water bath at 100°C for one hour. After cooling to 25°C, the final electrical conductivity (EC2) was recorded. Ion leakage percentage was calculated using Equation (4).

Equation 4:

$$\text{Ion leakage} = (EC1 / EC2) \times 100$$

Sensory evaluation. Sensory evaluation was conducted as a preliminary descriptive assessment to compare the overall sensory quality of the samples during storage. The evaluation was performed by three trained evaluators from the Department of Food Science, who were familiar with the sensory attributes of citrus fruits. Before the evaluation, the assessors received training sessions focused on the identification and scoring of key sensory attributes, including appearance, color, aroma, texture, and overall acceptability. The training involved discussion of attribute definitions, use of reference samples, and calibration to ensure consistency among evaluators. Samples were coded with random three-digit numbers and presented in a randomized order under controlled laboratory conditions (ambient temperature and neutral lighting). The evaluators scored each attribute using a five-point hedonic scale, where 1 indicated very poor quality and 5 indicated excellent quality. The mean scores of the evaluators were used for statistical analysis.

Statistical analysis

All experimental data were analyzed using SAS software (version 9.4, SAS Institute Inc., Cary, NC, USA). Prior to statistical analysis, data were tested for normality using the Shapiro–Wilk test and for homogeneity of variances using Levene's test.

The effects of treatments, storage time, and their interactions were evaluated using factorial analysis of variance (ANOVA). When significant differences were detected, mean comparisons were performed using the least significant difference (LSD) test at a 5% probability level ($p \leq 0.05$). Graphs were prepared using appropriate statistical software.

RESULTS AND DISCUSSION

Effect of treatments on fruit skin firmness. Variance analysis of the data indicated that the effect of sodium alginate, garden thyme essential oil, storage time, and their interaction was significant at the 1% level (Table 1). The results of mean comparisons showed that after 30 days, the lowest firmness was observed in the control group, and the highest firmness at this time was observed in the As1E1 treatment. After 60 days of storage, the lowest firmness was seen in the control treatment, and the highest firmness was also observed in the As1E1 treatment (Figure 1). It appears that the treatments had a positive effect on the percentage of weight loss, which may be due to the role of sodium alginate in stabilizing the cell membrane. Sodium alginate enriched with essential oils significantly increases the shelf life of fruits. Sodium alginate containing essential oils acts as a barrier to reduce gas exchange, thus preventing tissue softening by slowing down respiration [Díaz-Mula et al. 2012]. A study found that grapes treated with a combination of sodium alginate and essential oils showed higher firmness compared to untreated samples [Wang et al. 2020]. In pineapple, fruit coated with sodium alginate along with lemongrass essential oil and sodium alginate enriched with antioxidants and olive oil showed higher firmness compared to the control group [Azarakhsh et al. 2014, Ramana Rao et al. 2016]. In some treatments, fruit firmness measured after 30 days of storage was slightly higher than that at harvest. This phenomenon, while seemingly counterintuitive, has been previously observed in edible coating studies [Rojas-Graü et al. 2008, Zhang et al. 2024]. The apparent increase can be attributed to reduced moisture loss from the peel surface, leading to a drier and more rigid outer

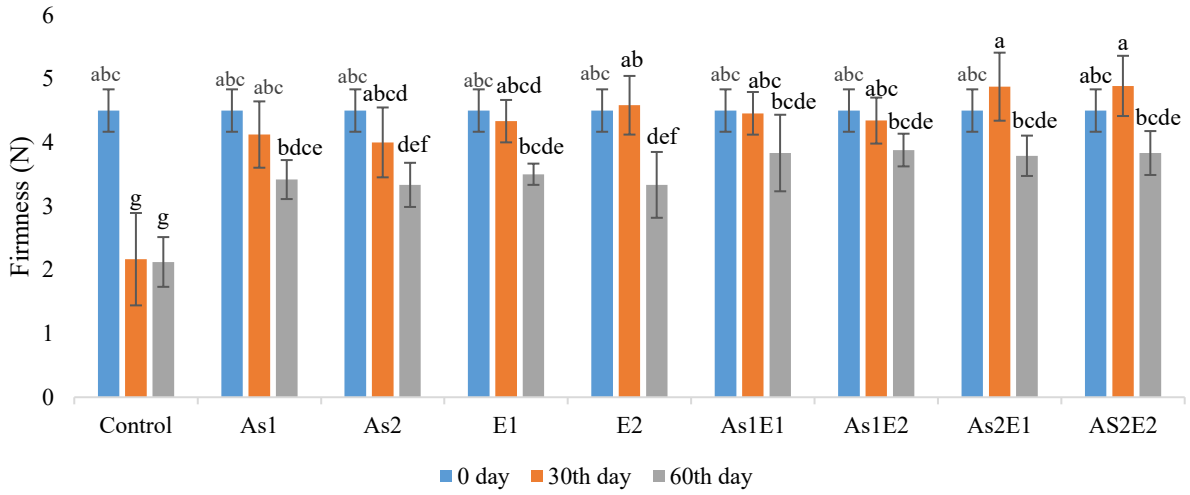
layer, as well as structural changes induced by the alginate-calcium crosslinking network that increase the peel's resistance to penetration [Zhang et al. 2024]. Importantly, this reflects changes in peel physical properties rather than an actual increase in flesh firmness. Similar observations have been reported in other fruits coated with hydrocolloid-based edible coatings, where surface drying and coating matrix formation resulted in higher penetrometer resistance during early storage [Adjouman et al. 2018].

Table 1. Analysis of variance for some traits evaluated in the study

Source of variation	df	Firmness	Ion leakage	Weight loss	TSS	TA
Treatments	8	9.90**	268.56**	55.73**	1.11**	0.23**
Time	2	812.57**	3024.54**	123967.26**	150.57**	0.93**
Treatment × time	16	3.11**	160.79**	53.59**	0.64*	0.11**
Error	54	0.49	21.28	2.5	0.32	0.16
Total error	80	–	–	–	–	–
CV (%)	–	3.15	6.42	1.99	3.85	16.89

*significant at 5% level,**significant at 1% level

Figure 1. Effects of sodium alginate and thyme essential oil treatments on fruit firmness during storage (0, 30, and 60 days). Values represent mean ± standard error (SE; n = 3). Means followed by the same letters are not significantly different at p ≤ 0.05 according to the LSD test



Effect of treatments on ion leakage. According to the results in the analysis of variance table, the effect of the experimental treatments and their interaction on the ion leakage trait was significant at the 1% level (Table 1). The results of mean comparisons showed that the highest ion leakage was observed in the control treatment at 60 days of storage, with significant differences compared to all other treatments at 30 days of storage, except for the control. The lowest ion leakage was observed in the As2E2 treatment (Figure 2). Ion leakage is an indicator used to assess the integrity of the cell membrane and its permeability. Maintaining the structural integrity of cells during the fruit ripening process is crucial for cell survival. Increased ion leakage is mainly due to cell degradation and increased membrane permeability [Sinha et al. 2022]. However, the sodium alginate coating with garden thyme essential oil, both individually and in combination, was effective in reducing ion leakage. Similar results were observed in peaches coated with sodium alginate along with rhubarb extract [Li et al. 2019].

Effect of treatments on weight loss. According to the analysis of variance table, the effect of sodium alginate, garden thyme essential oil, storage time, and their interactions on the percentage of weight loss in oranges was significant at the 1% level (Table 1). The results of mean comparisons also showed that after 30 days of storage, the

control sample had the highest weight loss, and the lowest percentage of weight loss was observed in the As2E1 treatment. In the samples after 60 days of storage, the highest weight loss was observed in the control, and the lowest weight loss was observed in the As2E2 treatment (Figure 3). The edible coating of sodium alginate acts as a semi-permeable barrier to limit water exchange. This reduces moisture loss, respiration rate, oxidative reactions, and delays the physiological aging of fruits [Wang et al. 2020]. Garden thyme essential oil was added to sodium alginate to delay moisture loss and preserve other sensory characteristics. Adding essential oils and surfactants improves the water retention ability of the coating [Shakerardekani et al. 2021]. The results of this study are consistent with the findings of Shakerardekani et al. [2021] on pistachio (*Pistacia vera*), Utami et al. [2023] on strawberries (*Fragaria* sp.), Linh et al. [2024] on Darabi (*Citrus maxima*), and Dulta et al. [2022] on oranges (*Citrus sinensis* L.).

Figure 2. Effects of sodium alginate and thyme essential oil treatments on ion leakage during storage (0, 30, and 60 days). Values represent mean ± standard error (SE; n = 3). Means followed by the same letters are not significantly different at $p \leq 0.05$ according to the LSD test

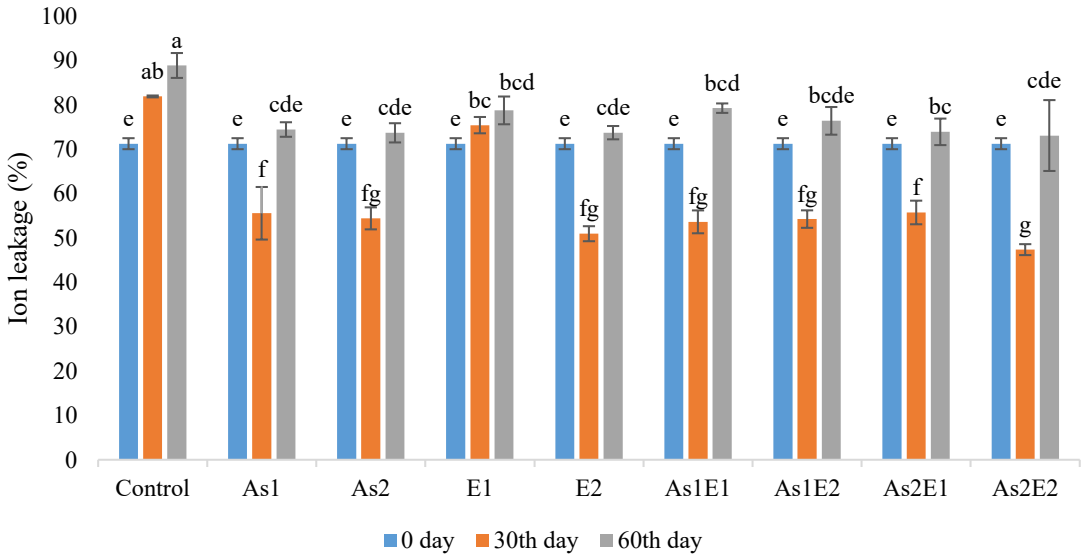
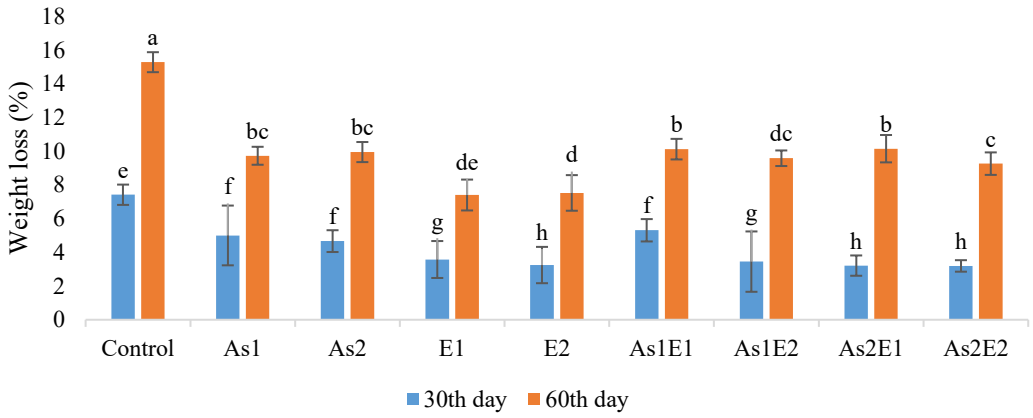


Figure 3. Effects of sodium alginate and thyme essential oil treatments on weight loss percentage during storage (30 and 60 days). Values represent mean ± standard error (SE; n = 3). Means followed by the same letters are not significantly different at $p \leq 0.05$ according to the LSD test



Effect of treatments on total soluble solids (TSS). Based on the results shown in the analysis of variance table, the effect of sodium alginate, garden thyme, and storage time was significant at the 1% level, while their interactions were significant at the 5% level on the total soluble solids (TSS) content (Table 1). The results of mean comparisons indicated that the control treatment at 60 days of storage had the highest TSS, while the lowest TSS was observed in the As1E1 treatment. In the 30-day storage samples, the control also had the highest TSS (Figure 4). The treatments showed a slight increase in TSS compared to the control, which might be due to the formation of a physical barrier by the coating materials, reducing transpiration losses. The highest TSS in the control may be attributed to faster metabolic activities through respiration and transpiration in the control compared to the other treatments. Similar observations have been reported by Rokaya et al. [2016] for different mandarin orange varieties and Thapa et al. [2020] for sweet oranges.

Figure 4. Effects of sodium alginate and thyme essential oil treatments on total soluble solids during storage (30 and 60 days). Values represent mean ± standard error (SE; n = 3). Means followed by the same letters are not significantly different at $p \leq 0.05$ according to the LSD test

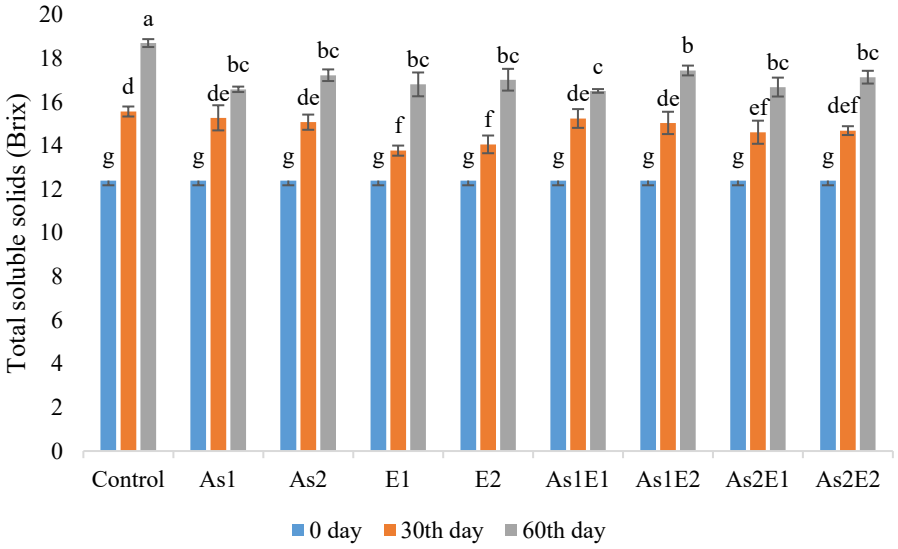
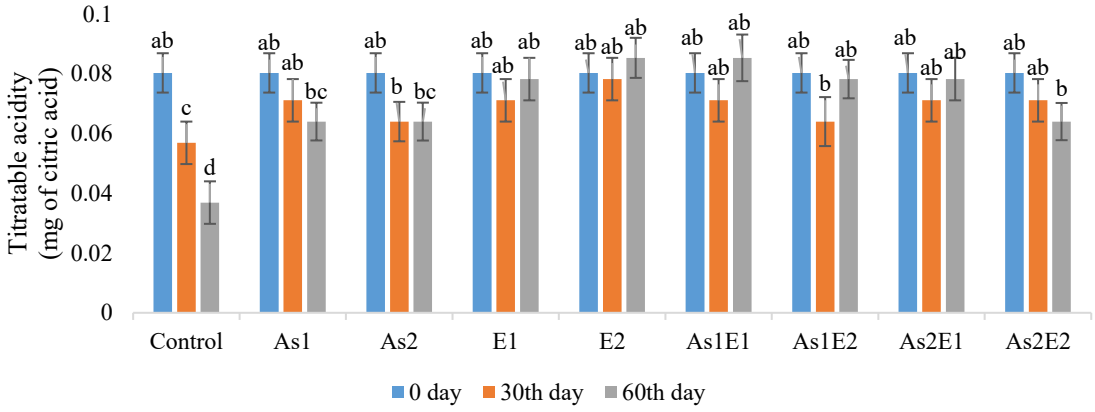


Figure 5. Effects of sodium alginate and thyme essential oil treatments on TA during storage (30 and 60 days). Values represent mean ± standard error (SE; n = 3). Means followed by the same letters are not significantly different at $p \leq 0.05$ according to the LSD test



Effect of treatments on titratable acidity (TA). According to the results from the analysis of variance, the effect of sodium alginate, garden thyme essential oil, storage time, and the interaction between the treatments and time were significant at the 1% level on titratable acidity (TA) – as shown in Table 1. Based on the results of mean comparisons, the highest titratable acidity was observed in the E2 and As1E1 treatments, which showed a signif-

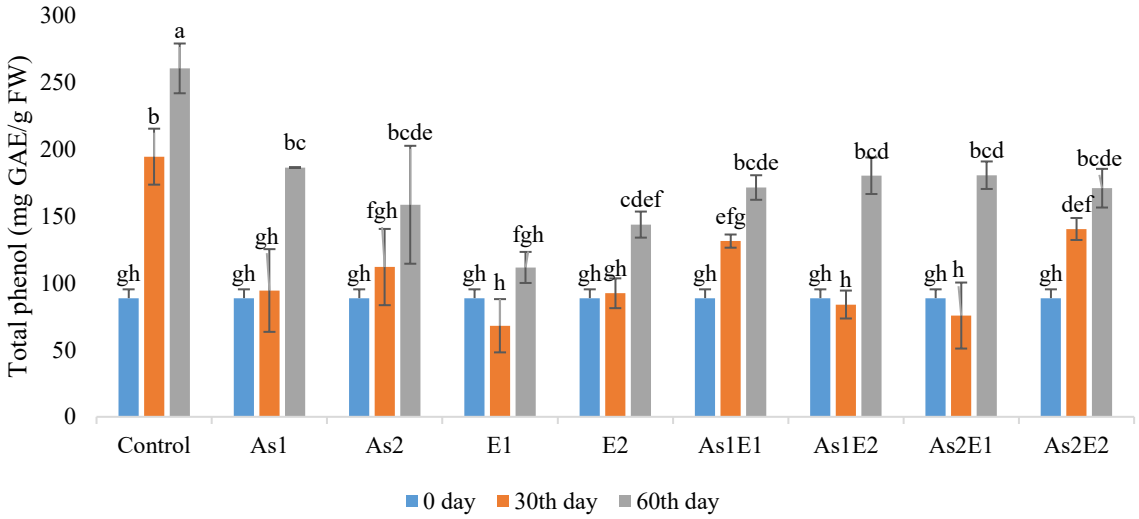
icant difference compared to the control at both 30 and 60 days of storage (Figure 5). In this study, the titratable acidity in all coated treatments was lower than in the control. This might be due to reduced acid utilization during respiration in fruits treated with sodium alginate and garden thyme essential oil, while faster acid utilization during respiration was observed in the control fruits during storage. Rokaya et al. [2016] and Khorram et al. [2017] reported the highest TA in the control compared to fruits treated with different coating materials in mandarin and Kinnow mandarin, respectively [Rokaya et al. 2016, Khorram et al. 2017]. Although titratable acidity (TA) values at 60 days of storage were higher in some treatments compared to the initial harvest, this increase should not be attributed to heterogeneity of fruit maturity at harvest. All fruits were harvested at the same commercial maturity stage. The observed retention or increase in TA during storage can be explained by the reduced respiration rate and delayed organic acid degradation in coated fruits. Similar behavior has been reported in previous studies, where edible coatings slowed metabolic processes and preserved organic acids during storage [Diaz-Mula et al. 2012, Chiabrando and Giacalone 2015].

Table 2. Analysis of variance for some evaluated traits in the study

Source of variation	df	Sensory evaluation	Total phenols	Flavonoids	Antioxidant capacity	Ascorbic acid
Treatments	8	0.76*	1627.59**	0.23**	18.71**	11.43**
Time	2	6.02**	70370.73**	7.96**	6296.11**	1631.38**
Treatment × time	16	0.52**	1700.03**	0.14**	11.29**	10.23**
Experimental Error	54	0.47	780.02	0.22	0.4	3.54
Total error	80	–	–	–	–	–
CV (%)	–	16.8	22.49	19.41	1.82	1.99

*significant at 5% level,**significant at 1% level

Figure 6. Effects of sodium alginate and thyme essential oil treatments on total phenol during storage (30 and 60 days). Values represent mean ± standard error (SE; n = 3). Means followed by the same letters are not significantly different at $p \leq 0.05$ according to the LSD test

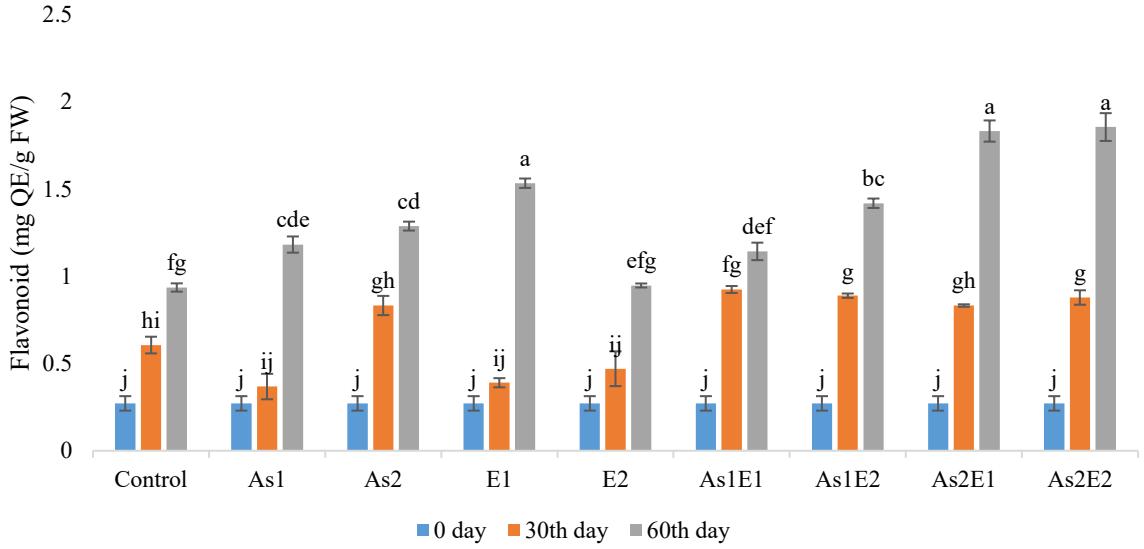


Effect of treatments on total phenol content. The results of the analysis of variance showed that the effect of sodium alginate and garden thyme at the 5% level, as well as storage time and the interaction of time and treatments, was significant at the 1% level for total phenol content (Table 2). The mean comparison results indicated that in the samples after 30 days of storage, the highest phenol content was observed in the control treatment, and the lowest phenol content was in the E1 treatment. In the samples after 60 days, the highest total

phenol content was observed in the control, while the lowest total phenol content was observed in the garden thyme treatment with a concentration of 150 mg/L (Figure 6). Edible coatings reduce the loss of polyphenols and maintain a higher antioxidant capacity during post-harvest storage of cherries [Díaz-Mula et al. 2012]. Studies on plums and apricots have reported that coatings reduce the ripening rate, thereby delaying the onset of aging and reducing cellular structural degradation. Additionally, coatings reduce respiration, decrease the available oxygen for metabolic activities in the fruit, and consequently reduce the activity of phenol oxidase and peroxidase [Ghasemnezhad et al. 2010, Kumar et al. 2017, Thakur et al. 2018]. A continuous increase in phenolic compounds was observed in cherries coated with alginate during cold storage. Thus, the coating allows for the accumulation of phenolic compounds throughout storage without any reduction [Díaz-Mula et al. 2012].

Effect of treatments on total flavonoids. According to the results of the analysis of variance, the effect of sodium alginate, garden thyme, storage duration, and their interaction was significant at the 1% level for flavonoid content (Table 2). The mean comparison results showed that in the samples after 30 days of storage, the highest flavonoid content was observed in the As1E1 treatment, while the lowest flavonoid content was in the As1 treatment. In the samples after 60 days of storage, the highest flavonoid content was observed in the As2E2 treatment, and the lowest flavonoid content was in the E2 treatment (Figure 7). Research has shown that flavonoid content can be influenced by fruit ripening, post-harvest treatments, and extraction processes [Tai et al. 2014, Addi et al. 2022]. In the present study, the results showed that flavonoid content increased over time. This might be due to the increase in flavonoid levels during storage, which enhances antioxidant properties and helps combat oxidative stress. Additionally, sodium alginate coating combined with garden thyme essential oil maintains flavonoid content by controlling the activity of the mentioned enzymes. These results align with studies on flavonoids in citrus fruits [Díaz-Mula et al. 2012, Thakur et al. 2018, Riva et al. 2020, Addi et al. 2022].

Figure 7. Effects of sodium alginate and thyme essential oil treatments on total flavonoid during storage (30 and 60 days). Values represent mean ± standard error (SE; n = 3). Means followed by the same letters are not significantly different at $p \leq 0.05$ according to the LSD test



Effect of treatments on antioxidant capacity and ascorbic acid content. The results from the analysis of variance indicated that the effect of sodium alginate, garden thyme essential oil, storage duration, and their interaction on the antioxidant capacity of the fruits was significant at the 1% level (Table 2). The mean comparison results showed that with the increase in storage time, the antioxidant capacity also increased, and at each time point, the control sample differed significantly from the other treatments (Figure 8). According to the variance table, the effect of sodium alginate, garden thyme essential oil, storage duration, and their interaction on ascorbic acid (vitamin C) content in oranges was significant at the 1% level (Table 2). The mean comparison results indicated that after 60 days, the highest ascorbic acid content was observed in the As2E2 treatment, while the lowest content was in the control sample. For the 30-day storage samples, no significant differences were observed among the treatments (Figure 10).

Figure 8. Effects of sodium alginate and thyme essential oil treatments antioxidant capacity during storage (30 and 60 days). Values represent mean ± standard error (SE; n = 3). Means followed by the same letters are not significantly different at $p \leq 0.05$ according to the LSD test

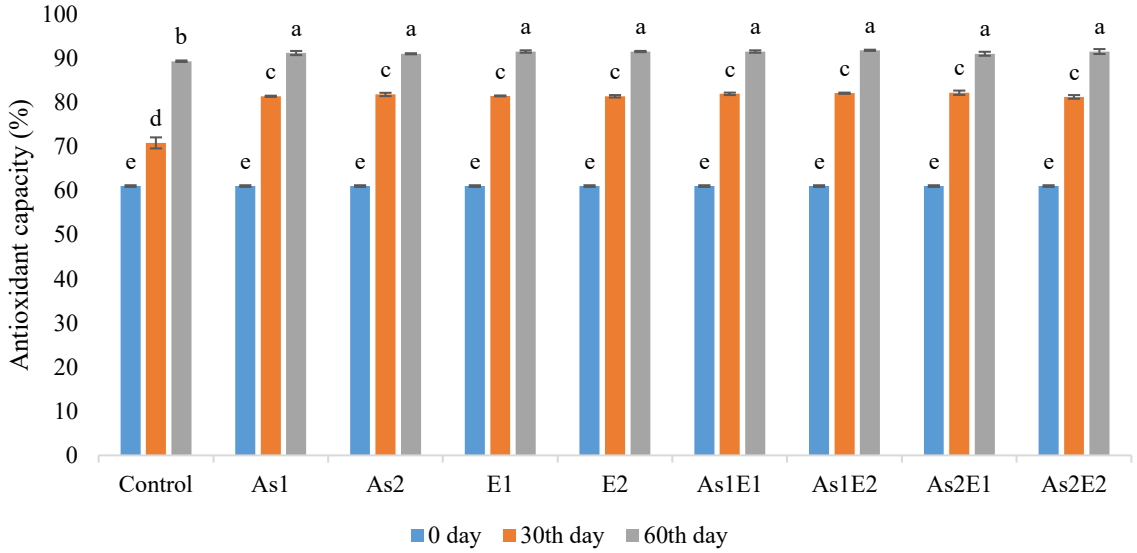
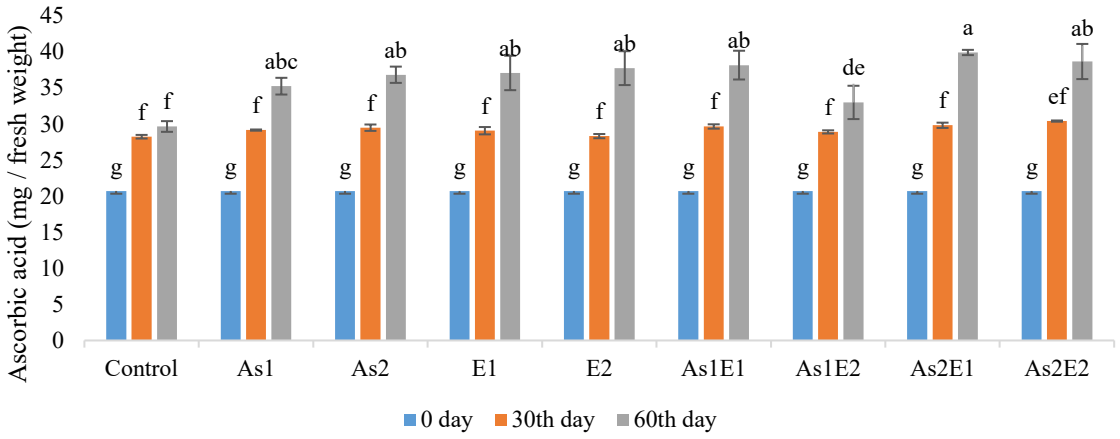


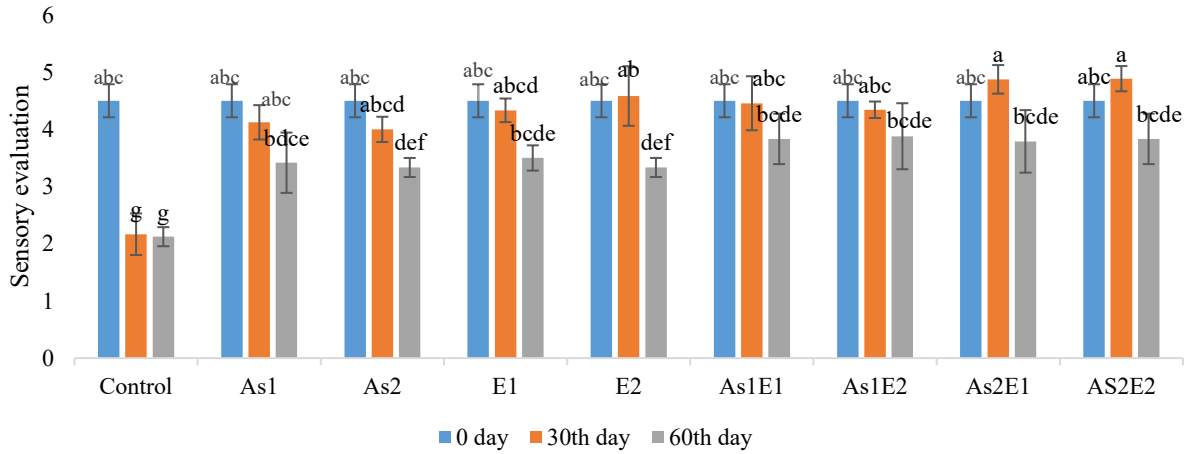
Figure 9. Effects of sodium alginate and thyme essential oil treatments on ascorbic acid during storage (30 and 60 days). Values represent mean ± standard error (SE; n = 3). Means followed by the same letters are not significantly different at $p \leq 0.05$ according to the LSD test



Fruits are rich in minerals, vitamins, and antioxidants that protect against cancer and cardiovascular diseases. However, improper storage of fruits has adverse effects on their quality, such as browning, off-flavors, loss of soluble solids, and decreased antioxidant activity [Barzegar et al. 2018]. Rahemi et al. [2023] reported that during storage, ascorbic acid is highly susceptible to degradation due to oxidation compared to other nutrients. By maintaining membrane stability, the binding of free radicals and reactive oxygen species to the membrane surface can be prevented, thereby preserving antioxidants such as ascorbic acid. The reduction in antioxidant capacity of the fruits during storage can be explained by the fact that ascorbic acid is converted by the enzyme ascorbate oxidase into dehydroascorbate, and by phenol oxidase [Linh et al. 2024]. A study on cherries showed that sodium alginate coating was effective in preserving antioxidants in cherries, with an increase in anthocyanins, phenolics, delayed loss of titratable acidity, and ascorbic acid content during 21 days of storage at 4°C. It was also reported that sodium alginate coating significantly reduced active oxygen levels, decreased the activity of defense enzymes, and preserved total soluble solids, titratable acidity, and ascorbic acid content, which resulted in increased antioxidant

activity [Chiabrando and Giacalone 2015]. It was also reported that sodium alginate edible coatings combined with essential oils exhibited higher antioxidant activity. Similarly, sodium alginate coatings enriched with citral and eugenol helped preserve antioxidant activity, anthocyanins, and phenolics in raspberries and fresh apples during refrigeration storage. Sodium alginate, by creating a semi-permeable barrier around the fruit and inhibiting ethylene production, helps preserve the antioxidant activity of fruits [Guerreiro et al. 2016, Wang et al. 2020].

Figure 10. Effects of sodium alginate and thyme essential oil treatments on sensory evaluation during storage (30 and 60 days). Values represent mean ± standard error (SE; n = 3). Means followed by the same letters are not significantly different at $p \leq 0.05$ according to the LSD test



Effect of treatments on sensory evaluation. The results from the analysis of variance table indicated that the treatments of sodium alginate, garden thyme, and their combination were significant at the 5% level, while storage time and their interaction were significant at the 1% level for sensory evaluation (Table 2). The results of mean comparisons showed that the lowest score was observed in the control treatment at both 30 and 60 days of storage, while the highest score was observed in the As2E2 treatment at 30 days of storage, with a significant difference compared to the control (Figure 10). The agricultural and food sectors are required to evaluate the organoleptic properties of oranges through sensory analysis. This type of analysis is related to consumer perception, as food is evaluated through the senses using organoleptic properties such as appearance, smell, aroma, texture, and taste [Villaseñor-Aguilar et al. 2024]. In this experiment, the sensory properties of the oranges were evaluated using a panel test. The results showed that sensory scores decreased over time, and the application of treatments at all levels improved the overall quality of the samples. Furthermore, the results indicated that the combined treatments of sodium alginate and garden thyme essential oil were the most effective. These results are consistent with findings from research by Megha et al. [2023] on pears, Fatemi et al. [2011] on oranges, and Shabanian et al. [2015] on oranges.

CONCLUSION

Washington navel oranges, although a non-climacteric fruit, have a high rate of perishability. One of the strategies to increase shelf life in postharvest processes is treating fruits with edible coatings. In the present study, sodium alginate coatings combined with garden thyme essential oil effectively preserved the physicochemical and antioxidant quality of Washington navel oranges during storage. The treatments reduced quality deterioration, as evidenced by lower firmness loss, reduced ion leakage, and better retention of total soluble solids, titratable acidity, phenolic compounds, flavonoids, and ascorbic acid.

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CONFLICT OF INTEREST

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

DATA AVAILABILITY

The data generated or analyzed during this study are included in this published article.

AUTHORS' CONTRIBUTIONS

H.H.: conducting laboratory work, providing fruits. R.E.: selecting the topic, leading and guiding the team, analyzing the data. M.B.: writing the article.

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RESEARCH PAPER

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BIOTECHNOLOGICAL MODULATION OF METABOLITE PROFILES IN *Ganoderma resinaceum* CULTIVATED ON AGRICULTURAL RESIDUES

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ABSTR ACT

Ganoderma resinaceum is a medicinal macrofungus recognised for its diverse pharmacologically active metabolites, including amino acids, organic acids, and polysaccharides with antioxidant, anti-inflammatory, and anticancer potential. This study aimed to elucidate how substrate formulation based on agricultural residues can modulate the metabolic composition of *G. resinaceum* under controlled cultivation conditions. Nine substrate mixtures were prepared using chickpea (*Cicer arietinum*), pea (*Pisum sativum*), and poppy (*Papaver somniferum*) stalks, as well as corncobs (*Zea mays*), in combination with beech sawdust and wheat bran. The fruiting bodies were analysed for amino acid, organic acid, and sugar profiles using high-performance liquid chromatography (HPLC). The results demonstrated that substrate composition markedly influenced metabolite accumulation. Chickpea- and pea-based substrates promoted the biosynthesis of key amino acids, whereas poppy stalk affected organic acid balance, and corncob formulations modified sugar metabolism. The findings indicate that metabolite production in *G. resinaceum* is strongly substrate-dependent, reflecting both nutrient availability and biochemical adaptability of the fungus. Overall, the study highlights a sustainable biotechnological approach to enhance bioactive metabolite production through tailored substrate design. The outcomes provide a foundation for future optimisation of *G. resinaceum* cultivation toward pharmaceutical and nutraceutical applications.

Keywords: *Ganoderma resinaceum*, agricultural residues, substrate optimization, metabolite profiling, amino acids, fungal biotechnology

INTRODUCTION

Ganoderma species have been used in traditional medicine for centuries, particularly across East Asia. These mushrooms contain substantial amounts of peptides, proteins, and minerals, making them an important nutritional source [Paliya et al. 2014, Rašeta et al. 2020]. Beyond their nutritional value, *Ganoderma* species are rich in bioactive compounds that have been widely investigated for their antioxidant, anti-inflammatory, and anticancer properties [Wachtel-Galor et al. 2011, Kozarski et al. 2020, Sułkowska-Ziaja et al. 2023a]. However, despite extensive research on various *Ganoderma* species, studies focusing specifically on the bioactive composition of *Ganoderma resinaceum* remain limited.

Ganoderma species are known to contain a wide spectrum of bioactive molecules, with more than 600 chemical compounds identified to date, including alkaloids, terpenoids, nucleobases, nucleosides, and polysaccharides

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[Paliya et al. 2014, Chen et al. 2018, Ren et al. 2021], proteins [Obodai et al. 2017, Sułkowska-Ziaja et al. 2023b], flavonoids and phenolic acids [Gong et al. 2019, Ahmad et al. 2021], as well as steroids and triterpenes [Niu et al. 2007, Wang et al. 2020, Galappaththi et al. 2023]. Among these, triterpenes are considered the principal pharmacologically active constituents [Gong et al. 2019, Sułkowska-Ziaja et al. 2023b]. Importantly, the chemical profile of *Ganoderma* mushrooms can vary significantly depending on species, cultivation conditions, substrate composition, developmental stage, tissue type, and storage environment [Wang et al. 2020, Amiri-Sadeghan et al. 2022].

In Türkiye, agricultural production generates substantial quantities of crop residues that may serve as valuable raw materials for fungal cultivation. According to FAO statistical data, the annual production in 2023 reached approximately 6.5 million tonnes of maize (*Zea mays*), 580 thousand tonnes of chickpea (*Cicer arietinum*), 235 thousand tonnes of pea (*Pisum sativum*), and 90 thousand tonnes of poppy (*Papaver somniferum*). These figures highlight the considerable amount of residual biomass available from chickpea stalk, pea stalk, poppy stalk, and maize cobs in Türkiye [FAO 2024]. Such residues represent an underutilised yet promising resource for developing sustainable substrate formulations in mushroom biotechnology.

Although *Ganoderma* species are widely recognized for their medicinal and economic value, their cultivation in Türkiye remains limited and is currently restricted to small-scale production by a few enterprises. Consequently, most *Ganoderma*-based products, including teas, capsules, powders, extracts, and functional formulations, are largely imported. In contrast, global production of *Ganoderma* – particularly *Ganoderma lucidum* – has increased substantially in recent years, reaching approximately 110,000–120,000 tons annually, with China accounting for the majority of production (around 70–85%) [FAO 2024, Bennett 2026]. The global market value of *Ganoderma*-based products has been estimated to exceed USD 2–4 billion, driven by increasing demand for nutraceuticals and functional foods [Andersen 2026]. Major producing countries include China, South Korea, Japan, the United States, and India, where *Ganoderma* cultivation is well established both for pharmaceutical and functional food industries. These global trends highlight the significant economic potential of *Ganoderma* cultivation and underline the importance of developing locally adapted production strategies in Türkiye.

The use of agricultural by-products as substrates for mushroom cultivation offers multiple advantages, including local availability, cost-effectiveness, and environmental sustainability [Chang and Miles 2004]. Optimising substrate composition can enhance both yield and the synthesis of bioactive metabolites in cultivated fungi [Royse et al. 2017]. Similar to other *Ganoderma* species, *G. resinaceum* produces amino acids, organic acids, and polysaccharides; however, detailed information on its specific metabolite composition remains scarce [Elisashvili 2012, Stojkovic et al. 2014, Rashad et al. 2019].

Therefore, the present study aimed to investigate the potential use of selected agricultural residues as cultivation substrates for *G. resinaceum* and to evaluate their effects on its biochemical composition. By identifying how substrate composition influences the biosynthesis of amino acids, organic acids, and sugars, this work provides novel insights into the biotechnological modulation of metabolite production in *G. resinaceum*. Furthermore, the findings contribute to the development of sustainable and value-added bioprocesses for the large-scale cultivation of medicinal fungi, supporting future applications in pharmaceutical biotechnology and functional food production.

MATERIAL AND METHODS

Fungal material

The spawn of *Ganoderma resinaceum* used in this experiment was obtained from the Atatürk Horticultural Central Research Institute (Yalova, Türkiye). The strain employed in the study was designated as isolate 44.3 (collection no. GBK035), originally collected on 30 August 2008 from a horse chestnut (*Aesculus hippocastanum*) stump in Yalova. The culture is currently maintained in the institutional fungal collection.

Substrate formulation and analysis

Substrate formulations were prepared using stalks of pea (*Pisum sativum*), poppy (*Papaver somniferum*), and chickpea (*Cicer arietinum*), as well as corn (*Zea mays*) cobs, beech (*Fagus orientalis*) sawdust, and wheat (*Triticum aestivum*) bran, resulting in a total of nine different media compositions (Table 1). Agricultural wastes were reduced to a particle size range of 1–2 cm. Substrate components were weighed, moistened, and homogenized according to the formulations (Table 1). Beech sawdust and wheat bran were selected as the control substrate formulation, as they are among the most commonly used lignocellulosic substrates in the commercial cultivation of *Ganoderma* species [Chang and Miles 2004, Amiri-Sadeghan et al. 2022].

Table 1. Composition of different substrate formulations used in the cultivation experiments of *Ganoderma resinaceum*

Substrate	Content
S1	40% beech sawdust + 40% chickpea stalk + 20% bran
S2	60% beech sawdust + 20% chickpea stalk + 20% bran
S3	40% beech sawdust + 40% pea stalk + 20% bran
S4	60% beech sawdust + 20% pea stalk + 20% bran
S5	40% beech sawdust + 40% poppy stalk + 20% bran
S6	60% beech sawdust + 20% poppy stalk + 20% bran
S7	40% beech sawdust + 40% corncob + 20% bran
S8	60% beech sawdust + 20% corncob + 20% bran
S9 (Control)	80% beech sawdust + 20% bran

Nitrogen (N) content of dried and ground substrate samples was determined using the Kjeldahl method [Kacar 1972], while moisture and ash contents were analyzed according to AOAC [2019]. Carbon (C) content was estimated as 50% of organic matter [Cormican and Staunton 1991], and the C : N ratio was calculated.

Cultivation and fruiting

The substrates were moistened to approximately 60%, and pH was adjusted to 8.0–8.5 by adding lime prior to sterilisation. Each prepared substrate was packed into 1.5 kg heat-resistant polypropylene bags, into which a plastic aeration tube was inserted centrally. The bags were sealed with rings and lids, then sterilised at 121 °C and 1.2 atm for 120 min. After cooling to room temperature, 75 g of spawn was inoculated into each bag. Incubation was carried out in a controlled room at 25–26 °C and 65–70% relative humidity. Following complete mycelial colonization (18–26 days after spawning), the bags were transferred to the cultivation room and opened to initiate fruiting. Environmental conditions were maintained at 25–26 °C, 70–80% relative humidity, and a light intensity of 400 lux for 10 h day^{-1}. Fruiting bodies were harvested at maturity (51–53 days after spawning), when the margins of the basidiocarps turned reddish and reached the desired size (Figure 1). Mature fruiting bodies of suitable size were harvested and used for subsequent analyses.

Preparation of fruiting body samples

Fruiting bodies of *G. resinaceum* from each treatment were washed with tap water, followed by 0.1 N HCl and deionised water, and then blotted dry with filter paper. Samples were oven-dried at 65 °C for 48–72 h until constant weight was achieved, and subsequently ground using a laboratory mill (Spice and Herb Grinder, model IC-25B) to pass through a 20-mesh sieve. The resulting powders were used for subsequent analyses.

Amino acid, organic acid and sugar analysis

Amino acid and organic acid analyses of dried fruiting body samples were performed according to the methods described by Olgun et al. [2016], and Henderson et al. [1999].

For amino acid analysis, 5 mg of dried fruiting body sample was mixed with 5 mL of 0.1 N HCl. The mixtures were homogenized using an IKA Ultra Turrax D125 Basic homogenizer and incubated at 4 °C for 12 h. The homogenized samples were vortexed and centrifuged at 1200 rpm for 50 min. The resulting supernatants were filtered through 0.22 µm Millex Millipore filters and transferred to vials for high-performance liquid chromatography (Agilent, 1200 HPLC) analysis. Amino acid concentrations were determined after 26 min of HPLC derivatization and expressed as pmol µL^{-1}.

For organic acid analysis, 5 mg of homogenized fruiting body sample was extracted with 10 mL of deionized water. The extracts were centrifuged at 1200 rpm for 50 min, filtered through 0.22 µm Millex Millipore filters, and transferred to vials. Organic acids were analyzed using an Agilent 1200 HPLC system equipped with a Zorbax Eclipse-AAA column (4.6 × 250 mm, 5 µm). Detection was carried out at 220 nm using a UV detector. The flow rate was set at 1 mL min^{-1}, with the column maintained at 25 °C. Organic acid contents were determined using 25 mM potassium phosphate (pH 2.5) as the mobile phase and expressed as ng µg^{-1}.

Figure 1. Different cultivation phases of *Ganoderma resinacium*. A. Mycelium growing phase. B. Primordia phase. C. Basidiocarp

Sugar analysis of dried fruiting body samples was performed using Agilent 1200 HPLC coupled with evaporative light scattering detection (ELSD), following the method modified by Ma et al. [2014]. Results were expressed as percentages.

Statistical analysis

Experimental data were analysed using the JMP statistical software package (Version 5.0, SAS Institute Inc., Cary, NC, USA) based on a randomised complete block design in triplicate. Analysis of variance (ANOVA) was performed to assess differences among treatments, and mean comparisons were conducted using Tukey's honestly significant difference (HSD) test at a significance level of $P < 0.01$.

RESULTS

Substrate physicochemical properties

The physicochemical characteristics of the different substrate formulations are presented in Table 2. Following sterilisation, pH values ranged from 5.67 (S8) to 6.30 (S3), indicating a slightly acidic character suitable for fungal growth. Moisture content varied between 58.14% (S1) and 63.74% (S2), reflecting optimal water-holding capacity for mycelial colonisation. Carbon and nitrogen contents ranged from 45.59% (S1) to 48.13% (S8) and 0.77% (S9) to 1.52% (S3), respectively. The C/N ratio varied from 30.14 (S3) to 62.12 (S9), while ash content, representing the mineral fraction of the substrate, ranged between 3.74% (S8) and 8.82% (S1).

Table 2. Chemical composition of different substrate formulations

Substrate	pH	Moisture (%)	Carbon – C (%)	Nitrogen – N (%)	C/N	Ash (%)
S1	5.75 f	58.14 i	45.59 i	1.18 b	38.63 d	8.82 a
S2	5.68 g	63.74 a	47.17 f	1.02 c	46.24 c	5.65 d
S3	6.30 a	62.10 d	45.79 h	1.52 a	30.14 e	8.41 b
S4	5.95 d	58.76 h	47.10 g	1.21 b	38.93 d	5.80 c
S5	5.94 d	63.24 b	47.24 e	1.06 c	44.62 c	5.53 e
S6	5.88 e	61.34 e	47.73 c	0.86 d	55.32 b	4.54 g
S7	6.16 b	62.74 c	47.66 d	1.02 c	46.71 c	4.69 f
S8	5.67 g	61.26 f	48.13 a	0.83 d	58.33 b	3.74 i
S9	5.98 c	61.25 g	47.86 b	0.77 e	62.12 a	4.28 h

*Mean values marked with a different letter in each column are statistically different from each other ($p \leq 0.01$)

Amino acids profiles

The concentrations of amino acids in *G. resinaceum* cultivated on different substrates are presented in Table 3. Substrate composition significantly influenced amino acid accumulation ($p < 0.01$). Chickpea-based substrates (S1–S2) promoted the biosynthesis of aspartic acid, tyrosine, tryptophan, and phenylalanine, whereas pea stalk substrates (S3–S4) favoured the accumulation of alanine, valine, isoleucine, leucine, sarcosine, and proline. The addition of 20% poppy stalk (S6) resulted in the highest overall amino acid concentrations, while corncob substrates (S7–S8) produced moderate levels. Control substrate S9 consistently yielded the lowest amino acid contents.

Organic acid profiles

Table 4 summarises the organic acid composition of *G. resinaceum* fruiting bodies. Substrates containing chickpea stalks (S1–S2) yielded the highest tartaric acid contents, along with elevated levels of butyric, malic, lactic, citric, and succinic acids. Pea stalks substrates (S3–S4) produced moderate levels of the same acids, whereas poppy stalks substrates (S5–S6) showed reduced organic acid yields. Corncob substrates (S7–S8) enhanced the accumulation of oxalic, propionic, lactic, maleic, and succinic acids, comparable to or higher than the control (S9).

Sugar profiles

The sugar composition (sucrose, glucose, fructose, rhamnose, xylose, and arabinose) of *G. resinaceum* cultivated on various substrates is presented in Table 5. Substrate composition significantly affected sugar accumulation ($p < 0.01$). Chickpea stalk substrates (S1–S2) yielded the highest glucose (23.17%) and fructose (19.32%) levels, while S2 produced the highest sucrose concentration (12.53%). Pea stalk substrates (S3–S4) favoured the biosynthesis of rhamnose and xylose, and poppy stalk (S6) resulted in the greatest arabinose enrichment. Corncob substrates yielded relatively low sugar contents.

DISCUSSION

The results demonstrate that substrate composition exerts a decisive influence on the biochemical profile of *G. resinaceum*, affecting amino acid, organic acid, and sugar metabolism. Substrates containing chickpea, pea, and poppy stalks were particularly effective in promoting amino acid biosynthesis. Chickpea stalks, rich in protein and free amino acids such as glutamic acid and aspartic acid [Jukanti et al. 2012, Begum et al. 2023], enhanced the synthesis of aromatic amino acids (phenylalanine, tyrosine, tryptophan). Pea stalk, which contains high levels of lysine and leucine [Day 2013, Nosworthy et al. 2017], favoured the accumulation of branched-chain amino acids (valine, isoleucine, leucine) and aliphatic amino acids (alanine, proline). Moderate supplementation with poppy stalk (20%) significantly increased total amino acid content, whereas higher levels (40%) were inhibitory. This dual effect likely reflects the balance between nutrient availability and the presence of alkaloids and phenolic compounds, which may stimulate metabolism at low concentrations but hinder enzymatic activity and nutrient uptake at higher doses [Jonathan and Fasidi 2001, Chang and Miles 2004, Nastišin et al. 2025].

Table 3. Amino acid contents of *G. resinaceum* grown on different substrates (pmol µl^{-1})

Amino acid	Chickpea salk		Pea stalk		Poppy stalk		Corncob		Control
	S1 (40%)	S2 (20%)	S3 (40%)	S4 (20%)	S5 (40%)	S6 (20%)	S7 (40%)	S8 (20%)	S9
Aspartic acid	1372.63 ±177.74ab	1213.17 ±66.07 bc	948.88 ±36.64 de	696.70 ±13.03e	700.97 ±68.94 e	1516.07 ±128.15 a	1278.42 ±75.15 abc	1081.80 ±108.19 cd	125.48 ±8.36 f
Glutamic acid	644.13 ±77.34 c	466.89 ±44.93 d	704.16 ±98.25 bc	693.14 ±3.21 bd	577.47 ±18.94 cd	1017.92 ±16.11 a	824.91 ±48.49 b	675.54 ±80.11 bc	136.34 ±9.51 e
Serine	980.59 ±93.75 abc	757.89 ±93.35 c	1004.43 ±102.22 ab	754.13 ±98.59 c	767.85 ±31.11 bc	1128.04 ±95.35 a	951.22 ±55.91 abc	788.42 ±85.56 bc	474.96 ±67.43 d
Asparagine	1538.15 ±101.13 cde	1369.39 ±51.51 de	1253.14 ±26.29 e	1659.03 ±72.22 cd	1313.04 ±12.98 e	2569.60 ±217.20 a	2166.80 ±127.36 b	1834.03 ±183.37 c	402.47 ±70.16 f
Glutamine	668.52 ±93.28 d	443.45 ±18.28 de	685.97 ±64.84 d	598.23 ±25.61 d	490.26 ±46.96 de	2799.32 ±236.62 a	2360.52 ±138.75 b	1915.39 ±245.76 c	123.41 ±12.09 e
Histidine	659.21 ±84.16 de	406.31 ±22.36 e	673.49 ±72.16 d	566.61 ±26.11de	508.30 ±16.46 de	3098.85 ±142.01 a	2556.88 ±150.29 b	2050.91 ±118.65 c	150.07 ±22.83 f
Threonine	401.07 ±24.99 d	395.57 ±31.25 d	397.31 ±32.98 d	384.92 ±63.12 d	343.10 ±64.82 d	3461.98 ±292.63 a	2919.31 ±171.59 b	2326.17 ±256.25 c	90.43 ±26.99 d
Alanine	494.03 ±75.11 d	564.64 ±18.84 d	2036.88 ±150.33 a	1585.27 ±83.67 b	1468.38 ±90.55 bc	1751.52 ±148.05 b	1476.96 ±86.82 bc	1248.58 ±125.02 c	176.57 ±19.04 e
Tyrosine	1247.19 ±95.16 ab	1389.87 ±78.86 a	1139.00 ±93.41 b	824.09 ±22.82 c	765.50 ±32.02 cd	827.77 ±69.97 c	698.01 ±41.03 cd	592.80 ±59.17 d	621.35 ±35.50 d
Cystine	426.22 ±58.59 cd	310.26 ±28.44 d	520.13 ±51.44 bc	356.68 ±35.41 d	332.96 ±73.88 d	756.63 ±63.96 a	638.02 ±37.50 ab	529.87 ±56.80 bc	9.82 ±2.21 e
Valine	638.10 ±116.08 a	414.94 ±70.88 bcd	610.14 ±152.18 ab	558.14 ±57.17 abc	511.91 ±73.38 a-d	421.50 ±35.63 a-d	355.43 ±20.89 cd	294.03 ±32.31 d	14.03 ±0.32 e
Methionine	779.89 ±111.61 bc	662.35 ±22.60 c	1271.45 ±71.00 a	868.06 ±168.54 bc	768.45 ±154.43 bc	1186.69 ±100.30 a	1000.67 ±58.82 ab	839.14 ±85.77 bc	18.95 ±5.46 d
Tryptophan	975.53 ±104.18 a	858.11 ±67.92 ab	682.93 ±151.92 bc	430.12 ±15.19 de	384.66 ±26.33 e	838.99 ±70.92 ab	707.48 ±41.58 bc	598.84 ±59.88 cd	13.97 ±4.40 f
Phenylalanine	2734.03 ±563.03 a	2811.73 ±96.43 a	2287.25 ±246.28 ab	1973.52 ±158.15 b	1721.63 ±110.57 bc	1198.27 ±101.29 cd	1010.44 ±59.39 d	845.32 ±87.23 d	28.61 ±2.56 e
Isoleucine	496.31 ±84.12 cde	335.06 ±51.39 e	1376.21 ±150.18 a	1251.77 ±84.70 ab	1072.32 ±63.67 b	688.74 ±58.22 c	580.78 ±34.14 cd	464.72 ±67.69 de	9.39 ±0.65 f
Leucine	598.64 ±53.15 c	366.69 ±10.59 d	993.51 ±96.44 a	942.39 ±17.67 a	884.25 ±50.38 a	838.66 ±70.89 ab	707.20 ±41.57 bc	572.66 ±74.84 c	18.76 ±6.75 e
Lysine	586.19 ±82.62 b	412.47 ±12.04 c	500.16 ±89.17 bc	1227.31 ±59.58 a	1109.08 ±24.80 a	604.92 ±51.13 b	510.10 ±29.98 bc	425.77 ±44.40 c	8.55 ±0.82d
Hydroxyproline	353.80 ±87.49 bc	287.80 ±8.15 bc	610.40 ±26.85 a	506.44 ±28.98 a	537.23 ±27.90 a	385.00 ±32.54 b	324.65 ±19.08 bc	263.78 ±33.45 c	5.93 ±1.64 d
Sarcosine	834.55 ±45.04 c	861.03 ±90.06 c	1769.94 ±285.73 a	1441.18 ±83.67 b	1235.56 ±78.44 b	905.51 ±76.54 c	763.57 ±44.88 c	637.97 ±66.21 c	19.28 ±7.25 d
Proline	354.81 ±90.27 b	294.81 ±50.94 b	402.30 ±47.46 ab	367.88 ±35.19 ab	357.91 ±45.19 b	501.24 ±42.37 a	422.67 ±24.84 ab	346.65 ±40.61 b	22.35 ±2.02 c

*Mean values marked with a different letter in each row are statistically different from each other. Values are expressed as mean ± standard deviation (SD) of three independent replicates

Table 4. Organic acid contents of *G. resinaceum* grown on different substrates (ng µg^{-1})

Organic Acid	Chickpea stalk		Pea stalk		Poppy stalk		Corncob		Control
	S1 (40%)	S2 (20%)	S3 (40%)	S4 (20%)	S5 (40%)	S6 (20%)	S7 (40%)	S8 (20%)	S9
Oxalic acid	31.49 ±4.27 c	24.95 ±3.78 c	23.83 ±3.59 c	24.44 ±1.14 c	23.03 ±2.02 c	21.26 ±1.10 c	245.48 ±1.69 a	211.03 ±11.18 b	263.70 ±16.89 a
Propionic acid	46.29 ±9.79 bc	38.12 ±5.77 c	36.40 ±5.49 c	37.32 ±2.97 c	36.47 ±5.54 c	31.61 ±2.88 c	111.99 ±1.72 ab	155.92 ±34.84 a	152.22 ±33.53 a
Tartaric acid	701.54 ±115.37 a	544.36 ±82.41 ab	519.80 ±78.39 ab	27.75 ±4.05 c	31.68 ±4.02 c	28.94 ±4.10 c	381.53 ±44.44b	427.89 ±69.05 b	438.73 ±59,25 b
Butyric acid	370.46 ±60.92 a	287.46 ±43.52 ab	274.49 ±41.39 b	23.81 ±1.04 c	19.87±2.61 c	20.20 ±4.64 c	224.13 ±6.33 b	271.37 ±21.63 b	247.32 ±36.81 b
Malonic acid	525.38 ±86.40 a	407.66 ±61.72 ab	389.27 ±58.70 b	37.27 ±10.95 d	34.32 ±2.58 d	28.73 ±5.41 d	137.92 ±4.89 cd	184.11 ±49.81 c	144.02 ±4.38 cd
Malic acid	287.09 ±47.21 a	222.77 ±33.72 ab	212.71 ±32.08 abc	22.38 ±0.33 d	21.67 ±5.26 d	18.09 ±3.10 d	116.67 ±12.25 cd	167.09 ±36.20 bc	127.82 ±29.98 bc
Lactic acid	244.59 ±40.22 a	189.80 ±28.73 a	181.23 ±27.33 a	19.15 ±1.01 b	18.78 ±2.19 b	17.04 ±0.80 b	181.04 ±37.27 a	217.63 ±26.71 a	208.01 ±34.60 a
Citric acid	193.60 ±40.95 a	159.45 ±24.14 ab	152.26 ±22.96 ab	21.18 ±5.35 c	23.41 ±4.81 c	23.13 ±4.48 c	121.41 ±7.50 b	117.34 ±19.76 b	108.23 ±20.63 b
Maleic acid	232.22 ±38.19 a	180.19 ±27.28 ab	172.06 ±25.95 ab	16.81 ±8.53 b	10.42 ±5.22 b	11.48 ±1.31 b	181.61 ±9.30 ab	284.21 ±47.13 a	291.16 ±21.30 a
Fumaric acid	399.22 ±65.65 b	309.78 ±46.90 bc	295.80 ±44.61 c	40.52 ±3.54 def	34.28 ±4.64 f	36.42 ±5.68 ef	135.06 ±3.00 de	601.44 ±44.72 a	136.62 ±11.85 d
Succinic acid	432.43 ±71.11 a	335.55 ±50.80 a	320.41 ±48.32 a	26.22 ±1.25 b	28.15 ±5.99 b	29.64 ±2.20 b	327.31 ±12.23 a	303.77 ±39.68 a	371.90 ±54.78a

*Mean values marked with a different letter in each row are statistically different from each other. Values are expressed as mean ± standard deviation (SD) of three independent replicates

Table 5. Sugar contents (%) of *G. resinaceum* grown on different substrates

Sugar	Chickpea stalk		Pea stalk		Poppy stalk		Corncob		Control
	S1 (40%)	S2 (20%)	S3 (40%)	S4 (20%)	S5 (40%)	S6 (20%)	S7 (40%)	S8 (20%)	S9
Sucrose	10.65 ±0.04 ab	12.53 ±1.21 a	9.88 ±1.55 abc	11.43 ±2.08 ab	8.81 ±0.62 abc	7.51 ±1.80 bc	7.32 ±1.57 bc	8.92 ±1.62 abc	6.23 ±1.42 c
Glucose	23.17 ±3.67 a	12.59 ±2.07 b	10.68 ±0.48 b	12.37 ±1.40 b	9.47 ±1.56b	8.87 ±1.30 b	8.02 ±1.46 b	10.01 ±1.14 b	6.47 ±3.32 b
Fructose	19.32 ±5.89 a	11.84 ±1.92 b	10.43 ±0.71b	11.64 ±1.44 b	9.31 ±1.02 b	7.69 ±1.24 b	7.92 ±1.43 b	9.42 ±1.16 b	6.52 ±2.56 b
Rhamnose	10.22 ±3.48 ab	13.64 ±1.88 a	11.96 ±1.48 ab	13.37 ±1.58 a	11.48 ±0.87 ab	10.76 ±0.15 ab	10.29 ±1.19 ab	10.82 ±1.28 ab	8.56 ±5.09 b
Xylose	5.59 ±1.40 ab	7.32 ±1.43 a	6.42 ±0.52 ab	7.18 ±0.97 a	5.56 ±1.34 ab	4.13 ±0.81 b	4.71 ±1.20 ab	5.81 ±1.22 ab	4.93 ±0.52 ab
Arabinose	9.81 ±2.98 ab	13.68 ±2.21 ab	11.40 ±0.64 ab	13.44 ±1.51 ab	12.14 ±1.98 ab	14.88 ±2.41 a	10.21 ±1.20 ab	10.87 ±1.22 ab	9.17 ±0.52b

*Mean values marked with a different letter in each row are statistically different from each other. Values are expressed as mean ± standard deviation (SD) of three independent replicates

Organic acid profiles varied markedly among substrate types. Chickpea and pea residues enhanced the synthesis of tartaric, malic, citric, and succinic acids—key intermediates of the tricarboxylic acid (TCA) cycle [Vohra and Satyanarayana 2001, Nelson et al. 2017]. The observed enhancement suggests that nitrogen-rich legume residues stimulate fungal energy metabolism and carbohydrate oxidation. Conversely, poppy stalk reduced organic acid accumulation, potentially due to its alkaloid and lignin content, which may inhibit central carbon metabolism [Duke 2017]. Corncob substrates, rich in lignocellulosic carbon, induced increased oxalic and propionic acid formation, possibly reflecting altered carbon fluxes in response to structural carbohydrate degradation.

Sugar accumulation patterns revealed distinct substrate effects. Chickpea-based substrates enhanced glucose, fructose, and sucrose, consistent with the high starch and soluble carbohydrate content of chickpea residues [Jukanti et al. 2012]. Pea stalk substrates favoured rhamnose and xylose accumulation, likely due to hemicellulose degradation [Rehman et al. 2015]. Poppy stalk increased arabinose content, consistent with its pectic and arabinoxylan components [Kocabaş et al. 2020]. Corncob substrates, high in lignin, supported limited sugar release and metabolism [Limayem and Ricke 2012].

Despite the long-standing medicinal use of *Ganoderma* species, systematic investigations into the chemical composition and biological activities of *G. resinaceum* remain limited [Chen et al. 2017, Chen et al. 2018]. *Ganoderma* species contain a wide array of bioactive compounds, including triterpenes (e.g., ganoderic acids), polysaccharides, peptides, sterols, and alkaloids, which exhibit diverse pharmacological effects such as anticancer, antioxidant, anti-inflammatory, neuroprotective, hypoglycemic, hypotensive, antimicrobial, and antiviral activities [Stojkovic et al. 2014, Rashad et al. 2019, Kozarski et al. 2020, Sułkowska-Ziaja et al. 2023b, Rašeta et al. 2024, Ren et al. 2025]. Nevertheless, challenges remain in compound isolation, purification, and evaluation of synergistic effects, along with clinical standardization and safety testing. Notably, Peng et al. [2024] reported hepatoprotective properties of *G. resinaceum* triterpenoids, independent of antioxidant mechanisms.

Similar substrate-dependent variations in metabolite profiles have also been reported in other cultivated fungi. For instance, studies on *Pleurotus* spp. and *Lentinula edodes* have demonstrated that substrate composition significantly influences amino acid and organic acid contents, with nitrogen-rich substrates generally enhancing amino acid biosynthesis, while lignocellulosic substrates modulate carbon metabolism and organic acid production [Royse 2002, Elisashvili 2012]. In *Pleurotus ostreatus*, substrates supplemented with leguminous residues were found to increase protein and amino acid content, consistent with the results observed in the present study. Similarly, lignin-rich substrates such as corncob have been reported to alter carbohydrate metabolism and reduce soluble sugar accumulation in various basidiomycetes [Elisashvili 2012]. These findings indicate that the substrate-driven metabolic responses observed in *G. resinaceum* are in line with general physiological patterns reported in other edible and medicinal fungi.

Overall, the present results highlight how substrate formulation can be used to modulate fungal metabolism for biotechnological applications. By selecting appropriate agricultural residues, metabolite profiles in *G. resinaceum* can be tailored to enhance the yield of specific amino acids or organic acids with potential pharmaceutical relevance. For instance, amino acids such as phenylalanine and tryptophan serve as precursors of bioactive secondary metabolites with antioxidant and immunomodulatory effects. Organic acids such as succinic and malic acid play important roles as intermediates in biosynthetic and energy pathways, supporting their use in pharmaceutical and nutraceutical production. Overall, this study highlights the potential of agricultural residues as sustainable and low-cost substrates for optimising the biochemical and therapeutic potential of medicinal fungi.

CONCLUSION

The results of this study demonstrate that the metabolite composition of *Ganoderma resinaceum* is strongly influenced by substrate formulation. Substrate-dependent amino acid enrichment reflects both the intrinsic composition of agricultural residues and the fungus's capacity to assimilate available nitrogen sources, with chickpea and pea stalk significantly enhancing essential and aromatic amino acids. Organic acid production was closely associated with substrate characteristics and TCA cycle activity, while sugar profiles varied according to substrate type, with chickpea, pea, and poppy stalk promoting distinct sugar fractions. Overall, these findings indicate that substrate optimisation is an effective strategy for modulating metabolite production in *G. resinaceum*.

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CONFLICT OF INTEREST

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

AUTHORS' CONTRIBUTIONS

M.K.S.: conceptualization, methodology, investigation, mushroom cultivation, formal analysis, statistical analysis, writing – original draft preparation. N.C.: conceptualization, methodology, supervision, writing – review and editing. M.T.: sample preparation, laboratory analyses, data curation.

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