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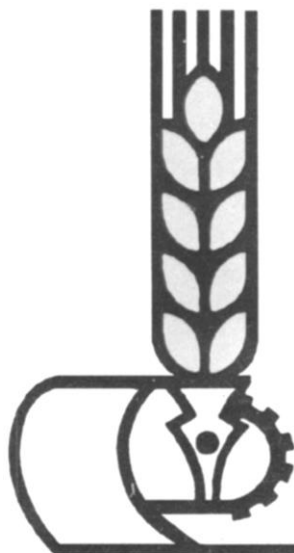
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CONTENTS

AGRICULTURAL ECONOMICS, EXTENSION AND RURAL DEVELOPMENT

Determining the Attitudes of Farmers Engaged in Livestock Production towards the Contracted Production Model: An Analysis of Risks, Expectations, and Sustainability
Adem Aksoy, Ferda Nur Özdemir, and Ümit Avcioglu 269

Equilibrium between Education and Research in Agricultural Higher Education: A Pathological Analysis.
Fatemeh Soleymani Sardo, Masoud Bijani, Esmail Karamidehkordi, and Fatemeh Sepahvand 285

FISHERIES

Research Note: Histopathological Changes Gill of Common Carp in the Confront of the Chlorpyrifos and Salinity.
Mona Hasankhany, Tina Hasankhani, Seyed Ali Akbar Hedayati, and Mohammad Mazandarani 299

FOOD SCIENCE AND TECHNOLOGY

Aroma of Yogurt from Cow Milk with a Combination of Several Probiotic Bacteria.
Suryono Suryono, Anton Apriyantono, and Ester Restiana Endang Gelis 307

Black, Q12, and Titicaca Quinoa Protein Isolate: Nutritional and Physicochemical Properties.
Seyed Saeed Sekhavatzadeh, and Saeid Hosseinzadeh 319

Enhancing the Shelf Life and Sensory Properties of Rainbow Trout Fillets through Sodium Alginate Coating Containing *Eryngium campestre* Extract at 4 °C.
Mohammad Abedi, Hossein Tajik, and Tooraj Mehdizadeh 335

PLANT SCIENCE

ABA Accumulation and *PsAO* Gene Expression in Field Pea under Water Deficit.
Gordana Petrović, Radmila Stikić, Tomislav Živanović, Dušica Jovičić, Aleksandra Ilić, Gordana Timotijević, and Jelena Samaržić 351

Effects of Starvation, Dietary Regimes, and Temperature Stress on Hemocyte Profiles and Phenoloxidase Activity in Larvae of *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae).
Jamaneh Karimian and Maryam Ajamhassani 367

Foliar Nutrient Concentrations and Antioxidant Activity of Tea (*Camellia sinensis* L. (O) Kuntze) Planted in Peninsular Malaysia and its Relation to Soil Edaphic Factors.
Wisnu Eko Murdiono, Nur Amirah Syafiqah Salman, Uma Rani Sinniah, Elisa Azura Azman, Mohd Izuan Effendi Halmi, Jean Wan Hong Yong, Abbey Maleyki Mhd Jalil, and Khairil Mahmud 385

Continued on back cover

Continued from front cover:

PLANT SCIENCE

Influence of Pre-Imaginal Stages of *Bemisia tabaci* on Development, Life Table Parameters, and Predation Rate of *Euseius scutalis* (Acari: Phytoseiidae).

Mohammad Gravandian, Parviz Shishehbor, Mehdi Esfandiari, and Elham Riahi 401

Synthesis of Poly- γ -Glutamate in Solid-State Fermentation and Its Application in Biocontrol.

Sareh Hashemi, Masoud Ahmadzadeh, Hossein Saremi, Soleiman Ghasemi, and Azad Omrani 415

Role of Melatonin and Pomegranate Seed Oil in Maintaining the Quality of Mexican Limes during Storage

Mahbobeh Mohammadi, Somayeh Rastegar, and Abbas Rohani 429

SOIL AND WATER

Co-Composting of Municipal Solid Waste with Activated Biochar: A Promising Approach to Improve the Quality of Compost.

Kamal Khalkhal, Adel Reyhanitabar, Shahin Oustan, and Nasser Aliasgharzad 449

Determining the Attitudes of Farmers Engaged in Livestock Production towards the Contracted Production Model: An Analysis of Risks, Expectations, and Sustainability

Adem Aksoy¹, Ferda Nur Özdemir^{1*}, and Ümit Avcioğlu¹

ABSTRACT

The transition of small-scale farmers from traditional production methods to Contract Farming (CF) enhances the potential for increased profits. By offering higher profits to producers, contract farming encourages greater product diversity and provides the opportunity to grow more valuable products. The primary objective of this study was to identify what kind of contract farming model producers are likely to adopt for their continued production. The Analytical Hierarchy Process (AHP) was used as a methodology in the study. In the region, 70% of the producers utilized female animals for fattening purposes. It was found that 73.2% of producers lacked knowledge about contract farming. Livestock operators in production contracts prioritized price guarantees (26.4%), followed by cash prices (24.8%), livestock supply (21.2%), input support (12.5%), advance payments (10.3%), and organized production (4.8%). If contract farming is to be implemented in the region, policymakers should prioritize price guarantees in the model, ensuring that these guarantees are not set below the market price.

Keywords: Analytical Hierarchy Process, Contract production, Livestock breeding, Sustainable production.

INTRODUCTION

Achieving the Sustainable Development Goals (SDGs) is essential for ensuring economic growth in developing countries, preventing persistent poverty, and securing food supply chains (Ton *et al.*, 2018; Vamuloh *et al.*, 2020). Within the agricultural sector, particularly in countries such as Turkey, addressing these goals holds significant importance. To ensure access to safe and reliable food for a growing global population, governments have increasingly encouraged large-scale enterprises to invest in small-scale farms that operate under traditional agricultural methods (Ray *et al.*, 2021). In this context, Contract Farming (CF) has emerged as a key strategy for enhancing sustainability in agriculture.

Many studies emphasize that CF serves as a critical mechanism for supporting agricultural development in developing economies (Da Silva and Ranking, 2013; Eaton and Shepherd, 2001; Minot and Ronchi, 2015; Otsuka *et al.*, 2016; Schipmann and Qaim, 2010).

FAO defines contract farming as agricultural production conducted under an agreement between buyers and producers that sets the conditions for the production and marketing of farm products (Jing *et al.*, 2023). A similar definition describes CF as a commercial arrangement between firms and groups of producers (Ton *et al.*, 2018). CF facilitates market access for small-scale farmers while improving access to essential inputs and financial credit (Da Silva and Ranking, 2013; Eaton and Shepherd, 2013). Transitioning from traditional farming to CF

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enables smallholder farmers to enhance earnings, diversify their product range, and cultivate higher-value crops. This transition allows them to operate at more competitive price points in global markets, fostering opportunities to compete with large-scale agribusinesses (Glover and Kusterer, 2016; Runsten, 1992; Sharma, 2014).

Private firms, as well as partnerships between corporations, governments, and Non-Governmental Organizations (NGOs), frequently implement CF arrangements. Additionally, modern market systems necessitate greater coordination along the value chain, with CF standing out as a viable institutional framework to meet this demand (Reardon and Berdegue, 2003). Studies in developing nations have explored contract farming participation rates (Günden and Miran, 2008), as well as its impact on production and income levels. While much of the literature focuses on high-value crops such as horticulture, flowers, palm oil, and coffee (Blouin and Macchiavello, 2019; Cahyadi and Waibel, 2016; Gatto *et al.*, 2017; Macchiavello and Morjarria, 2015; Michelson, 2013; Wang *et al.*, 2014), CF has also proven beneficial for smallholder farmers, often increasing incomes by 25-50% compared to conventional farming (Ton *et al.*, 2018). When examining the literature, particularly studies focused on the livestock sector, CF has emerged as a model that provides financial security to producers in the livestock sector, enhancing income stability and reducing market uncertainties. The literature highlights that contract farming facilitates producers' access to technology, improves animal health and welfare, and enhances product quality (Hernandez *et al.*, 2007; Key and Runsten, 1999). For instance, a study conducted in India found that contract dairy farming reduces production costs and strengthens farmers' bargaining power, thereby increasing their income (Birthal *et al.*, 2005). Similarly, research conducted in the Philippines demonstrated that contract pig farming improves access to veterinary services, thereby reducing animal disease

prevalence (Swinnen and Maertens, 2007). However, for this model to be effectively implemented, small-scale farmers must fully comprehend the contract terms, and price mechanisms must be transparently established (Warning and Key, 2002). These findings underscore the crucial role of CF in improving efficiency and sustainability in the livestock sector.

Despite its advantages, smallholder farmers may struggle to meet CF requirements due to stringent quality standards, leading corporations to favor larger-scale enterprises. Even when smallholders are encouraged to engage in CF, they remain highly vulnerable to unfavorable market conditions (Sudha, 2013). Several studies have examined the barriers preventing small-scale farmers from adopting CF, identifying key determinants such as perceived risks, access to credit and markets, expected benefits, and land tenure rights (Baker *et al.*, 2017; Lee *et al.*, 2016). Although smallholders contribute 28-31% of global crop production and 30-34% of the total food supply, their participation in CF remains minimal, often below 5% (Amanor, 2012; Azumah *et al.*, 2017; IFAD, 2013; Nguyen *et al.*, 2015; Ton *et al.*, 2018).

The literature also examines the environmental, economic, and social implications of CF, highlighting challenges that hinder its effectiveness for small farmers. In Vietnam, for example, research indicates that while contract livestock farmers experience economic benefits, increased farming activities have also led to heightened environmental pollution (Takahashi *et al.*, 2020). Studies analyzing CF's impact on crop diversity suggest that CF is more effective in competitive markets or production processes requiring technical expertise, such as poultry farming (Ragasa *et al.*, 2018; Simmons *et al.*, 2005). Additionally, research investigating CF's spillover effects on labor markets suggests that the adoption of labor-intensive technologies in contract crop production increases labor demand within contract farming households (Bellemare, 2018).

In Turkey, research on CF has primarily focused on its historical development at both global and domestic levels, the challenges small farmers face, and macro-level obstacles to CF expansion (Aydın, 2007; Pakdemirli, 2020). Regional studies have identified implementation challenges, including the fragmented nature of small farms, their lack of bargaining power, and limited awareness of how CF systems function (Konak *et al.*, 2000). The literature also underscores that CF in the livestock sector facilitates better management of production and marketing processes, reducing uncertainties in agricultural production (Öztürk, 2020). Furthermore, this system incentivizes producers to maintain high-quality standards by enabling buyers to specify demand and quality requirements (Çelik, 2019). Consequently, CF is recognized as a crucial mechanism for sustainable livestock production and food security. However, studies addressing the economic dimensions of CF in Turkey remain relatively scarce. Given the prominence of livestock farming in Turkey's agricultural sector, particularly in the eastern province of Erzurum, there is a pressing need to transition to a more efficient production model.

Recent sharp increases in input prices and fluctuations in meat prices have adversely affected small enterprises in Turkey. If this economic instability persists, maintaining consumer access to meat and supporting small-scale businesses to sustain production will become increasingly critical. This study aims to identify the contract farming model that livestock businesses in Erzurum would be most likely to adopt. The study's originality stems from its focus on the first application of contract farming in Turkey's livestock sector, allowing for the design of a contract model aligned with farmers' expectations and offering policy recommendations to relevant institutions and organizations.

Establishing a successful CF model in the livestock sector could provide preliminary insights for contract farming models in other agricultural branches. Given the current debate surrounding red meat imports, meeting the growing population's demand

for red meat through domestic production is paramount. Importing red meat or live animals would not only fail to resolve Turkey's livestock sector challenges but could also further destabilize the industry, potentially forcing small family-run farms out of business. Therefore, the primary objective of this study was to examine how livestock producers in Erzurum can sustain production through a CF model.

MATERIALS AND METHODS

The primary data source for this study consisted of responses collected from a 2023 survey conducted with 138 livestock producers across various districts of Erzurum. Secondary data were obtained from the relevant online resources, FAO publications, TURKSTAT reports, and other national and international studies.

Method for Selecting the Study Population

To ensure a representative sample, districts were selected based on regional similarities within Erzurum Province. The selection process incorporated the perspectives of officials from the Provincial Directorates of the Ministry of Agriculture and Forestry. The study included the following districts: Narman and Oltu from the northern region, Çat from the southern region, Horasan, Köprüköy, and Pasinler from the eastern region, Aşkale from the western region, and Yakutiye, Palandöken, and Aziziye from the central region (Figure 1).

Method for Determining Sample Size

This study aimed to identify the factors influencing contract farming adoption by conducting face-to-face interviews with livestock producers engaged in animal husbandry in Erzurum, in 2023. To achieve this objective, the sample size was determined using the proportional sampling

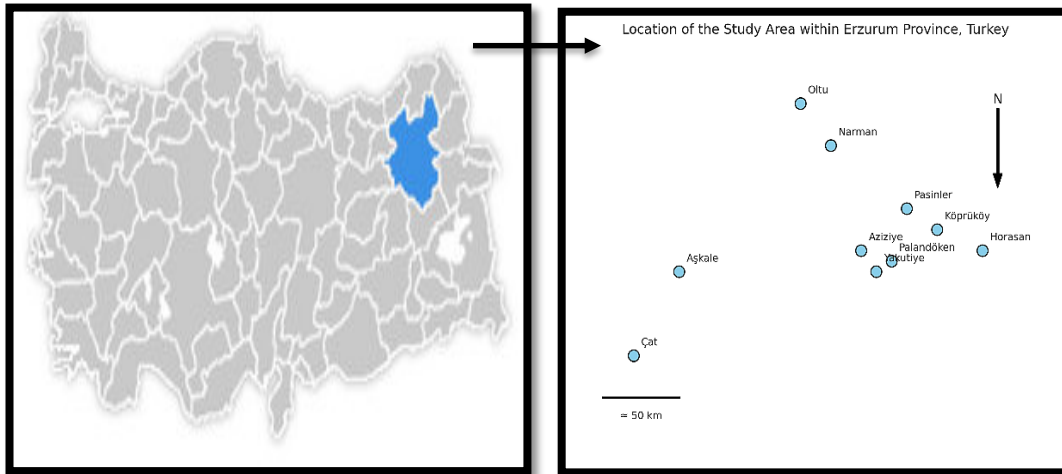


Figure 1. Description of the study areas.

method. A 90% confidence interval and a 5% margin of error were applied to ensure statistical reliability. The formula used to determine the sample size is provided below (Newbold, 1995; Miran, 2010).

$$n = \frac{Np(1-p)}{(N-1)\sigma_p^2 + p(1-p)} \quad (1)$$

The variance is calculated as follows.

$$\sigma_p^2 = \left(\frac{r}{Z_{\alpha/2}}\right)^2 \quad (2)$$

$$\sigma_p^2 = \left(\frac{0.05}{1.64}\right)^2 = 0.000923$$

Where, n: Sample size; N: Number of enterprises engaged in animal husbandry (53676); p: The proportion of farmers who prefer contract fattening, (set as 0.15), and σ^2 : Variance (0.000923).

There was a total of 53,676 livestock producers in Erzurum Province. With a confidence interval of 90% and an error of 5%, the sample volume was found to be 138.

Methodology for Questionnaire Design

Ensuring a conducive environment is essential for obtaining unbiased and reliable information from farmers regarding their agricultural activities. Farmers often exhibit skepticism toward inquiries from public officials and private sector representatives,

which may lead to reluctance in providing accurate responses (Aksoy, 2008; Erkuş, 1977). To mitigate this issue, previous studies on the subject were carefully reviewed and incorporated into the development of the questionnaire forms.

Analytical Hierarchy Process (AHP) Method

The Analytical Hierarchy Process (AHP) was developed by Thomas L. Saaty in the 1970s as a structured decision-making framework for solving complex problems involving multiple criteria. This model is based on a hierarchical structure, wherein objectives, criteria, sub-criteria (if applicable), and alternatives are systematically organized and their interrelationships analyzed (Ballica, 2020; IFAD, 2013). The AHP methodology enables the quantification of decision-making factors by assigning percentage weights to various influencing criteria, provided that a well-defined decision hierarchy exists (Yaralıoğlu, 2001). Due to its versatility and applicability, the AHP method has been widely utilized in diverse decision-making scenarios (Vaidya, 2006).

The AHP is widely utilized for addressing multi-criteria decision-making problems by structuring decisions hierarchically, incorporating a goal, criteria, and

alternatives. However, certain simplified implementations focus solely on pairwise comparisons of alternatives, excluding explicit criteria. These adaptations, facilitated by AHP's inherent flexibility, are particularly useful in specific decision-making contexts and are referred to as "single-level AHP" or "criterion-free AHP" (Vaidya and Kumar, 2006). In alignment with the objectives of this study, a criterion-free single-level AHP approach was adopted. The AHP model applied in this study is illustrated in Figure 2.

To address the decision-making problem within this study using the Analytical Hierarchy Process (AHP), the necessary steps are outlined and defined below. Each stage is accompanied by the relevant formulation and explanation:

Stage 1: Define the Decision-Making Problem

Stage 2: Comparison Matrix between Factors is created

Stage 3: Percentage importance distributions of factors are determined

Stage 4: Consistency in factor comparisons is measured

Stage 5: For each factor, the percentage importance distributions at m decision points are found

Stage 6: Find the distribution of results at decision points.

AHP provides decision-makers with an objective and structured framework for analyzing various alternatives. This method is particularly useful when evaluating multiple alternatives that must be ranked based on pairwise comparisons. In this study, six alternatives were identified as key

factors influencing producer participation in the contract farming model. Since AHP is the most suitable method for making pairwise comparisons and determining the relative importance of these alternatives, it was employed as the primary decision-making approach in this research.

RESULTS AND DISCUSSION

Descriptive Analysis Results

In agricultural enterprises, labor is one of the fundamental production factors, predominantly provided by family members. Face-to-face interviews with producers revealed that reliance on family labor is an economic necessity for managing routine daily tasks that require minimal time, particularly in livestock operations. As farm size increases, the use of external labor tends to rise alongside family labor. The average family size was calculated to be 5.44 individuals (Table 1).

A similar study on dairy farms in Erzurum reported an average family size of 5.81, with 86% of participants having social security coverage (Kılıçtek and Aksoy, 2022). Furthermore, 60% of the surveyed breeders indicated that livestock breeding was profitable; however, only 18% had received prior training related to their work.

Another study investigating the impact of contract farming on food security highlights that household resilience to food insecurity is significantly influenced by variables such as education level, asset ownership (total livestock), access to public services (e.g.,

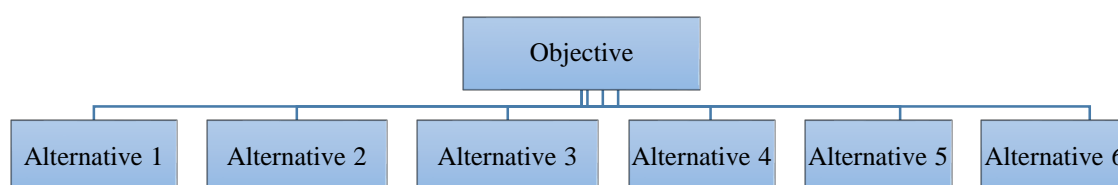


Figure 2. A simple AHP model.

**Table 1.** Main characteristics of the operator.

Operator characteristics	Min.	Max.	Mean
Number of family members	0	20	5.44
Marital status (Single= 0, Married= 1)	0	1	0.95
Registration in the animal registration system	0	1	0.93
Membership in any cooperative	0	1	0.56
Agricultural credit utilization	0	1	0.66
Non-agricultural work	0	1	0.32
Any Social Security	0	1	0.91
Number of the family labor force	0	8	2.21
Foreign labour force	0	1	0.28
Do you think fattening is a profitable business?	0	1	0.60
Do you plan to continue fattening?	0	1	0.88
Have you received training on cattle fattening?	0	1	0.18

Source: Research findings.

Table 2. Basic features related to livestock enterprises.

Features related to the business	Min.	Max.	Mean
Average annual expenditure on livestock (TL)	1.000	6.200.000	800.395
Annual non-agricultural income of the enterprise (TL)	0	2.500.000	105.109
Annual income of your enterprise from crop production (TL)	0	5.000.000	274.891
Total amount of agricultural land (da)	0	5.000	212
Annual amount of support received for crop production (TL)	0	550.000	20.503
Annual amount of support received for animal production (TL)	0	200.000	9.321
Is there a manure scraper in the enterprise? (No= 0, Yes= 1)	0	1	0.11
Is there an automatic drinker in the enterprise? (No= 0, Yes= 1)	0	1	0.76
Is there a scratcher in the enterprise? (No= 0, Yes= 1)	0	1	0.12
Is there a quarantine area in the enterprise? (No=0, Yes=1)	0	1	0.21
Are safety measures related to diseases implemented in the enterprise? (No= 0, Yes= 1)	0	1	0.73
Is there ventilation in the enterprise? (No= 0, Yes= 1)	0	1	0.95
Is technical support received for the enterprise? (No= 0, Yes= 1)	0	1	0.64
Do you have a feed depot? (No= 0, Yes= 1)	0	1	0.89
Is factory feed used? (No= 0, Yes= 1)	0	1	0.96
Barn type (Closed= 0, Semi-open= 1)	0	1	0.01
Stop type (Free stop= 0, Bound= 1)	0	1	0.86

Source: Research findings.

microfinance services), social support, and both income and food availability (total calorie intake and farm income) (Gelata *et al.*, 2024).

Similarly, studies have emphasized the significance of farmer and farm characteristics in contract farming participation (Kuhfuss and Subervie, 2018; Mack *et al.*, 2020). Key factors such as gender, age, education, farming experience, and land size have been identified as critical

determinants in contract farming adoption. Moreover, the research underscores the importance of demographic variables including gender, education level, family size, and marital status, in shaping farmers' decisions to engage in contract production (Calvet *et al.*, 2019; Kuhfuss and Subervie, 2018; Mack *et al.*, 2020).

The annual average livestock expenses of the enterprises analyzed in this study were determined to be 800,394.9 TL, while the

average plant production income was recorded as 274,891 TL (Table 2).

An examination of the infrastructure of the surveyed businesses revealed that 11% had manure scrapers, 76% had automatic irrigation systems, 21% had quarantine areas, and 89% had feed storage facilities. Similar studies have indicated that, due to the climatic conditions of the region, nearly all barns operate within closed and connected systems. Assessing the economic and environmental impacts of these structures is crucial for ensuring their long-term sustainability (Gibon *et al.*, 1999; Lebacqz *et al.*, 2013; Lovarelli *et al.*, 2020).

In alignment with the European Union's "Farm to Fork" strategy, ensuring the technological compliance of such barn structures with economic efficiency and environmental sustainability standards is of paramount importance (European Commission, 2017) (Table 2).

A range of studies examining factors that influence the success of contract farming (CF) highlights that geographical conditions, farm type, household asset accumulation, social capital availability, and firm characteristics serve as critical determinants (Barret *et al.*, 2012; Lambrecht and Ragasa, 2018; Bellemare and Bloem, 2018). The

capacity to engage in contract farming models.

The total number of cattle in the livestock farming enterprises analyzed in this study was categorized into three strata. Among the enterprises, 34.8% fell within the first stratum (1–30 cattle), 45.6% in the second stratum (31–70 cattle), and 19.6% in the third stratum (71 and above).

Contract farming is a model that has been implemented in both developed and developing countries for many years. Evaluating and refining this model is essential to enhance its effectiveness and expansion, both globally and in Turkey (Ağır and Akbay, 2017). Within this study, it was determined that 73.2% of livestock breeders in the research area lacked knowledge about contract farming (Table 3). Additionally, only 9.4% of the operators had prior experience with contract fattening. In contrast, a study conducted among cattle breeders in Adana Province found that 39.35% of the participants were engaged in contract fattening (Ağır, 2018).

These findings highlight a significant lack of awareness regarding contract farming in the research area. Notably, 47.1% of the producers expressed a willingness to participate in contract production, indicating

Table 3. General opinions of enterprise owners about contract production (%).

Business owners' level of knowledge about contract production				
	Strata 1	Strata 2	Strata 3	Mean
No.	77.1	76.2	59.3	73.2
Yes	22.9	23.8	40.7	26.8
Total	100.0	100.0	100.0	100.0
Previous contracted production status of enterprise owners				
No.	93.8	95.2	74.1	90.6
Yes	6.3	4.8	25.9	9.4
Total	100.0	100.0	100.0	100.0
Willingness of enterprise owners to make contract production				
No.	66.7	49.2	37.0	52.9
Yes	33.3	50.8	63.0	47.1
Total	100.0	100.0	100.0	100.0

Source: Research findings.

findings emphasize the significance of assessing farm characteristics when determining farmers' willingness and

potential for further adoption with targeted awareness and support programs.



Econometric Model Results

Table 4 presents the descriptive statistics of the alternatives considered in contract farming, as analyzed using the AHP method.

Table 4. Descriptive Statistics of AHP alternative.

Alternative	Average	Geometric average	Harmonic mean	Standard deviation
Price guarantee	0.264	0.21927	0.16254	0.140
Advance Price	0.248	0.21999	0.18579	0.112
Advance payment	0.103	0.08885	0.07354	0.057
Input support	0.125	0.10298	0.08010	0.078
Organized production	0.048	0.02848	0.02291	0.076
Livestock supply	0.212	0.16226	0.11351	0.139

Source: Research findings.

In the context of contract farming, 26.4% of livestock enterprises prioritized price guarantees, 24.8% valued advance pricing, 21.2% emphasized livestock supply, 12.5% focused on input support, 10.3% preferred advance payments, and 4.8% considered organized production as a key factor.

These findings indicate that price guarantees (26.4%) and advance pricing (24.8%) are the most critical factors influencing decision-making. The higher average values of these two alternatives compared to others suggest that enterprises prioritize these considerations in their decision-making processes. Livestock supply (21.2%) ranks third, while input support (12.5%), advance payments (10.3%), and organized production (4.8%) hold lower importance.

Furthermore, the standard deviation values highlight the variability in the importance of each criterion. Price guarantees (0.140) and livestock supply (0.139) exhibit the highest variability, whereas cash payment (0.057) shows the least variability. This suggests that while some alternatives are consistently prioritized, others vary significantly across enterprises.

This analysis provides valuable insights into the factors that enterprises prioritize under CF conditions. The findings emphasize the significance of price guarantees and advance pricing in the

decision-making process. A similar study on farmer cooperatives highlights that none of the surveyed farmers preferred to contract with buyers, with this lack of coordination making compliance with food safety standards challenging (Jia and Huang,

2011). Likewise, a study conducted in the United States underscores how CF has enabled production control in the poultry, egg, and swine industries, leading to substantial improvements (Martinez, 2002). Additionally, CF accounts for 75% of poultry production in Brazil, 90% of cotton and milk production in Vietnam, and 50% of tea production globally (da Silva and Ranking, 2013; MacDonald, 2011).

Table 5 shows the results of pairwise comparisons of alternatives considered in contract manufacturing. The difference between each alternative pair was statistically evaluated using the Friedman test. Test statistic, standard error and significance values are given. It is observed that there was a significant difference between most alternative pairs. These pairs with a significance value below 0.05 show a statistically significant difference. For example, the difference between the pairs "Organized Production - Price Guarantee" (Test Statistic= 3.290, Significance= 0.000) and "Organized Production-Cash Price" (Test Statistic= 3.210, Significance= 0.000) is quite significant. However, it was observed that there was no significant difference between some alternative pairs. For example, there was no significant difference in the pair "Cash Price - Price Guarantee" (Significance= 0.723). This may indicate that these two alternatives were

perceived by the participants at similar levels of importance.

According to the Friedman test results given below the table, the test statistic was 309.951 and the significance value (Asymptotic Sig.) was 0.000. This result shows that there was a significant difference between the alternatives in general. The highest test statistic is seen in the

elements highlight the role of contract farming in mitigating risks and providing stability for agricultural enterprises. Over the past 15 years, research has predominantly focused on the benefits of CF, particularly for family-run farms.

Empirical studies suggest that farmers engaged in CF differ significantly from their small-scale counterparts. Several positive

Table 5. Pairwise comparison results of alternatives.

Alternative 1-Alternative 2	Test statistic	Std. error	Significance
Organized Production-Advance Payment	1.420***	0.225	0.000
Organized Production-Input Support	1.736***	0.225	0.000
Organized Production-Breeding Animal Supply	-2.518***	0.225	0.000
Organized Production-Advance Price	3.210***	0.225	0.000
Organized Production-Price Guarantee	3.290***	0.225	0.000
Advance Payment-Input Support	-0.315	0.225	0.162
Advance Payment-Livestock Supply	-1.098***	0.225	0.000
Advance Payment-Advance Price	1.790***	0.225	0.000
Advance Payment-Price Guarantee	1.870***	0.225	0.000
Input Support-Livestock Supply	-0.783	0.225	0.001
Input Support-Advance Price	1.475***	0.225	0.000
Input Support-Price Guarantee	1.554***	0.225	0.000
Livestock Supply-Advance Price	0.692***	0.225	0.002
Livestock Supply-Price Guarantee	0.772***	0.225	0.001
Advance Price-Price Guarantee	0.080	0.225	0.723

Friedman's Test Statistic= 309,951, Asymptotic Sig. (2-sided test)= 0.000

Note: *** 1%, ** 5%, * 10% indicate significance level.

"Organized Production - Price Guarantee" and "Organized Production - Cash Price" pairs. This means that the difference between these pairs was the most significant. The lowest test statistic was calculated for the "Advance Price - Price Guarantee" pair, indicating that there was almost no difference between these alternatives.

These results reveal that there are significant differences between the alternatives in contracted production. In particular, alternatives such as price guarantee and cash price stand out and there are significant differences between organized production and some alternatives. This shows that businesses attach more importance to certain alternatives and that some alternatives have similar values.

Organized production processes and price guarantees emerge as the most critical factors for farmers in adopting CF. These

outcomes have been documented, including increased production efficiency (Mishra *et al.*, 2018), enhanced food security (Jagri Binpori *et al.*, 2021), higher earnings (Ruml and Qaim, 2021), poverty alleviation (Cahyadi and Waibel, 2016), income growth (Dubbert *et al.*, 2023), improved output quality (Adabe *et al.*, 2019), higher profit margins (Madani *et al.*, 2018), and increased yields (Prasetyo *et al.*, 2022).

Additionally, CF ensures consistent product quality, reduces production costs, secures access to high-quality inputs at competitive prices, and streamlines procurement processes. These advantages collectively enhance the appeal of contract farming and contribute to its increasing adoption among agricultural producers (Gelata *et al.*, 2024).

The table 5 resulting from this analysis summarizes the functioning of the contract



production model in the livestock sector and the relationships between the parties. The table structures the interactions between farmers, companies and contracts in terms of alternative, objectives, commitments and possible outputs.

Farmers' alternatives include items such as organized production, advance payment, input support, cash price, price guarantee and livestock support. These alternatives help farmers stabilize the production process. The companies' commitments include regular procurement, support, and quality assurance. These commitments help companies to create a sustainable supply chain. The objective of the contracts is linked to financial security, risk minimization and low cost, which creates a basis for mutual benefit for both the farmers and companies.

Possible outputs of this model include increased yields, profitability, food security and sustainability. In summary, the contract production model provides financial security

CONCLUSIONS

The findings of this study show that animal husbandry, an important sub-sector of agriculture in developing countries such as Turkey, is managed by small family businesses that are highly sensitive to economic fluctuations. These businesses face limited access to financial services and high production costs. Contract Farming (CF) offers the most effective solution to protect these businesses, increase their productivity, and enable profitable production. The study revealed that 73.2% of cattle breeders in Erzurum did not have knowledge about CF. Among those who do, 26.4% prioritized price guarantees, 24.8% valued advance pricing, 21.2% preferred livestock procurement, 12.5% emphasized input support, 10.3% considered advance payment, and 4.8% evaluated organized production within the contract model. However, the "Contract Livestock Project" of the Ministry of Agriculture and Forestry

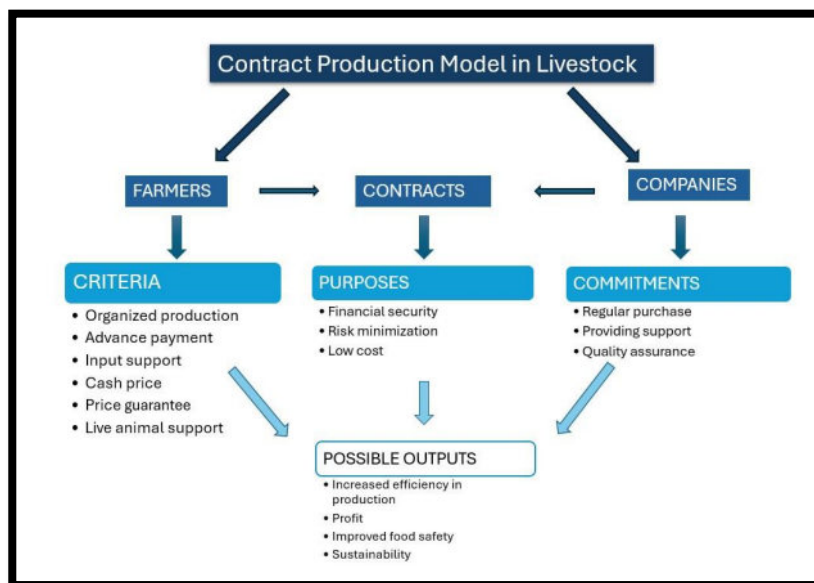


Figure 3. Estimated contract production model design as a result of the analysis.

for both farmers and companies, supports sustainable production conditions in the sector, and offers the potential to increase food security (Figure 3).

was not successful, as it did not sufficiently align with these priorities. In this context, farmers' readiness for contract production was assessed, the alternatives they

prioritized within the contract were identified, and a sample contract model was developed.

In regions where animal husbandry is the primary economic activity, implementing a specialized contract farming model could provide significant benefits. To effectively implement this model, policymakers should prioritize price guarantees above market rates to ensure income stability and should also ensure that payments are made within five business days. Adapting contract terms to local priorities in different regions will further increase the adoption and impact of the model.

Additionally, considering the ongoing decline in the cattle numbers, expanding this research to other representative provinces could help policymakers develop more durable livestock support strategies.

The limited number of empirical studies conducted on this subject in Turkey presents a wide range of opportunities for future research. Future studies could focus on developing a comprehensive model for CF in each province, tailored to regional products or general agricultural production. Furthermore, supporting the effects of CF on the production process with empirical evidence, rather than limiting studies to model design, would contribute to the creation of a more effective and sustainable structure in practice. In this regard, future research could evaluate the adaptation of the CF model across different product groups and farmer segments, as well as examine the financial and production performances of farmers. Such studies could provide critical data to establish CF as a permanent structure in the Turkish agricultural sector. Additionally, further research in this area would significantly contribute to both national and international literature on contract farming.

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تعیین نگرش کشاورزان فعال در دامپروری نسبت به مدل تولید قراردادی: تحلیلی از ریسک‌ها، انتظارات و پایداری

آدم آکسوی، فردا نور اوزدمیر، و اومیت آوچی اوغلو

چکیده

برای کشاورزان خرده‌پا، گذراز روش‌های تولید سنتی به کشاورزی قراردادی (CF) (Contract Farming= پتانسیل زیاد شدن سود را افزایش می‌دهد. کشاورزی قراردادی با ارائه سود بیشتر به تولیدکنندگان، تنوع بیشتر محصول را تشویق می‌کند و فرصت پرورش محصولات ارزشمندتر را فراهم مینماید. هدف اصلی این پژوهش شناسایی نوع مدل کشاورزی قراردادی (CF) بود که تولیدکنندگان احتمالاً برای ادامه تولید خود می‌پذیرند. در این مطالعه از فرآیند تحلیل سلسله مراتبی (AHP) به عنوان روش استفاده شد. در این منطقه، 70 درصد از تولیدکنندگان از دام‌های ماده برای پرواربندی استفاده می‌کردند. مشخص شد که 73.2 درصد از تولیدکنندگان در مورد کشاورزی قراردادی نا آگاه بودند. متصدیان دام در قراردادهای تولید، تضمین قیمت (26.4 درصد) را اولویت‌بندی کردند و به دنبال آن قیمت‌های نقدی (24.8 درصد)، تامین دام (21.2 درصد)، حمایت ورودی (12.5 درصد)، پرداخت‌های پیشرفته (10.3 درصد) و تولید سازمان‌یافته (4.8 درصد) قرار گرفتند. اگر قرار شد که کشاورزی قراردادی در منطقه اجرا شود، سیاست‌گذاران باید تضمین‌های قیمتی را در مدل در اولویت قرار دهند و اطمینان حاصل کنند که این تضمین‌ها پایین‌تر از قیمت بازار نخواهد بود.

Equilibrium between Education and Research in Agricultural Higher Education: A Pathological Analysis

Fatemeh Soleymani Sardo¹, Masoud Bijani^{1*}, Esmail Karamidehkordi¹, and Fatemeh Sepahvand²

ABSTRACT

Education and research are fundamental pillars of educational systems, including agricultural higher education institutions. However, an inadequate balance between these components can lead to a deviation from the primary goals of these institutions. This study aimed to conduct a pathological analysis of the equilibrium between education and research in the higher education system of agriculture in Iran. "Three-pronged pathology model" was utilized, comprising components of "context" (including knowledge, moral, individual-psychological, satisfaction, and the thematic nature), "behavior" (including planning, coordination, control, and monitoring), and "structure" (including demonstrative, diversity, consequence, and political). This quantitative study was applied in purpose non-experimental in design, retrospective in timing, survey-based in data collection, and descriptive-causal comparative in data analysis. The data collection instrument was a researcher-developed questionnaire, with face and content validity confirmed by experts. The reliability of the questionnaire was established using Cronbach's alpha for each variable ($0.704 \leq \alpha \leq 0.902$). The statistical population comprised faculty members from agricultural departments of public universities in Iran ($N= 3,335$), with a sample of 307 selected. Results indicated a tendency among faculty members towards research activities. The greatest gap and imbalance between education and research were observed in the areas of context, followed by structure and behavior. This indicates that the existing context and structure push faculty members towards research activities for their sustainability and advancement in the agricultural higher education system. This poses a serious issue that must be addressed by policymakers and decision-makers.

Keywords: Agricultural faculty member, Gap analysis, Pathology, Teaching and research balance, Three-pronged model.

INTRODUCTION

In today's rapidly changing world, universities and higher education institutions continuously reassess their positions in response to societal demands. They require a distinctive and clear identity to make appropriate decisions concerning these changes (Zubrick *et al.*, 2001); because the current developments in higher education

have impacted the relationship between education and research (Gutiérrez *et al.*, 2023). These changes include a shift towards mass education, a reconsideration and alteration of scheduling, the nature of education, the political context, and perspectives regarding the existence of knowledge in education and research (Brew, 2010). In this context, universities seek to strengthen the link between education and research to enhance the quality of both

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(Hajdarasic *et al.*, 2015). This interaction creates a space for the transfer of knowledge and research experiences educationally (Gutiérrez *et al.*, 2023). Research focuses on discovering and expanding knowledge, while education relates to distributing and empowering learners (Hattie and Marsh, 1996). Some scholars consider teaching to be the core of educational institutions, while others emphasize the connection between education and research (Brew, 2010).

The balance between education and research in educational systems, particularly in agriculture, is one of the fundamental challenges that requires attention and thorough examination (Doss *et al.*, 2022). In today's world, where rapid changes and the need for innovation in agriculture are acutely felt (Soleimani Sardo *et al.*, 2024), faculty members, as the key components of this system, play a crucial role in establishing and maintaining this balance (Gutiérrez *et al.*, 2023). However, significant gaps exist between the current and the desired states in this area, which can negatively impact the quality of education and research (Hattie and Marsh, 1996). Research indicates that many agricultural faculty members face challenges such as resource shortages, time pressures, and inadequate support (Khan *et al.*, 2023). These challenges affect the quality of education (Chaka, 2022) and may also lead to a decline in research quality (Hasan *et al.*, 2020), ultimately impacting sustainable agricultural development (Akinhanmi, 2023). For example, a lack of financial resources and educational equipment can hinder the provision of quality education and effective research (Lee, 2004). Moreover, time pressures resulting from high workloads can prevent faculty members from focusing on in-depth and quality research (Flores *et al.*, 2020). In this regard, a precise understanding of the faculty members' perspectives on this balance and a pathological analysis of the current situation can help identify the existing strengths and weaknesses (Okoduwa, 2018).

Achieving equilibrium between education and research is crucial in agricultural higher education, yet Iranian institutions face an increasing imbalance. Historically, faculty have been expected to excel in both domains. However, in recent years, a shift has occurred due to evolving incentives and competitive pressures. Policies prioritizing research output, coupled with the need for rapid promotion and participation in international scientific competitions, have inadvertently incentivized faculty to focus more on research. Consequently, education risks becoming a routine task, receiving less attention and dedication. This trend threatens the holistic development of students and the long-term vitality of agricultural knowledge transfer in Iran (Soleimani Sardo *et al.*, 2024).

Undoubtedly, education and research are two fundamental pillars in educational systems, including agricultural higher education institutions. However, an inappropriate balance between these two components leads to a deviation from the primary objectives of such institutions—objectives that foster growth, entrepreneurship, creativity, and ultimately the development of the agricultural sector. Currently, in Iran, agricultural education, which should primarily be skill-oriented, is often delivered in a theoretical manner due to a lack of resources. On the one hand, effective education relies on continuous research, and if education is not practical, there will be a subsequent lack of quality research conducted by trainees. Therefore, faculty members must maintain their clear identity and strengthen the effective linkage between education and research to address the existing challenges and enhance the quality of both education and research. This is a pressing issue that requires responsiveness to the challenges emerging in a changing world and the improvement of education and research quality, particularly in the realm of agricultural higher education. Although some previous studies have examined the balance between education and research among faculty members, this

topic has received less attention in the field of agricultural higher education. Accordingly, the purpose of the study was to analyze the pathological balance between the two pillars of education and research in the higher education system of agriculture in Iran.

To many people, the assertion that universities are established for education and research is indisputable; however, the significance and interrelations between these two domains remain subjects of discussion and examination. Researchers have analyzed this relationship in various ways. The perspective of Hattie and Marsh (1996) is recognized as one of the most important classifications in this area, highlighting three types of relationships between education and research: positive, negative, and neutral.

A negative relationship between education and research arises when individuals engaged in research devote most of their time, energy, and commitment to research, while those focused on education concentrate all their efforts in this area. From this viewpoint, concurrently achieving high productivity in both research and education becomes an intensive task, making excellence in both domains seem nearly impossible. Education necessitates a focus on knowledge transfer, whereas research concentrates on the discovery and generation of new results (Bajaj, 2022). These differences may diminish productivity in both fields and reflect the distinct personality traits of educators and researchers. Research and education have contradictory roles with varying expectations and commitments, resulting from different reward systems (Bowering *et al.*, 2021). These systems focus on the generation of new knowledge and its transfer, each requiring substantial time and commitment (Åkerfeldt *et al.*, 2020). Researchers need more time and resources for advancement in scientific fields, whereas educators require energy and time for effective teaching (Chen and Lee, 2022).

In a positive relationship, research and education are conducted simultaneously and

interactively, reinforcing one another. Researchers produce new knowledge by pursuing new questions that are directly utilized in educational activities. This aids educators in conveying contemporary knowledge to students, who, by acquiring the latest skills, contribute to the production of new knowledge (Hattie and Marsh, 1996).

In arguments regarding the absence of a relationship between education and research, it is believed that research and education are two distinct activities. Research involves the discovery of new knowledge, while education refers to the transfer of that knowledge to others. These two domains require different skills and approaches, with individuals evaluated separately in each (Nguyen and Miller, 2023). This separation can have its advantages. For instance, it allows educators to be more influenced by the actual needs of learners and can enhance the quality of education and learning through teachers' focus on instruction, while researchers can concentrate all their energy on research activities to contribute to new knowledge production (Bozeman *et al.*, 2013).

A review of the theoretical literature indicates the importance and emphasis on the relationship and connection between education and research in higher education systems (Karim *et al.*, 2024). Research motivation among faculty members leads to improved educational quality, while strengthening education enhances research productivity. A sole focus on either education or research can decrease the quality of the other. Researchers believe that effective teaching is essential for success in research, and on the other hand, the relationship between research and teaching must be developed effectively to optimize the learning process (Alshammari, 2023).

Reflecting on the above, it can be understood that education and research are two fundamental pillars in any higher education system, including agricultural higher education, which require simultaneous attention. However, lack of balance between these two can lead to



adverse effects. To address such issues, there is an urgent need for “pathological analyses.” Pathology is the process of examining and analyzing the causes, symptoms, signs, and consequences of a problem across various scientific domains. The main goal of pathology is to identify the problems in the normal progression of phenomena and activities and to gain a better understanding of the causes or factors that lead to abnormalities in a system, which necessitates determining appropriate solutions to rectify these abnormalities and ensure the health and improvement of the system (Majidi *et al.*, 2017).

For pathological analysis in diverse contexts, various theories and models have been proposed including the following pathological models (Majidi *et al.*, 2018):

1. Lewin and Lovit's organizational model,
2. Nadler-Tushman's organizational model,
3. McKinsey 7S model,
4. Harrison's individual and group behavior model,
5. The four-frame model,
6. The pathological three-pronged model.

One of the best models for pathological analysis that has already been used in the agricultural higher education system is the three-pronged model. The theoretical framework of this research is based on the “three-pronged” model, which encompasses the components of “context,” “content,” and “structure” (Figure 1). Behavioral factors include motivation and satisfaction of human resources, while structural factors relate to the internal relationships of the system (Kundi *et al.*, 2023). Contextual factors also include the environment and external conditions that affect other factors (Zhang and Shin, 2015).

MATERIALS AND METHODS

This quantitative research is applied in purpose, non-experimental in design and variable control, retrospective in timing, survey-based in data collection, and descriptive-causal comparative in data analysis. The data collection instrument was a researcher-developed questionnaire, whose face and content validity were confirmed by a panel of experts in the field of agricultural higher education. The reliability of the

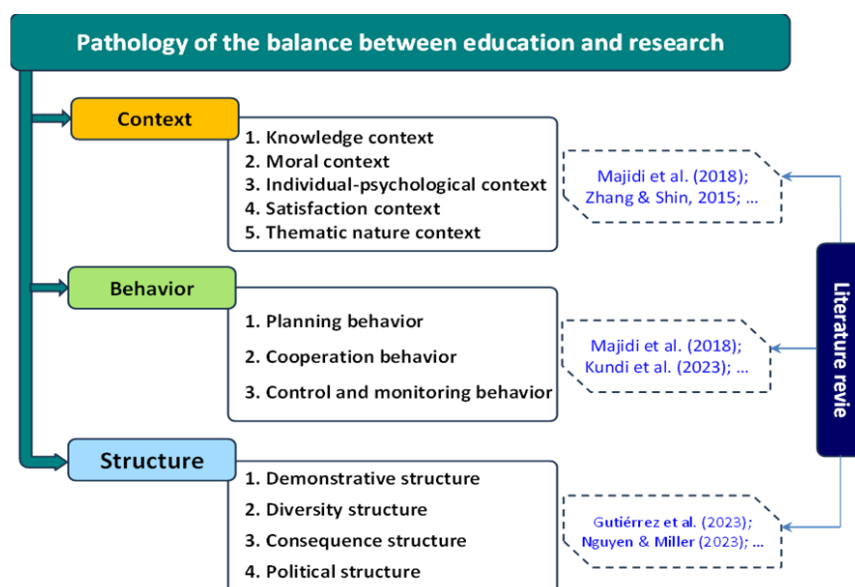


Figure 1. Three-pronged model as a research conceptual framework.

questionnaire was also established using Cronbach's alpha ($0.704 \leq \alpha \leq 0.902$) for the item categories of each variable, through a pilot test conducted outside the statistical population with 30 samples (Table 1).

The statistical population of the study included faculty members from agricultural departments of public universities in Iran ($N= 3,335$). Using the Krejcie and Morgan sampling table (Krejcie and Morgan, 1970), 345 individuals were selected as a sample. Ultimately, 307 completed questionnaires were returned, resulting in a response rate of approximately 89%. The sample selection occurred in three stages. Scientific poles in Iran were initially selected using stratified random sampling. Subsequently, universities within each pole were chosen via cluster sampling. Finally, stratified random sampling with proportional allocation was used to select agricultural education groups as strata.

The research questionnaire assessed the main components of the study (Figure 1), which includes context (encompassing knowledge, moral, individual-psychological, satisfaction, and thematic aspects), behavior (covering planning, coordination, control, and monitoring behaviors), and structure (including demonstrative, diversity, consequence, and political structures), each evaluated through a number of items (Table 1). Respondents were asked to express their opinions on the two pillars "Education" and "Research" using a 5-point Likert scale (Very low= 1, Low= 2, Medium= 3, High= 4, and Very high= 5). The collected data were analyzed using SPSS₂₇ software.

RESULTS AND DISCUSSION

Respondents' Descriptive Statistics

Approximately 87% (258) respondents were male, and the rest (about 13%) were female. Most of them (about 95%) were married. The average age of the respondents was approximately 48 years. The average work experience was about 19 years, with a

minimum of 1 year and a maximum of 46 years. About half of them held the rank of associate professor (Table 2).

Descriptive Statistics of the Components of the Three-Pronged Theory

Descriptive statistics measuring the items that make up the components of the three-pronged pathology theory are presented in Tables 3-5. The results indicate that, from the respondents' perspective, each item has a higher average in research compared to education. In fact, this reflects a greater inclination among faculty members to focus on research activities rather than educational activities. This finding differs from the results of Gutiérrez *et al.* (2023) but aligns with the research of Soleymani Sardo *et al.* (2024).

Comparative Analysis

To conduct a comparative analysis between the two pillars of education and research based on the theoretical framework presented (Figure 1), comparisons were made among the three components: "context" (including knowledge, moral, individual-psychological, satisfaction, and thematic nature), "behavior" (including planning, coordination, control, and monitoring), and "structure" (including demonstrative, diversity, consequence, and political) using the Paired-samples t-test for each of the stated components (Table 6).

The results of the paired comparisons in Table 6 indicate that the greatest disparity, from the respondents' perspective, lies within the context component, with a mean difference of -50.25. Following this, the structure component shows a mean difference of -48.34, while the behavior component exhibits the smallest difference at -39.75.

This statement of discrepancy is illustrated as a pyramid in Figure 2, based on the three



Table 1. Cronbach's Alpha test results for research variables.

Component	Variable		Number of items		Cronbach's Alpha (α)		Semantic judgment ^a
Context	Knowledge	In educcastion	6	12	0.753	0.835	Good
		In research	6		0.647		
	Moral	In educcastion	6	12	0.834	0.776	Acceptable
		In research	6		0.504		
	Individual-psychological	In educcastion	6	12	0.772	0.831	Good
		In research	6		0.580		
Satisfaction	In educcastion	6	12	0.711	0.772	Acceptable	
	In research	6		0.725			
Thematic nature	In educcastion	6	12	0.753	0.865	Good	
	In research	6		0.755			
Behavior	Planning	In educcastion	9	18	0.792	0.886	Good
		In research	9		0.779		
	Cooperation	In educcastion	7	14	0.844	0.845	Good
		In research	7		0.771		
Control and monitoring	In educcastion	5	10	0.852	0.902	Perfect	
	In research	5		0.826			
Structure	Demonstrative	In educcastion	6	12	0.809	0.755	Acceptable
		In research	6		0.735		
	Diversity	In educcastion	6	12	0.681	0.704	Acceptable
		In research	6		0.673		
	Consequence	In educcastion	3	6	0.651	0.708	Acceptable
		In research	3		0.770		
Political	In educcastion	8	16	0.654	0.816	Good	
	In research	8		0.762			

^a Source: Habibpour and Safari (2009): $0.90 \leq \alpha$: Perfect; $0.80 \leq \alpha < 0.90$: Good; $0.70 \leq \alpha < 0.80$: Acceptable; $0.60 \leq \alpha < 0.70$: With hesitation; $0.50 \leq \alpha < 0.60$: Weak; $\alpha < 0.50$: Not acceptable.

Table 2. Descriptive statistics of some personal and professional characteristics of the respondents.^a

Variable	Level	Frequency	Percent	Valid percent	Cumulative percent	Min	Max	Mode	Median	M	SD
Gender	Female	37	12.05	12.54				Gender			
	Gender	258	84.03	87.46							
	No response	12	3.92								
Age (year)	$X_i \leq 35$	19	6.19	6.31	6.31						
	$35 < X_i \leq 50$	151	49.20	50.17	56.48	34	70	41	45	48.15	9.362
	$50 < X_i$	131	42.67	43.52	100						
	No response	6	1.40								
Marital status	Single	25	8.14	8.14				Married			
	Married	267	86.97	95.11							
	No response	15	4.89								
Work experience (year)	$X_i \leq 5$	7	2.28	2.31	2.31						
	$5 < X_i \leq 10$	75	24.43	24.75	27.06						
	$10 < X_i \leq 15$	73	23.78	24.09	41.15						
	$15 < X_i \leq 20$	65	21.17	21.45	72.60	1	43	12	20	19.26	10.462
	$20 < X_i$	83	27.04	27.40	100						
Scientific rank	Assistant Professor	97	31.60	32.33	32.33			Associate Professor			
	Associate Professor	158	51.46	52.67	85.00						
	Professor	45	14.66	15.00	100						
	No response	7	2.28								

^a Min: Minimum, Max: Maximum, M: Mean; SD: Standard Deviation.

Table 3. Descriptive statistics and ranking of the items of the “context” component in the analysis of the balance between education and research.^a

In education			Variable	Row	Items	In research		
Rank	SD	M				M	SD	Rank
4	1.254	2.83	Knowledge	1	Your awareness of existing laws and regulations	4.58	0.751	1
1	1.039	3.11		2	Your level of expertise	4.57	0.733	2
6	1.045	2.68		3	Your level of knowledge in teaching/research techniques	4.58	0.751	1
4	0.964	2.70		4	The level of knowledge of other faculty members about laws, techniques and specialized subjects	4.57	0.733	2
3	1.017	2.83		5	The amount of notification of relevant laws and regulations by the university	4.58	0.751	1
2	1.375	3.52		6	Existence and provision of related workshops and training courses	4.06	0.395	3
	3.572	17.67	Mean		(Between 6 and 30)	26.97	2.581	
1	1.108	2.80	Moral	1	The existence of different conditions, criteria and ethical regulations	4.58	0.765	2
2	0.937	2.75		2	Variety of disciplinary regulations	4.57	0.733	3
5	0.964	2.20		3	Supervision and monitoring in compliance with the points of affairs	4.58	0.751	1
4	0.943	2.35		4	Disciplinary action by the university	4.57	0.733	3
6	0.999	2.17		5	Compliance with ethical and disciplinary principles (Such as copying and plagiarism) by you	4.58	0.751	1
3	0.971	2.52		6	Compliance with ethical and disciplinary principles by other colleagues	4.06	0.395	4
	3.217	14.79	Mean		(Between 6 and 30)	26.96	2.595	
3	0.943	2.44	Individual- psychological	1	Ease of doing things	4.29	0.834	6
1	1.126	2.78		2	A pleasure to do things for you	4.46	0.708	2
5	0.943	2.39		3	The level of interest and having a favorable attitude towards doing things	4.36	0.742	5
3	1.260	2.78		4	The level of motivation to do things	4.48	0.660	1
6	1.098	2.27		5	Attitudes and views of other colleagues towards educational/research matters	4.40	0.716	4
2	1.135	2.78		6	The sensitivity of the scientific community of your field in educational/research cases	4.45	0.673	3
	4.221	15.44	Mean		(between 6 and 30)	26.25	3.500	
3	1.227	2.71	Satisfaction	1	Your level of satisfaction with the results of doing things	4.29	0.834	6
2	1.124	2.73		2	The level of satisfaction with the variety of different activities	4.46	0.708	2
6	0.996	2.42		3	The fruitfulness of the results	4.36	0.742	5
4	1.047	2.67		4	How satisfied your colleagues are with the usefulness of the results	4.48	0.660	1
5	1.110	2.53		5	The existence of material incentives necessary to do things	4.40	0.716	4
1	1.319	2.92		6	The existence of spiritual incentives necessary to do things	4.45	0.673	3
	4.154	15.99	Mean		(between 6 and 30)	26.44	3.586	
2	0.979	2.57	Thematic nature	1	The concreteness of the results of doing things	4.46	0.708	2
4	0.956	2.37		2	Ease of doing things	4.36	0.742	4
3	0.879	2.56		3	It is possible to do things	4.48	0.660	1
1	1.103	2.71		4	Managing affairs	4.40	0.716	3
4	2.589	10.23	Mean		(Between 4 and 20)	17.70	2.391	
	10.527	74.06	Total mean		(Between 28 and 140)	124.31	11.332	

^aM: Mean; SD: Standard Deviation. Rank: The ranking is based on the mean. In the cases where the means were equal, the lower standard deviation was the higher ranking criterion.

mentioned components and their constituent variables, indicating that as one move from the base of the pyramid to its apex, the discrepancies diminish. This means that the existing contexts and structures direct the faculty members in agricultural higher education institutions towards a greater focus on educational activities. In fact, the current contexts and structures are designed to compel faculty

members to pursue this emphasis. This finding aligns with the studies conducted by Hattie and Marsh (1996), Brew (2010), and Hajdarpasic *et al.* (2015).

CONCLUSIONS

The equilibrium between education and research in the agricultural higher education



Table 4. Descriptive statistics and ranking of the items of the “behavior” component in the analysis of the balance between education and research.^a

In education			Variable	Row	Items	In research		
Rank	SD	M				M	SD	Rank
8	0.915	2.66	Planning	1	Appropriate design and planning by you	4.61	0.643	3
5	1.096	2.72		2	Appropriate design and planning by the university	4.62	0.663	2
1	1.049	2.96		3	Proper implementation of programs by you	4.62	0.618	1
7	1.053	2.69		4	Proper implementation of programs by the university	4.58	0.751	4
3	1.065	2.78		5	Proper control of program execution by you	4.57	0.733	5
4	0.973	2.75		6	Appropriate control of program implementation by the university	4.58	0.751	4
2	1.034	2.89		7	Appropriate evaluation of programs by you	4.57	0.733	5
9	0.908	2.59		8	Appropriate evaluation of programs by the university	4.58	0.751	4
6	0.951	2.71		9	The amount of follow-up and analysis of the effectiveness of activities	4.57	0.733	5
5.398		24.75	Mean	(Between 9 and 45)		41.34	4.1285	
5	0.985	2.55	Cooperation	1	The extent to which you cooperate with other people in carrying out activities at the university or national level	4.62	0.663	2
1	1.019	2.70		2	The extent of your cooperation with other people in carrying out activities at the international level	4.62	0.618	1
6	0.925	2.48		3	The extent to which other people cooperate with you in carrying out activities at the university or national level	4.58	0.751	3
7	1.107	2.47		4	The degree of cooperation of other people with you in carrying out activities at the international level	4.57	0.733	4
4	1.047	2.64		5	Easy to work with others	4.58	0.751	3
2	1.066	2.69		6	The willingness of other colleagues to cooperate	4.57	0.733	4
3	0.960	2.67		7	Your willingness to cooperate with other people in doing activities	4.58	0.751	5
4.161		18.21	Mean	(Between 7 and 35)		32.15	3.331	
1	0.967	2.92	Control and monitoring	1	The degree of control and monitoring of the realization of goals and strategies by you	4.58	0.751	1
2	1.085	2.79		2	The amount of control and monitoring of how you perform activities	4.57	0.733	2
5	0.880	2.61		3	The degree of control and monitoring of organizational culture by you	4.58	0.751	1
4	1.041	2.69		4	Monitoring the allocation and optimal consumption of resources by you	4.57	0.733	2
3	1.012	2.73		5	Existence and diversity in evaluation and feedback by you	4.62	0.618	3
3.575		13.73	Mean	(Between 5 and 25)		22.94	2.293	
			Total mean	(Between 21 and 105)		96.43	9.795	

^a M: Mean; SD: Standard Deviation. Rank: The ranking is based on the mean. In the cases where the means were equal, the lower standard deviation was the higher ranking criterion.

Table 5. Descriptive statistics and ranking of the items of the “structure” component in the analysis of the balance between education and research.^a

In education			Variable	Row	Items	In research		
Rank	SD	M				M	SD	Rank
1	1.098	2.69	Demonstrative	1	Benefiting from the benefits of publication or transfer of results	4.58	0.765	2
6	1.049	2.31		2	Ease of publishing, transferring or exchanging the results of activities at the provincial or national level	4.57	0.733	3
4	1.105	2.45		3	The possibility of seeing the results of activities at the provincial or national level	4.58	0.751	1
5	1.004	2.33		4	Ease of publishing, transferring or exchanging the results of activities at the international level	4.57	0.733	3
2	1.058	2.62		5	The possibility of seeing the results of activities at the international level	4.58	0.751	1
3	1.113	2.55		6	Existence of channels and ways of publishing, transferring or exchanging results (Magazines, books, website, conference, workshop, etc.)	4.06	0.395	4
3.642	14.96	Mean			(Between 6 and 30)	26.96	2.595	
1	1.019	2.63	Diversity	1	The amount of variety of laws, regulations and guidelines for doing different things	4.63	0.655	2
5	0.998	2.34		2	The possibility of working with other faculty members and experts at the university and national levels	4.61	0.643	5
4	1.082	2.44		3	The possibility of cooperation with other faculty members and experts at the international level	4.62	0.663	4
2	1.162	2.63		4	The amount of diversity in the choice of topics for activities	4.62	0.618	3
3	1.023	2.53		5	The variety of incentives offered at the university or national level	4.63	0.639	1
6	0.874	2.20		6	The amount of variety of incentives offered at the international level	4.06	0.395	6
3.688	14.78	Mean			(Between 6 and 30)	27.18	2.252	
1	0.933	2.45	Consequence	1	The existence of sufficient rules, regulations and guidelines in the university or Ministry of Ataf for publishing, transferring or exchanging results at the national level.	4.61	0.643	3
3	1.068	2.29		2	The existence of laws, rules of the Ummah and sufficient guidelines in the university or Ministry of Ataf for publishing, transferring or exchanging results at the international level.	4.62	0.663	2
2	0.843	2.34		3	Necessary infrastructure to monitor or follow up the educational/research successes of faculty members (For example, referrals)	4.62	0.618	1
2.066	7.08	Mean			(Between 3 and 15)	13.86	1.356	
6	0.927	2.38	Political	1	University policies to deliver different outcomes of activities	4.61	0.643	3
3	1.126	2.55		2	University policies to provide incentives for doing activities	4.62	0.663	2
5	0.918	2.39		3	The existence of a necessary policy structure to guarantee the implementation and continuation of activities by the university	4.62	0.618	1
4	1.037	2.47		4	Sustainable policy in maintaining the productivity of activities	4.58	0.751	4
1	0.966	2.63		5	The extent of your compatibility and coordination with existing regulations and policies	4.57	0.733	5
7	1.017	2.35		6	Availability of financial resources to do things	4.58	0.751	4
2	1.039	2.58		7	Availability of physical facilities and equipment to do things	4.57	0.733	5
8	0.908	2.26		8	Availability of sufficient human resources to do things	4.58	0.751	4
4.246	19.61	Mean			(Between 8 and 40)	36.76	3.694	
9.264	56.43				(Between 23 and 115)	104.77	9.573	

^a M: Mean; SD: Standard Deviation. Rank: The ranking is based on the mean. In the cases where the means were equal, the lower standard deviation was the higher ranking criterion.



Table 6. Paired-samples t-test results for comparing the components and variables used in the analysis of the balance between education and research.

Component	Variable	Mean of education	Mean of research	t	Sig.
Behavior	Control and monitoring	13.73	22.94	-9.21	0.008
	Cooperation	18.21	32.15	-13.94	0.000
	Planning	24.75	41.34	-16.59	0.000
	Total	56.68	96.43	-39.75	0.000
Structure	Consequence	7.08	13.86	-6.78	0.045
	Demonstrative	14.96	26.96	-12.00	0.000
	Diversity	14.78	27.18	-12.40	0.000
	Political	19.61	36.76	-17.15	0.000
	Total	56.43	104.77	-48.34	0.000
Context	Thematic nature	10.23	17.70	-7.47	0.030
	Knowledge	17.67	26.97	-9.30	0.008
	Satisfaction	15.99	26.44	-10.45	0.004
	Individual-psychological	15.44	26.25	-10.81	0.005
	Moral	14.79	26.96	-12.17	0.000
	Total	74.06	124.31	-50.25	0.030
Comparison of three components		187.12	325.51	-138.39	0.000

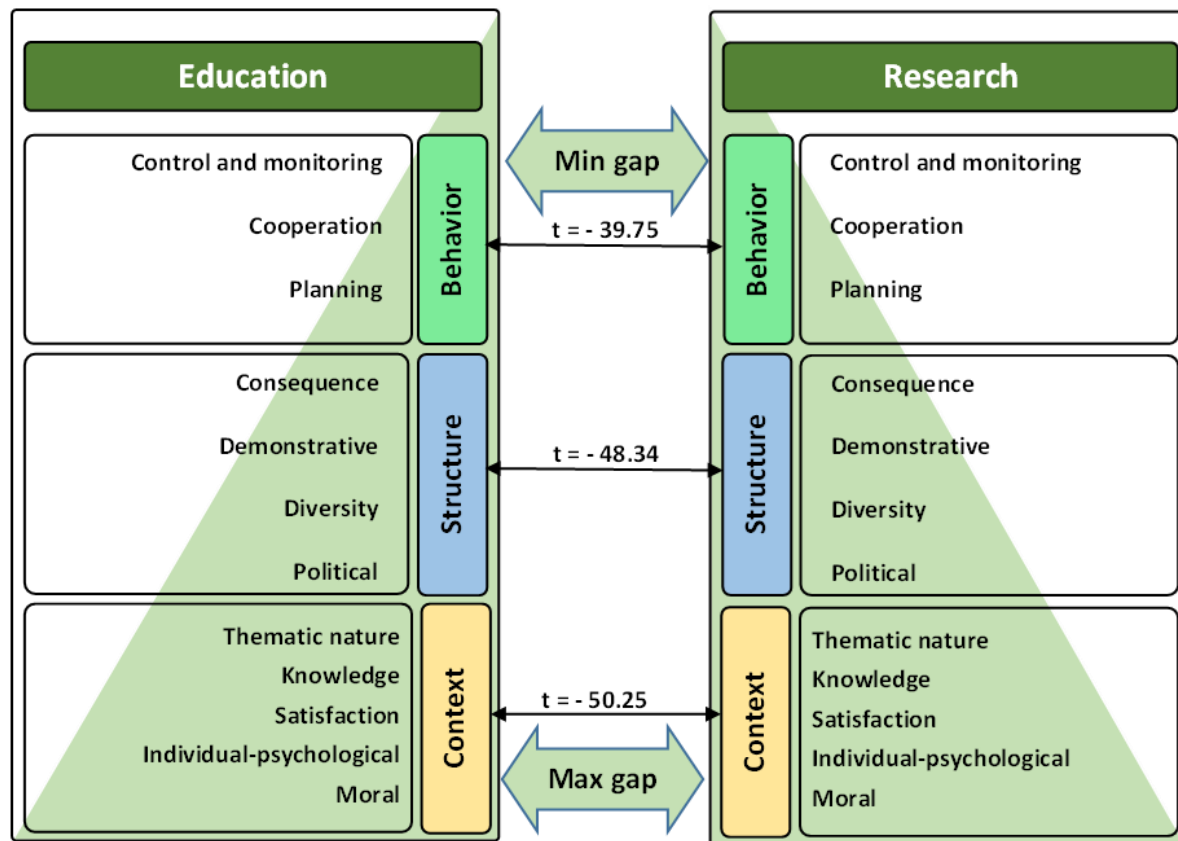


Figure 2. Pyramid analysis of the gap between the focus of agricultural faculty members on education and research.

system has become a complex and challenging issue as a key component of sustainable development in this field. This study, which conducts a pathological

analysis of this equilibrium, reveals a significant tendency among faculty members towards research activities, resulting in a noteworthy gap between education and

research. This situation is a consequence of the inefficiency of structures, contexts, and also unstable behaviors in planning and control. The results further indicate that education, as a fundamental pillar in the agricultural education system, has been marginalized due to insufficient attention from faculty members and policymakers. Specifically, the imbalance among context, structure, and behavior in this system has led to neglecting some essential aspects of education and, ultimately, diminished the quality of scientific and technological production in the agricultural sector. Therefore, policymakers and decision-makers in the agricultural higher education system need to address these gaps and work towards strengthening the interaction between these two key elements. In this regard, the following policy suggestions can be proposed:

Formulating Incentive Policies for Balancing Education and Research: Decision-making bodies should develop policies enhancing the balance between education and research. For instance, incentive programs could be established for faculty members actively engaged in both areas. These programs should include financial benefits, career advancements, and awards for those who proportionately dedicate their efforts to both domains.

Strengthening Research-Based Educational Programs: Universities should focus on developing educational programs centered on research, where students gain practical experience through group projects and research activities. This approach can enhance the educational and research quality and enable students to operate more effectively in various fields.

Creating a More Interactive Environment between Faculty Members and Students: Organizing workshops and joint meetings between faculty members and students can facilitate knowledge exchange and foster stronger connections between research and education. Such interactions can assist in refining educational programs

and better reflecting the real needs of students and the job market.

Continuous Monitoring and Evaluation of Educational and Research Outputs: Establishing performance evaluation systems to continuously assess educational and research outputs is essential. These systems can help identify strengths and weaknesses, leading to the formulation of appropriate policies based on the findings.

Despite its strengths, this study has limitations. One major limitation is that the findings are based solely on the opinions of agricultural faculty members. Consequently, the perspectives of other stakeholders, such as students, policymakers, and employers, have been overlooked, and potentially different viewpoints regarding the balance between education and research may not have been considered in this analysis. Furthermore, conducting such investigations using qualitative or mixed methods could provide a deeper understanding of the current situation and yield more reliable results. Future research should explore a broader range of stakeholder perspectives and utilize various methodologies to achieve a more comprehensive understanding.

ACKNOWLEDGEMENTS

The authors would like to express their sincere gratitude to all the faculty members who participated in this study by completing the research questionnaires. Their contributions have been invaluable to the success of this research. The insights and perspectives provided by the respondents on the balance between education and research in agricultural higher education are crucial for understanding the current dynamics within this field. Without their willingness to engage and share their experiences, this study would not have been possible. The authors appreciate the time and effort dedicated by the faculty members in providing thoughtful and comprehensive responses, which will ultimately aid in the development of policies aimed at enhancing



the equilibrium between education and research in agricultural institutions. Additionally, the authors would like to acknowledge and thank Tarbiat Modares University for its financial support in funding this project, which has made this research possible.

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تعداد آموزش و پژوهش در آموزش عالی کشاورزی: تحلیل آسیب شناختی

فاطمه سلیمانی ساردو، مسعود بیژنی، اسماعیل کرمی دهکردی، و فاطمه سپهوند

چکیده

آموزش و پژوهش از ارکان اساسی نظام های آموزشی از جمله مؤسسات آموزش عالی کشاورزی هستند. با این حال، تعداد ناکافی بین این مؤلفه ها



می تواند منجر به انحراف از اهداف اولیه این مؤسسات شود. این مطالعه با هدف تحلیل آسیب شناختی تعادل آموزش و پژوهش در نظام آموزش عالی کشاورزی ایران انجام شد. از "مدل آسیب شناسی سه وجهی" استفاده شد که شامل اجزای "زمینه" (شامل دانش، اخلاقی، فردی-روانی، رضایت و ماهیت موضوعی)، "رفتار" (شامل برنامه ریزی، هماهنگی، کنترل و نظارت) و "ساختار" (شامل نمایشی، تنوع سیاسی، پیامد) است. این مطالعه کمی از نظر هدف کاربردی، از نظر طراحی غیرتجربی، از نظر زمان بندی گذشته نگر، در جمع آوری داده ها پیمایشی و در تحلیل داده ها از نوع توصیفی-علی مقایسه ای است. ابزار گردآوری اطلاعات، پرسشنامه محقق ساخته بود که روایی صوری و محتوایی آن توسط خبرگان تایید شد. پایایی پرسشنامه با استفاده از آلفای کرونباخ برای هر متغیر ($0.704 \leq \alpha \leq 0.902$) به دست آمد. جامعه آماری شامل اعضای هیأت علمی گروه های کشاورزی دانشگاه های دولتی ایران ($N=3335$) با نمونه 307 نفر انتخاب شد. نتایج حاکی از گرایش اعضای هیئت علمی به فعالیت های پژوهشی بود. بیشترین شکاف و عدم تعادل بین آموزش و پژوهش در حوزه های بافت و پس از آن ساختار و رفتار مشاهده شد. این نشان می دهد که بافت و ساختار موجود، اعضای هیئت علمی را به سمت فعالیت های تحقیقاتی برای پایداری و پیشرفت در سیستم آموزش عالی کشاورزی سوق می دهد. این یک موضوع جدی است که باید توسط سیاست گذاران و تصمیم گیران مورد توجه قرار گیرد.

Research Note: Gill Histopathological Lesions of Common Carp in Exposed to Chlorpyrifos and Salinity

Mona Hasankhany^{1*}, Tina Hasankhani², Seyed Ali Akbar Hedayati¹, and Mohammad Mazandarani¹

ABSTRACT

The effect of poison to salinity were studied on gill tissue of common carp (*C. carpio*). Based on this, 250 common carp fry with an average weight of 21 ± 2 g were distributed in four treatments, each with three repetitions, including salinities of zero, 4, 8, and 12 ppt. After 7 days, one group was placed for 4 days in exposure to poison with an acute concentration of 150 ppm chlorpyrifos with the formulation of 40.8% EC, and the second group was placed for 7 d in the sub-acute concentration of 15 ppm chlorpyrifos. Histopathology of Gill tissue showed that the poison and salinity had such injuries as epithelial hypertrophy, lamellar aneurism, secondary connecting adjacent blades, distal hyperplasia, epithelial lifting, leukocyte infelt, and hyperplasia. Gill histopathological result showed some lesions at high concentrations as epithelial hypertrophy, distal hyperplasia and lamellar fusion, however at low concentrations lamellar aneurism, epithelial lifting and leukocyte infelt were recorded. Therefore, these pathological indicators can be used as biomarkers.

Keywords: Agriculture poison, Histological lesions, Toxicology.

INTRODUCTION

Chlorpyrifos, Dursban, Imidacloprid and Confidor insecticides are widely used in Iran (Shafiei *et al.*, 2023). Chlorpyrifos belongs to a group of organophosphorus pesticides, which exhibit a wide spectrum of biological activity (Perry *et al.*, 2020). Chlorpyrifos is the common name for the chemical 0,0-diethyl 0-(3,5,6-trichloro-2-pyridinyl)-phosphorothioate (Figure 1). Application of Chlorpyrifos for agricultural purposes results in its dispersion into various environmental components such as air, soil, rivers and lakes, disrupting ecosystem functioning (Mackay *et al.*, 2014). When pesticides enter water bodies, they can exert direct toxic effects on fish, resulting in impaired reproduction, loss of balance, impaired growth, disruptions of

physiological, convulsions and mortality (Esbaugh *et al.*, 2018). Gills are in contact with the external environment, they are directly affected by many stress factors and toxic substances in aquatic areas (Bury *et al.*, 2014). Increasing evidence indicates that fish gills are excellent biomarkers for analyzing the impact of contaminants in aquatic ecosystems due to their high permeability and large area of water exposure. Gill surfaces make up 50% of the total surface in fish. When gills are affected by pollutants in water, normal physiological activities such as respiration, excretion, and ion transport are inevitably affected, impacting fish survival and even causing death. Additionally, pesticides can bio-accumulate within the tissues of fish. As they move up the food chain, predatory fish such as larger species

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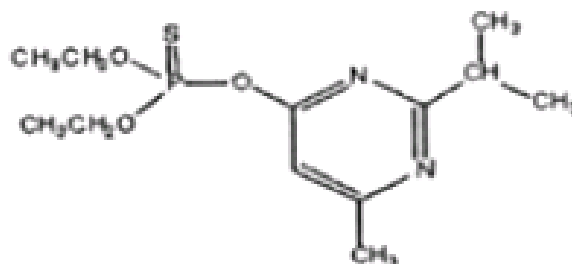


Figure 1. Biochemical structure of chlorpyrifos (Cocker *et al.*, 2002).

can accumulate significant levels of these chemicals, posing a threat to both their own health and that of species (Rohani, 2023).

Common carp (*C. carpio*) is commercially an important species and cultured throughout the world. It belongs to Cyprinidae family and distributed in all watersheds. *C. carpio* are important components of freshwater ecosystems and represent one of the major sources of nutrients for people in Asia, especially China. *C. carpio* is relatively insensitive and can survive and accumulate contaminants at heavily polluted sites, which is why it is used as a bio-indicator in environmental toxicology (Sanoesi *et al.*, 2020). Therefore, our aim was to detect the remarkable histopathological alterations in gills of *C. carpio* exposed to chlorpyrifos and salinity.

MATERIALS AND METHODS

Materials

This research was conducted in Shahid Fazli Aquaculture Hall, Faculty of Fisheries, Gorgan University of Agricultural Sciences and Natural Resources. Two hundred and fifty common carps, ranging 21 ± 2 g in weight, were transferred from the rearing place of the private center to Veniro. Common carp were divided into four treatments, conducting three phases of trials with each phase consisting of 84 fish in three replicates: It is important to note that the fish did not receive any poison through-out the first phase. The only variable factor was

different concentrations of salinity i.e. 0, 4, 8 and 12 ppt, for 7 days. Salinity treatments include 0, 4, 8 and 12 ppt, for treatments 1, 2, 3, and 4 respectively, were exposed to 15 ppm chlorpyrifos for 7 days. For the next phase of the experiment; salinity 0, 4, 8 and 12 ppt, were placed for 4 days in acute expose of 150 ppm chlorpyrifos. Fish were subjected. Fish were subjected to a one-week period of acclimatization prior to the start of the experiment. Fish were fed with commercial pellets twice daily at 3% body weight (Hasankhani *et al.*, 2023). Feeding was discontinued 24 hours prior to the test, and the water was changed every 24 hours from the prepared stocks. Physicochemical conditions were monitored daily. The water temperature was $21 \pm 1^\circ\text{C}$ and pH was kept at 7.6 ± 1 . No mortality was observed during this period.

Salty Caspian Sea Water

The sea water salinity from the shores of Port Turkmen was checked with a salinometer (Atago Refractometer, Japan) (Ataimehr *et al.*, 2009) to match the salinity intended for the experiment (Moustakas *et al.*, 2004). Then, juvenile fish were transferred to tanks individually and were exposed to 15 and 150 ppm of chlorpyrifos at varying salt concentration of 0, 4, 8 and 12 ppt.

Effective physicochemical parameters of water including pH, dissolved oxygen, and temperature were recorded daily (Huyben *et al.*, 2018).

Determination of Acute and Sub-Acute LC50 Toxicity Concentrations

In this study, the statically acute (150 ppm) and sub-acute (15 ppm) toxicity (Hasankhany *et al.*, 2020) of chlorpyrifos on *C. carpio* was determined according to standard methods OECD in 4 and 7 days (Hedayati *et al.*, 2013; Gao *et al.*, 2020).

Histopathological Examination

The second gill arches from opercula cavities were harvested. Gills from each fish were fixed in 10% neutral buffered formalin. The tissues were then dehydrated in graded series of ethanol, embedded in paraffin and sectioned at 5 μm , and were stained with (H&E) stains (Hedayati *et al.*, 2013). The slides were observed under a light microscope at 40X magnification and were photographed with Nikon, TS100 digital camera attached to the microscope.

Statistical Analysis

The test results were calculated in Excel and LC50 with PROBIT software. The calculated LC50 was found to be 150 ppm for 96 h. The following method was used to describe the severity of the pathological change: (-) No alteration, (+) Mild alteration, (++) Moderate alteration, and (+++) Severe alteration. These symptoms indicate the severity of complications (Riba *et al.*, 2005; Roy *et al.*, 2013).

RESULTS AND DISCUSSION

Fish Behavior

Fish applied with 150 ppm chlorpyrifos did not show any change in food starting from day 1.

Moreover, their aggression and remaining motionless on the bottom were also observed. The progressive darkening in the body

coloration with slight mucus secretion was recorded as a clinical sign of toxicity (Mazandarani *et al.*, 2015). The calculated LC50 was found to be 150 ppm for 96 hours. Mortality was observed during this period. A decline in the rate of swimming after 4 days was studied in *C. carpio*, when exposed to chlorpyrifos, as the concentration increased from 0.1 to 2.2 mg L^{-1} (Xing *et al.*, 2015) and the 96 hours LC (50) of chromium salt, potassium dichromate was determined to be 41.75 mg L^{-1} (Mishra and Mohanty, 2008). The chlorpyrifos (5 ppm) exposed group shows loss of balance, swimming pattern, food search behavior and convulsions were noticed on after 10 days exposure (Stalin *et al.*, 2019). On exposure to chlorpyrifos, the Japanese medaka showed abnormality in swimming (Khalil *et al.*, 2013).

Histopathological Findings

Histopathologic studies showed a series of changes in gill tissue, including primary lamellar edema, hyperplasia, severe secondary lamellar fusion, and clubbing (Schlenk and Benson, 2001) (Figure 2). Moreover, the imposed stress was responded in a tissue-specific manner and histological lesions became more severe with increasing concentration (Dogan *et al.*, 2022).

Figure 2 illustrates an increase in the lamellar fusion originating from the mucus cells located at the base of the lamellae, leading to the merging between secondary lamellae (Kakade *et al.*, 2020). Another damage that occurred was hyperplasia. The interlamellar space, which is the aqueduct and the mucus or mucus production space, can be blocked due to hyperplasia of epithelial cells originating from the primary filaments. Gill is usually the first target tissue for waterborne contaminants, and prolonged exposure results in the absorption of these pollutants through the gills, producing visible damage effects in this tissue.

Through increasing the duration of exposure and an increasing dose of the poisoning agent, epithelium thoroughly

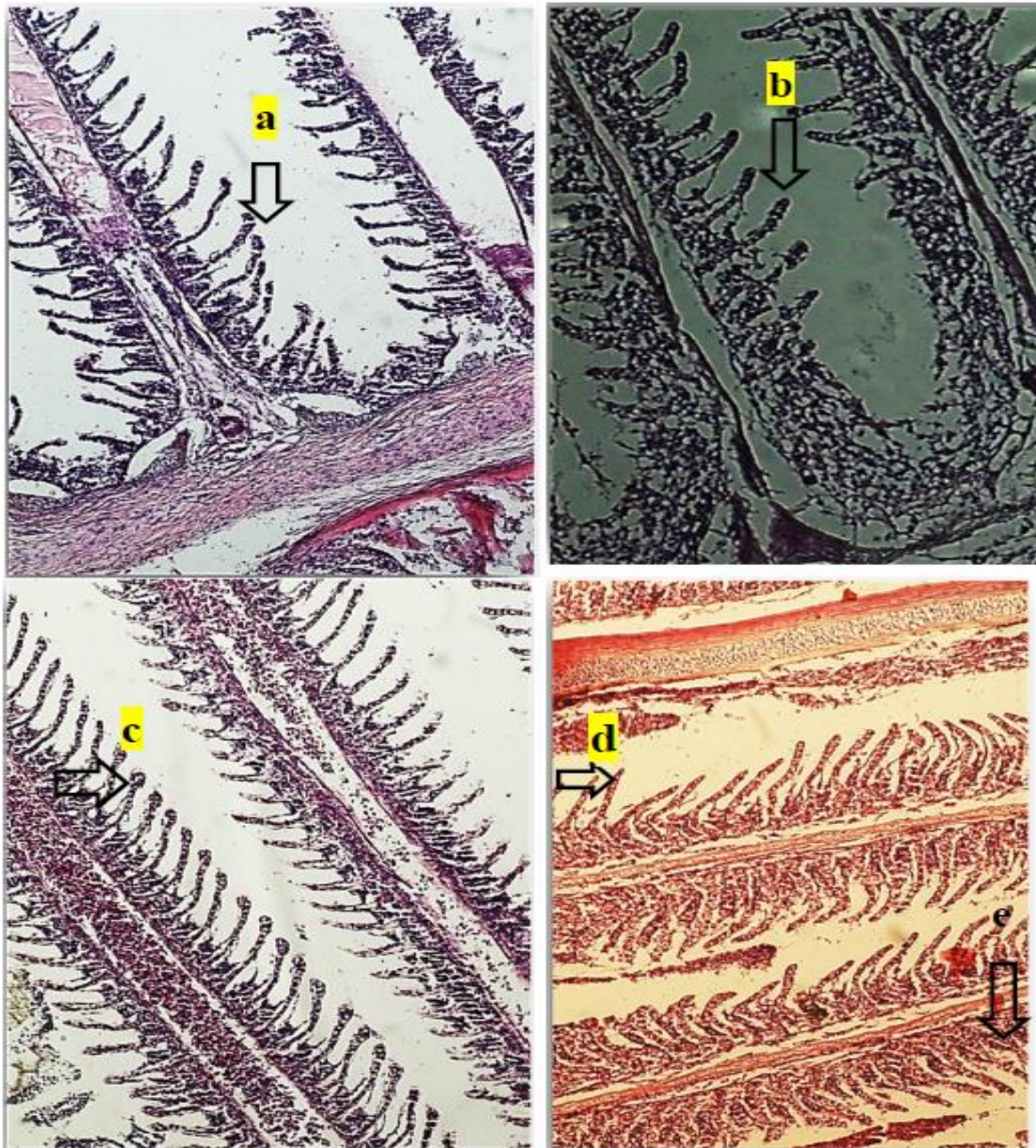


Figure 2. Light microscope image gill of common carp in the exposure to the salinity and chlorpyrifos. [Nikon Eclipse TS100 (40X)]: (a): Up and down (arrow), Epithelial hypertrophy, (b): Up and down (arrow), Epithelial lifting, (c): Left to right (arrow), Distal hyperplasia, (d): Left to right (arrow), Lamellar aneurism, (e): Up and down (arrow), Lamellar fusion.

separates and necrosis of gill tissues is performed. The pronounced degenerative changes observed in gill indicate the vulnerability of the tissue, possibly due to its role as the first contact and entry point for the pesticide. Consequently, chlorpyrifos exerted its toxic effects by altering normal behavior,

causing neurotoxicity and disturbing osmoregulation.

Complications from Gill Histological Studies

Histopathological results indicated that gill was the primary target tissue affected by chlorpyrifos and salinity. No histopathological changes were observed in the gill of the control fish. Epithelial hypertrophy, epithelial lifting, lamellar fusion and distal hyperplasia were observed in the 4 and 8 ppt salinity and chlorpyrifos groups. Exposure to 8, 12 ppt and 15 ppm salinity and chlorpyrifos resulted in lamellar fusion, distal hyperplasia, epithelial hypertrophy, lamellar aneurism and epithelial lifting (Table 1). Also, epithelial

exhibited lamellar aneurism, rupture of the lamellar epithelium (Pal *et al.*, 2012), necrosis, epithelial lifting, epithelial hypertrophy, lamellar fusion, (Stalin *et al.*, 2019), hyperplasia (Samanta *et al.*, 2015) and excessive secretion of mucus. Alteration in pattern histopathology of gill was studied in *Channa punctatus*, after acute exposure to hexavalent chromium (Mishra and Mohanty, 2008). Katuli *et al.* (2014) reported that the impact of diazinon and sodium dodecyl sulfate leads to severe necrosis lamellar in gill tissues of *Rutilus rutilus* and *Scophthalmus maximus*. Similarly, diazinon exposure in gills of *Scatophagus argus* exhibited, epithelial lifting, hyperplasia and lamellar fusion (Ghasemzadeh *et al.*, 2015). These

Table 1. Common complications of carp gill tissue exposure to salinity and chlorpyrifos poison.^a

Treatments (T)	0 ppt				4 ppt				8 ppt				12 ppt			
	0 ppm				15 ppm				150 ppm							
	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4	T1	T2	T3	T4
Lamellar fusion	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(++)	(+++)
Distal hyperplasia	(-)	(+)	(+)	(+)	(+)	(+)	(++)	(++)	(+)	(+)	(++)	(++)	(+)	(+)	(++)	(+++)
Lamellar aneurism	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(++)
Epithelial hypertrophy	(-)	(-)	(+)	(+)	(-)	(+)	(++)	(++)	(+)	(+)	(++)	(++)	(+)	(+)	(+++)	(+++)
Leukocyte infiltr	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)
Epithelial lifting	(-)	(-)	(+)	(+)	(-)	(-)	(+)	(+)	(-)	(-)	(+)	(+)	(-)	(-)	(+)	(++)

^a No complications (-), 1 to 3 complications (+), 3 to 5 complications (++), 5 to 11 complications (+++). (Riba *et al.*, 2005; Roy *et al.*, 2013).

hypertrophy, lamellar aneurism, epithelial lifting, lamellar fusion, leukocyte infiltr and distal hyperplasia were observed in the 12 ppt, 150 ppm salinity and chlorpyrifos groups. Exposure to 8 ppt, 150 ppm salinity and chlorpyrifos resulted in lamellar fusion, distal hyperplasia, epithelial hypertrophy, lamellar aneurism and epithelial lifting and lamellar fusion, distal hyperplasia, lamellar aneurism and epithelial hypertrophy were observed in the 4 ppt, 150 ppm salinity and chlorpyrifos groups (Table 1). The most common gill changes at all doses of chlorpyrifos and salinity were lamellar fusion, distal hyperplasia and epithelial hypertrophy.

Histopathological alterations in gill of common carp, *C. carpio*, intoxicated with sub-lethal concentrations of chlorpyrifos

changes lead to reduced oxygen consumption in fish and, ultimately, their death.

CONCLUSIONS

In the present study, gill histological changes have been related to poison and salinity concentrations. It can be concluded that gill alterations as a result of salinity and Chlorpyrifos exposition of fish may serve as a sensitive biomarker for the toxicity of sublethal concentrations of Chlorpyrifos as well as other pollutants. Exposure to low concentrations altered biological parameters in fish, but long-term exposure to high concentrations caused death.



ETHICAL APPROVAL

The experimental procedure was performed according to the Guide for the Care and Use of Laboratory Animals and was approved by the research committee of Gorgan University of Agricultural Sciences and Natural Resources (Ethical Approval Code: 9721313104).

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یادداشت پژوهشی: تغییرات هیستوپاتولوژیک آبشش ماهی کپور معمولی در مواجهه با کلرپیریفوس و شوری

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چکیده

در این مطالعه، اثر سم بر شوری بر بافت آبشش ماهی کپور معمولی (C. *carpio*) بررسی شد. بر این اساس، ۲۵۰ قطعه بچه ماهی کپور معمولی با وزن متوسط 21 ± 2 گرم در چهار تیمار هر کدام با سه تکرار شامل: شوری صفر، ۴، ۸ و ۱۲ ppt به مدت ۷ روز توزیع شدند. سپس یک گروه به مدت ۴ روز در معرض سم با غلظت حاد ۱۵۰ ppm کلرپیریفوس با فرمولاسیون ۴۰.۸٪ EC و گروه دوم به مدت ۷ روز در معرض غلظت تحت حاد ۱۵ ppm کلرپیریفوس قرار گرفتند. هیستوپاتولوژی بافت آبشش نشان داد که سم و شوری آسیب‌هایی مانند هیپرتروفی اپیتلیال، آنوریسم لایه‌ای، اتصال ثانویه تیغه‌های مجاور، هیپرپلازی انتهایی، بلند شدن اپیتلیال، نفوذ لکوسیتی و هیپرپلازی ایجاد کرده‌اند. نتایج هیستوپاتولوژی آبشش در غلظت‌های بالا به عنوان هیپرتروفی اپی تلیال، هیپرپلازی دیستال و جوش خوردن لایه‌ای برخی ضایعات را نشان داد، اما در غلظت‌های پایین آنوریسم لایه‌ای، جدا شدن اپیتلیال و نفوذ لکوسیت‌ها ثبت شد. بنابراین، این شاخص‌های پاتولوژیک می‌توانند به عنوان نشانگرهای زیستی مورد استفاده قرار گیرند.

Aroma of Yogurt from Cow Milk with a Combination of Several Probiotic Bacteria

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ABSTRACT

This study aimed to investigate the effect of different probiotic bacteria on aroma of yogurt through a unique incubation process. Probiotic bacteria examined were *Lactobacillus*, *Streptococcus*, and *Bifidobacterium*. Additionally, yogurt production process included sterilizing skim milk, introducing bacterial cultures, and incubating at specific temperatures. Aroma components of yogurt were analyzed using gas chromatography and mass spectrometry. The result showed that yogurt comprised various aroma compounds classified into four categories, namely, acids, alcohols, ketones, and aldehydes. The dominant acid components found were acetic, octanoic, decanoic, and dodecanoic acids. Among the components, alcohol affected aroma despite being present in minimal quantities. Ketones such as acetoin and diacetyl were identified along with aldehydes including octadecanal and dodecanal in yogurt. This study provided valuable insights into the effect of probiotic bacteria on aroma profile of yogurt, assisting manufacturers in refining production processes to prepare product appealing to consumers.

Keywords: Aroma compounds, Gas chromatography, Mass spectrometry, Yogurt production.

INTRODUCTION

Yogurt is a popular fermented milk product known for the nutritional value and unique aroma, which varies depending on the used ingredients. The major ingredient in yogurt is primarily cow milk, and live cultures such as *Lactobacillus bulgaricus* and *Streptococcus thermophilus* are commonly used during the production. Additional flavorings or sweeteners may be added to customize the taste of yogurt (Nair and Peerkhan, 2022; Trentin *et al.*, 2022; Wihansah *et al.*, 2022; Fadhlurrohman *et al.*, 2023).

Aroma of yoghurt plays a crucial role in the acceptance by consumers and this varies significantly based on factors such as the type of probiotic bacteria used in fermentation, milk type, and the incubation methods applied. According to Chen (2017), yogurt production is a complex process that

generates a distinctive aroma. Zhao *et al.* (2023) reported that factors such as the production process, extraction method, starter culture concentration, and incubation time could further affect aroma.

Aktar (2022) stated that aroma produced was affected by variations in the use of bacteria. Krastanov *et al.* (2023) found that the starter culture (bacteria) affected the sensory characteristics, including aroma and taste of yogurt.

A key factor in shaping the preferences of consumers for yogurt product is aroma, while the perceptions greatly determine purchase decisions and continual product enjoyment. This shows the need to comprehend the various aroma components of yogurt as well as the effect on consumers' preferences in the food and beverage industry. Aroma is affected by several factors, including the type of bacteria in the starter culture, the

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processing method, the source of milk and chemicals, and any additional ingredients used. According to Eker *et al.* (2020), aroma is essential in shaping the preferences of consumers and the general sensory experience of yogurt.

Probiotic is a group of bacteria commonly found in fermented milk product that provides numerous health benefits to humans. According to Latif *et al.* (2023), probiotic is live microorganism that offers health advantages to the host when consumed in sufficient quantities. The seven primary microbial genera majorly used in probiotic product are *Lactobacillus*, *Bifidobacterium*, *Saccharomyces*, *Streptococcus*, *Enterococcus*, *Escherichia*, and *Bacillus*.

Probiotic bacteria used in this study were *Lactobacillus*, *Streptococcus*, and *Bifidobacterium*, which provide health benefits and produce various aroma components. The combination of the three bacteria is expected to help with the production of high-quality yogurt due to containing a harmonious blend. According to Siddiqi *et al.* (2024), *L. bulgaricus* and *S. thermophilus* bacteria contribute to a decrease in pH, which is important for the texture and flavor of yogurt. *Bifidobacterium* is among the dominant colonies in the human gut, specifically in infants (He *et al.*, 2023; Znamenska and Vorobiova, 2024). Kamara *et al.* (2016) reported that yogurt fermented with mixed cultures of *L. bulgaricus*, *S. thermophilus*, and *L. acidophilus* showed antibacterial activity against *E. coli* and *Bacillus subtilis*. A study by Elbarbary (2014) found that the inclusion of bifidobacteria spp. in stored yogurt inhibited the growth of *E. coli* and *Staphylococcus aureus*.

During yogurt fermentation, probiotic bacteria used are essential in developing the unique aroma of the finished product and various strains can generate different aroma compounds. Therefore, selecting the appropriate probiotic combination is crucial in achieving the desired aroma.

Several previous investigations focused on different culture treatments and temperature variations (Yu *et al.*, 2016; Swelam *et al.*,

2019; Jegal *et al.*, 2019; Adrianto *et al.*, 2020). However, a unique method was used in this study where *Bifidobacterium* was incubated with *L. bulgaricus* and *S. thermophilus* cultures, as well as in a separate manner.

This study aimed to examine the effect of various probiotic bacteria on yogurt aroma. Understanding the effect of microbial factors can help yogurt producers enhance manufacturing processes to prepare product with an appealing aroma that resonates with consumers. Aroma profile in this study is determined by the presence of volatile components.

MATERIALS AND METHODS

Material and Equipment

Commercial powdered skim milk was used in yogurt production, along with bacteria strains *L. bulgaricus* 18 Visbyvac Serie 50 No. 700398 (*L. bulgaricus*), *S. thermophilus* A Vysbivac No. 640638 (*S. thermophilus*), and *Bifidobacterium longum* BF1 (*B. longum*). Additionally, the laboratory equipment used were a rotary evaporator, gas chromatograph (Shimadzu 17 A), and mass spectrometer (Shimadzu QP 5000), as well as Erlenmeyer tubes and other necessary glass tools.

Yogurt Preparation and Production

Skim milk powder was dissolved in distilled water until a specific gravity of 1.027 equivalent to fresh milk was attained. The mixture was sterilized at 121°C for 15 minutes in an autoclave. After cooling to 42°C, a commercial culture of *L. bulgaricus*/*S. thermophilus* was added at a concentration of 2% of the sterilized milk volume. The resulting mixture was incubated at 37°C for 20 hours, then, cooled to 37°C before adding *B. longum* to the milk. *B. longum* was selected over more commonly used probiotic due to the superior adaptability and health

benefits provided in the human gut (Sharma *et al.*, 2021; Li *et al.*, 2023; Li *et al.*, 2024).

Preparing Samples for the Analysis of Aromatic Compounds

The separation of volatile aroma components is conducted using the Likens-Nickerson equipment and SDE (Simultaneous Distillation Extraction) method. A Gas Chromatograph was used in combination with a Mass Spectrometer (GC-MS) to analyze aroma components following the method described by Shiratsuchi *et al.* (1994). A solution of 300 g yogurt dissolved in 900 mL distilled water was used to separate aroma components, with polyethylene glycol 4000 added to prevent foaming. Subsequently, extraction and distillation were carried out simultaneously for 1 hour using 30 mL of diethyl ether as the solvent. Anhydrous sodium sulfate was added to bind water, and the solution was concentrated with a rotary evaporator.

Nitrogen gas was used to obtain the distillate, which was injected into GC-MS apparatus. This process was duplicated and analyzed under the conditions presented in Table 1.

Yogurt was produced in this study using the incubation method with a freeze-dried *B. longum* bacterial culture. The incubation process of the culture was carried out for 20 hours at 37°C.

Seven combination treatments were performed using probiotic bacteria and the incubation method during yogurt production. These included the followings:

- 1) L= Single culture of *L. bulgaricus*,
 - 2) LB1= *L. bulgaricus*+*B. longum* incubated,
 - 3) S= Single culture of *S. thermophilus*,
 - 4) SB1= *S. thermophilus*+*B. longum* incubated,
 - 5) LS= *L. bulgaricus*+*S. thermophilus*,
 - 6) LSB1= *L. bulgaricus*+*S. thermophilus*+*B. longum* incubated,
 - 7) B= Single culture of *B. longum*,
- The results are reported descriptively.

Table 1. Condition of GC-MS Instrument Used.

Condition of GC-MS	Information
GC Conditions	
Brand of tools	: Shimadzu Model 17 A
Column type	: Shimadzu CBP20-M50_025. Column length 50 m (i.d. 0.22 mm and o.d. 0.33 mm). Hi-Cap series. Film layer thickness 0.25 µm
Carrier gas	: Helium, pressure 199 kPa
Initial temperature	: 50°C duration of 5 minutes
Final temperature	: 200°C duration of 30 minutes
The rate of temperature increase	: 4°C per minute
Sample volume.	: 1 µl
Injection method	: Grob Split-splless; Sampling time is 30 seconds
Temperature interface	: 230°C
Injector temperature	: 220°C
MS Conditions	
Brand of tools	: Shimadzu model QP 5000
Energy detector	: 1.1 kV
Mass range	: 33 – 400
Resolution	: 1000
Database.	: National Institute for Standard and Technology (NIST) library



RESULTS AND DISCUSSION

Yogurt manufactured contains a wide variety of aroma components grouped into acids, alcohols, ketones, and aldehydes. This study found that the entire components vary greatly in both quantity and type.

Group of Acids

The results showed that yogurt group had a dominant presence of acids in terms of aroma components. GC-MS analysis can be used to identify various acidic compounds, specifically volatile acids that evaporate when exposed to high GC temperatures and pressures. Table 2 presents the acidic compounds detected in the treated yogurt.

Yogurt is a dairy product containing various acids, such as lactic acids essential for the taste and texture. During fermentation process, Lactic Acid Bacteria (LAB), such as *L. bulgaricus* and *S. thermophilus*, consume the lactose in milk and convert it into lactic

acids. The tangy flavor of yogurt originates from lactic acids, which also helps prevent the growth of harmful bacteria and contributes to the thick product consistency. The performance of LAB starters directly affects the quality of yogurt, including the texture and gel properties (Zhang and Zhang, 2012). LAB from traditionally prepared dairy product, such as *Lactobacillus plantarum*, was found to enhance the condensed and textural integrity of yogurt (Li *et al.*, 2022).

Acids are long carbon chain fat molecules with a carboxylic acid group at the terminal portion. The milk used in yogurt production contains both saturated and unsaturated acids, which contribute to the characteristic taste and aroma. Additionally, acids play a role in the thickness and texture of yogurt. Some acids, such as butyric (butanoic) and caproic (octanoic) acids, can impart a buttery taste to yogurt, while others, including acetic and hexanoic acids, contribute to a soft and fruity aroma. According to Zhao *et al.* (2023) and Tian *et al.* (2019), aroma is influenced by various acids, including butyric, caproic,

Table 2. Group of acids.^a

No.	Types of acids	RT	LRI	LRI (Ref.)	Treatments						
					L	LB	S	SB	LS	LSB	B
1	Acetic acid	21.60	1457	1425*	1.37	2.11	0.87	5.95	3.06	3.28	2.04
2	Butanoic acid	27.30	1631	1652*	2.08	1.55	0.38	1.12	3.66	3.58	4.56
3	Hexanoic acid	33.60	1842	1849*	6.63	7.98	1.26	10.59	9.88	9.06	10.5
4	Octanoic acid	38.02	2010	2084*	19.72	20.22	6.73	15.11	22.9	20.35	16.5
5	Nonanoic acid	41.95	2166	2192*	0.68	0.99	0.33	0.47	0.82	2.92	0.41
6	Decanoic acid	44.88	>2200	2486*	20.72	19.94	18.7	14.95	14.6	15.38	14.4
7	Benzoic acid	50.83	>2200	2380*	0.15	1.81	0	0	0.16	1.12	0.32
8	Dodecanoic acid	52.15	>2200	2517**	1.06	3.79	27.7	10.4	0	0	20.3
9	Undecanoic acid	53.28	>2200	2365***	1.48	6.19	0.24	9.8	5.52	3.06	0.28
10	Tetradecanoic acid	64.11	>2200	2733**	3.06	1.03	13.4	5.25	0	0	8.91

^a L= Yogurt with single *L. bulgaricus* culture; LB= Yogurt with *L. bulgaricus* and *B. longum* cultures; S= Yogurt with single *S. thermophilus* culture; SB= Yogurt with *S. thermophilus*+*B. longum* cultures; LS= Yogurt with *L. bulgaricus*+*S. thermophilus* cultures; LSB= Yogurt with *L. bulgaricus*+*S. thermophilus*+*B. longum* cultures; B= Yogurt with single *B. longum* culture; RT= Retention Time; LRI= Linear Retention Index; Ref= Reference.

* Tian *et al.*, 2019; ** Shiratsuchi *et al.*, 1994; *** Marce *et al.*, 1976.

acetic, and hexanoic acids, which collectively contribute to the general flavor profile and sensory characteristics of yogurt.

Analysis of yogurt using GC-MS did not detect lactic acids, potentially due to the hydrophilic nature and presence in the form of ions in yogurt. GC-MS is more effective for detecting volatile non-polar compounds, leading to the unsuitability for detecting lactic acids. This result corresponded with reports from other studies (Zhao *et al.*, 2023; Liu *et al.*, 2022) that failed to detect lactic acids in yogurt.

The data in Table 2 show that the composition of acids varies across the different treatments, suggesting that each treatment has a unique pattern in terms of the type and concentration of acids. Some treatments are characterized by a high concentration of specific types of acids.

Yogurt aroma analysis in this study found that acetic acids were the first to appear, therefore, they are considered as the most volatile component (Zhao *et al.*, 2023) detected using GC-MS (Arslaner, 2020; Yüksel and Bakırcı, 2015; Cheng, 2010). According to Cheng (2010), acetic acids contribute to the vinegar-like, pungent, and acidic aroma of yogurt.

Table 2 shows that certain yogurt using different cultures and combinations (L, LB, B) produce 10 types of volatile acid

SB, LS, and LSB, generated fewer than 10 types of volatile acids. In this study, yogurt with a single culture of *L. bulgaricus* produced 11 acid group compounds. Liu *et al.* (2022) identified 12 aroma-active compounds in yogurt, including butanoic, acetic, and hexanoic acids, as the main aroma-active compounds in fermented milk produced by *L. bulgaricus*. The results showed that yogurt produced with a single culture of *L. bulgaricus* obtained these compounds. Treatments with a single culture of *S. thermophilus* (S), as well as incubation with *L. bulgaricus* (LS) and Bifidobacteria (SB), did not produce benzoic acid. According to Yu *et al.* (2016), benzoic acid production has been studied for various starter cultures and incubation temperatures without a report on the optimization process. The use of a single culture of Bifidobacteria in preparing yogurt produces 10 types of components, among which the most prominent are octanoic, decanoic, and dodecanoic acids. Tian *et al.* (2019) found that both octanoic and decanoic acids contributed to the distinctive taste of cheese, while octanoic acids produced a light creamy taste in yogurt.

The majority of treatments generated high levels of octanoic, decanoic, and dodecanoic compounds compared to the other groups. These acids are present in higher quantities in

Table 3. Group of alcohol.^a

No.	Types of alcohol	RT	LRI	LRI (Ref)*	Treatments						
					L	LB	S	SB	LS	LSB	B
1	2-Nonanol	22.87	1494	1097	2.06	1	0	0	1.12	0.8	0
2	2-Undecanol	29.38	1704	1301	5.06	1.1	0	0	1.89	0.9	0
3	2-Tridecanol	34.81	1885	1570	0	1.04	0	0	1.11	0	0
4	2-Decanol	43.62	2200	1198	2.15	1.41	0	0	0.79	0	0

^a L= Yogurt with single *L. bulgaricus* culture; LB= Yogurt with *L. bulgaricus* and *B. longum* cultures; S= Yogurt with single *S. thermophilus* culture; SB= Yogurt with *S. thermophilus*+*B. longum* cultures; LS= Yogurt with *L. bulgaricus*+*S. thermophilus* cultures; LSB= Yogurt with *L. bulgaricus*+*S. thermophilus*+*B. longum* cultures; B= Yogurt with single *B. longum* culture; RT= Retention Time; LRI= Linear Retention Index; Ref= Reference. *= Adams (2007).

components. Some treatments, including S,

yogurt due to the activity of probiotic bacteria



commonly used during fermentation. Probiotic bacteria, such as *Lactobacillus* and *Bifidobacterium*, convert lactose (milk sugar) into octanoic, decanoic, and dodecanoic acids. Barros *et al.* (2019) stated that the process of converting lactose into acids in yogurt included several steps. Fermentation of lactose leads to the production of acids, which contribute to the flavor and texture of yogurt.

Group of Alcohol

During the preparation of yogurt, microbes ferment lactose and produce acids as well as other compounds, including alcohol. The amount of alcohol produced is extremely low and not considered harmful to health. Additionally, the presence of alcohol can contribute to the unique aroma of yogurt. Hussain *et al.* (2010) found that *S. thermophilus* and *L. bulgaricus* produced alcohol-related compounds as well as saturated volatile free acids during yogurt manufacturing.

Alcohol compounds produced were very low, probably due to acids generated as the main product by bacteria during yogurt preparation, while more alcohol was obtained from fermentation with yeast. Nurhayati *et al.* (2022) found that yeast could generate alcohol, and several studies focused on the isolation and identification of various yeast species with the potential to produce ethanol. The profile of alcohol amount obtained from this study had the same pattern, where some treatments generated a high content of alcohol compounds. The three treatments that produced higher alcohol compounds included LB1, LS, and B.

Group of Ketones, Aldehydes, Furan Derivatives, and Ester Derivatives

Ketones are a type of compound included in the carbonyl group and not the major product formed during yogurt fermentation process. Table 4 shows that acetoin and

diacetyl are the most abundant compounds produced in ketone group.

Acetoin and diacetyl are both ketone compounds commonly used in foods and drinks to enhance specific flavors and aroma. For example, acetoin can impart a buttery taste to yogurt, while diacetyl provides an acidic or buttery flavor. This study showed that all treated yogurt produced acetoin, and diacetyl was only generated by six (L, S, SB, LS, LSB, B) out of the seven treatments. These two are the primary aroma compounds in yogurt playing a significant role in determining the general aroma and taste (Tian *et al.*, 2020). Acetoin is produced by *L. acidophilus* during fermentation, while diacetyl is formed through the lipolysis of milk fat as well as the microbiological transformation of lactose and citrate (Cheng, 2010). The odor threshold for acetoin and diacetyl in yogurt is higher than in water (Nadal *et al.*, 2009). Acetoin is commonly found in food fermentation product due to being a metabolic byproduct of microorganisms such as lactic acid bacteria. In yogurt, acetoin is typically present in higher concentrations than other ketone compounds, leading to easy detection with GC-MS and a more prominent appearance. This is attributed to acetoin being a compound produced by the metabolism of LAB commonly used in the production of yogurt.

Aldehydes are included in the group of carbonyl compounds, along with ketones. The main difference between ketones and aldehydes depends on the atoms attached to the carbonyl group. In ketones, the carbonyl group is bonded to two carbon atoms, while in aldehydes, it is bonded to one carbon and a hydrogen atom. Aldehydes are present in very small amounts as by-product during fermentation process, but not considered a major component or the main characteristic of yogurt.

Table 4. Groups of ketone compounds, aldehydes, furan derivatives, and ester derivatives.^a

No.	RT	LRI	LRI (Ref.)	Compound type	Treatments							
					L	LB	S	SB	LS	LSB	B	
					% area							
A				Ketones								
1	6.09	1014	981*	Diacetyl	0.07	0	2.14	1.7	0.9	1.34	2.4	
2	8.17	1089	1056*	2,3-pentanedione	0.06	0	0.67	0.89	0	0	0.4	
3	15.2	1280	1299*	Acetoin	1.6	3.44	3.4	19.5	2.2	8.38	4.6	
4	18.2	1362	1387*	2-nonanone	0.07	1.63	0	1	1.3	0	0	
5	18.4	1368		3-hydroxi-2-pentanone	0	0	0.27	0.91	0.9	0.61	0.4	
6	31.7	1774	1488*	2-tridecanone	1.56	0.54	0.25	0	0	0	0	
B				Aldehydes								
1	23.2	1504	1502**	Benzaldehyde	0.07	0	0	0.8	0	0	0	
2	33.5	1836	1410***	Dodecanal	6.63	0.67	0.18	0.4	0	1.22	0.2	
3	40.4	2103		Octadecanal	4.86	1.25	0.76	0.53	1.8	7.53	0.6	
C				Furan Derivatives								
1	21.6	1456	1474****	2-furancarboxaldehyde	0.71	0	0.19	0.96	0.9	0.68	1.2	
2	27.97	1652	1657*****	2-furanmethanol	0.21	0	0.47	0.99	0	0.69	2.3	
D				Ester Derivatives								
1	4.25	901	888*****	Ethyl Acetate	1.16	1.3	6.47	5.65	3.3	1.22	4.7	
2	58.1	2200	1356*	Butyl octyl ester	0	0	0	0	0	0	0.6	

^a L= Yogurt with single *L. bulgaricus* culture; LB= Yogurt with *L. bulgaricus* and *B. longum* cultures; S= Yogurt with single *S. thermophilus* culture; SB= Yogurt with *S. thermophilus*+*B. longum* cultures; LS= Yogurt with *L. bulgaricus*+*S. thermophilus* cultures; LSB= Yogurt with *L. bulgaricus*+*S. thermophilus*+*B. longum* cultures; B= Yogurt with single *B. longum* culture; RT= Retention Time; LRI= Linear Retention Index; Ref= Reference. *Tian *et al.*, 2019; **Sakho *et al.*, 1985; *** Zhang *et al.*, 2020; ****Wong and Tie, 1993; *****Shiratsuci *et al.*, 1994.

The results of yogurt aroma analysis conducted using GC-MS showed the presence of dodecanal, benzaldehyde, and octadecanal aldehyde groups. Both octadecanal and dodecanal were found to be the most dominant. Octadecanal was present in all treatments, while dodecanal was not detected in SB2 and S. These compounds have no significant effect on aroma of yogurt, and aldehydes are known for the stimulating and fruity aroma (Reineccius, 2006).

Furan derivatives found in yogurt are chemical compounds with furan rings in the structure. These have been detected in various foods, including coffee, canned/bottled product, cereal-based product, and thermally processed foods such as rye bread. However, the presence of furan derivatives in yogurt remains unclear. More investigations or special studies are needed to determine its presence in this food product (Arisseto, 2016; Tameko *et al.*, 2017). The heating process particularly carried out during milk pasteurization before

fermentation may lead to the formation of furan derivatives in yogurt (Batoool *et al.*, 2023). Furan derivatives can be formed during milk heating, specifically in the context of Ultra High Temperature (UHT) processing for yogurt production.

In LS, 2-furancarboxaldehyde increased and 2-furanmethanol decreased, which was a trend similarly observed in all yogurt treatments. These two compounds are primarily produced in yogurt with treatment B. The relationship between bacterial activity in producing 2-furancarboxaldehyde and 2-furanmethanol is not yet understood, as both compounds are formed during the heating process. According to Guo *et al.* (2019) and Asaduzzaman *et al.* (2021), the heating process of milk in yogurt production significantly affects furan formation. Furan has the potential to be a carcinogenic compound that can form during the thermal processing of heated food.

Ester derivatives are chemical compounds related to esters, which are organic groups



formed from the reaction between carboxylic acids and alcohols. The structures and properties of the derivatives can vary depending on the type of carboxylic acids and alcohol used in the synthesis. Moreover, esters are formed during the esterification process through the reaction of lactic acids with other acids in milk. These compounds play a role in giving yogurt a distinctive taste and aroma, although the contribution is not very significant. Farooq and Haque (1992) stated that sugar esters could improve the general quality of yogurt, enhancing the texture, taste, and mouthfeel. Vásquez-Trespalacios and Romero-Palacio (2014) found that yogurt drinks with added plant stanol esters significantly reduced total and LDL cholesterol.

Ethyl acetate, such as esters, is formed through an esterification reaction between carboxylic acids and alcohol, probably due to the better solubility of ethyl acetate compared to butyl octyl ester. Furthermore, it is suspected to be more volatile, leading to the dominance during analysis with GC-MS. Cheng (2010) described aroma of ethyl acetate as solvent-like and fruity, resembling pineapple. Ni *et al.* (2022) found this aroma to induce cytotoxicity in breast cancer without affecting normal cells.

The results will provide valuable insights into the production of yogurt with a distinct aroma, enhancing product competitiveness in a rapidly growing market. Additionally, this study can contribute to advancing knowledge on fermented milk product and the practical applications in the food and beverage industry.

CONCLUSIONS

In conclusion, yogurt is a fermented dairy product prioritized for the nutritional benefits and unique aroma, commonly influenced by the ingredients and fermentation process. The production of yogurt from cow milk often uses live cultures such as *L. bulgaricus* and *S. thermophilus*. Therefore, this study explores the effect of different probiotic bacteria

combinations on aroma of yogurt by identifying components such as acids, alcohols, ketones, and aldehydes. Octanoic, decanoic, and dodecanoic acids are the most prominent among the 10 types of acids found, contributing to aroma complexity. Aldehydes and furan derivatives formed during heating are not highly significant. The results show the importance of selecting specific probiotic combinations to achieve the desired aroma profile, which helps to improve yogurt marketability.

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بوی (عطر) ماست از شیر گاو با ترکیبی از چندین باکتری پروبیوتیک

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چکیده

این پژوهش با هدف بررسی تأثیر باکتری‌های پروبیوتیک (Probiotic Bacteria) مختلف بر بوی (عطر) ماست از طریق یک فرآیند آنکوباسیون منحصر به فرد انجام شد. باکتری‌های پروبیوتیک مورد بررسی شامل لاکتوباسیلوس (*Lactobacillus*)، استرپتوکوک (*Streptococcus*) و بیفیدوباکتریوم (*Bifidobacterium*) بودند. علاوه بر این، فرآیند تولید ماست شامل استریل کردن شیر بدون چربی، افزودن کشت‌های باکتریایی و گرمخانه‌گذاری در دماهای خاص بود. اجزای بوی (عطر) ماست با استفاده از کروماتوگرافی گازی و طیف‌سنجی جرمی مورد تجزیه و تحلیل قرار گرفتند. نتایج نشان داد که ماست حاوی ترکیبات معطر مختلفی است که در چهار دسته اسیدها، الکل‌ها، کتون‌ها و آلدئیدها طبقه‌بندی می‌شوند. اجزای اسیدی غالب شامل استیک اسید، اکتانویک اسید، دکانویک اسید و دودکانویک اسید بودند. در میان اجزا، الکل، با وجود مقادیر کم، برعطر تأثیر گذاشت. کتون‌هایی مانند استوئین و دی‌استیل به همراه آلدئیدهایی از جمله اکتادکانال و دودکانال در ماست شناسایی شدند. این پژوهش، داده‌های ارزشمندی در مورد تأثیر باکتری‌های پروبیوتیک بر پروفایل بو و عطر ماست ارائه داد و به تولیدکنندگان در اصلاح فرآیندهای تولید برای تهیه محصولی جذاب برای مصرف‌کنندگان کمک کرد.

Black, Q12, and Titicaca Quinoa Protein Isolate: Nutritional and Physicochemical Properties

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ABSTRACT

Quinoa is a pseudocereal plant that has been cultivated in Iran recently. The purpose of this research was to evaluate its properties for use in food. Quinoa Protein Isolates (QPIs) were obtained from Iranian Quinoa Seed cultivar (QS) varieties (Black-QS, Q12-QS, and Titicaca-QS). The Black-QPI and Titicaca (T)-QPI had a higher protein content: 87.30 ± 1.96 and $87.80 \pm 1.61\%$ w/w, respectively. The results showed foaming capacity (40.54%), stability (65.26% in 60 min), and oil absorption (3.02 mL g^{-1}) were significantly ($P \leq 0.05$) higher in Black-QPI. Textural parameters revealed that viscosity and shear stress were higher in Q12-QS than others. The amino acid profile showed that T-QS had a well-balanced profile with the highest content of tryptophan (8.23%). Consequently, the suitable nutritional and functional properties of *Titicaca* protein make it an appropriate candidate for use as a safe food additive.

Keywords: *Chenopodium quinoa* Willd., Titicaca properties.

INTRODUCTION

Quinoa (*Chenopodium quinoa* Willd.) is a gluten-free dicotyledonous pseudo-grain, and is consumed by people living in the Andean Region for a very long time. There has been a growing concern about plant-based diets, applied as an alternative protein source. Recently, plant proteins are introduced as proper alternatives to animal-based ones, due to their lower side effects as compared to those associated with the consumption of animal-based proteins (Alrosan *et al.*, 2022). Moreover, gluten-free pseudocereals (Amaranth, Buckwheat, and Quinoa) exist in human diets to have outstanding nutritional value. In addition, the potential health benefits of pseudocereals have been recently pointed out as important sources for the development of functional food research. The amino-acid composition

and bioavailability of crops' proteins are important factors to examine the quality of these protein sources (Martínez-Villaluenga *et al.*, 2020). The biological value of quinoa's dietary value (73%) is nearly comparable to beef (74%). The daily consumption of quinoa is suggested to patients suffering from Cardiovascular Diseases (CVDs), high blood cholesterol and glucose, plasma antioxidant activity, and systemic inflammation (Shahbaz *et al.*, 2023).

Due to its high protein content, quinoa is considered a good source of methionine (3.6%), histidine (2.9%), and lysine (5.4%), which currently attract worldwide attention. Protein isolate is the most refined, which constructed $90 \text{ g } 100 \text{ g}^{-1}$ of the total protein of quinoa (Gupta *et al.*, 2021). Although the proteins of these important pseudo-grains are rich in essential amino acids, their poor

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functional properties including solubility, foaming water binding, and emulsifying have been approved (Mir *et al.*, 2021). An 11S globulin called chenopod is predominantly present in the mature quinoa seed. Chenopod consists of approximately 37% total protein and 2S albumin, which are stabilized by disulfide bonds. In addition, quinoa seeds contain a low concentration of Prolamins (0.57% of the total protein), which makes them suitable for celiac patients (Dakhili *et al.*, 2019). The use of protein isolation has increased due to different factors, including bioactive components, good functionality, higher levels of proteins in the food industry, and lower content of anti-nutritional factors. The alkaline pH (8-11) is one of the most effective ways to obtain protein, while for the isoelectric precipitation of solubilizing proteins an acidic pH (4-6) is applied (Abugoch *et al.*, 2008; Vega-Gálvez *et al.*, 2010).

Research on the nutritional properties of quinoa grown in Iran is limited. For instance, the amounts of available carbohydrates, fat, protein, ash, and dry matter were reported as follows: 73.14 ± 1.59 , 6.09 ± 0.30 , 16.30 ± 1.52 , 4.43 ± 0.47 , and $90.30 \pm 0.89\%$, respectively. Analysis of the amino acid profile of quinoa revealed the highest levels of lysine (3.08%) and glutamic acid (1.230%). Linoleic acid content is 63.5% in fat (Sekhavatizadeh *et al.*, 2021).

Quinoa Protein Isolate (QPI) is an impressive and promising source of nutrient that makes it a suitable nutritional supplement for functional foods. The physicochemical properties of quinoa proteins isolated from other countries have been already determined, but proteins from Iranian quinoa varieties have not been described. Hence, systematic information about the functional, chemical, and physical properties of proteins is necessary to categorize their feasible application without compromising nutritional and health-related issues. While a few studies have already investigated the quinoa proteins, there is an

urgent need to further characterize the grains, flours, and protein isolates from Black quinoa grains (Ghumman *et al.*, 2021).

This study provides a comprehensive comparative analysis of three distinct quinoa varieties—Titicaca (T-QPI), Q12 (Q12-QPI), and Black (Black-QPI), which have not been thoroughly investigated in terms of their proximate composition and functional properties in prior literature. Our research provides a systematic evaluation of the chemical, nutritional, and functional characteristics of protein isolates derived from these specific genotypes, thereby contributing to the understanding of how genetic variability influences quality, and functionality of plant-based proteins.

MATERIALS AND METHODS

Materials

Methanol, sodium hydroxide, sulfuric acid, KH_2PO_4 , NaOH, hydrochloric acid, hexane, chloroform, standards including sodium acetate, boric acid, borate buffer, methyl red, methanol (HPLC grade) hydrochloric acid, and the additional standard reagents were purchased from Merck (Darmstadt, Germany). O-phthalaldehyde, 2-Mercaptoethanol, norvalin, and pepsin were obtained from Sigma Chemical Co (St. Louis, MO, USA).

Collection and Further Identification of Seeds

The three dried genera of quinoa (saponin-free) consisting of Black-QS, T-QS, and Q12-QS were harvested from growing plants at Zarghan Station, Zarghan City, Fars Province (southern Iran) (Figures 1-A, -B, and -C). Further identification of the plant was completed by the Fars Research Center for Agriculture and Natural Resources (FRCANR), Herbarium in Shiraz, Iran. A

representative sample was finally deposited in the FRCANR herbarium, Shiraz, Iran.

Flour Preparation

The procedure for washing the seeds involved washing them four or five times with cold water, or until no foam remained to eliminate the saponins, which were then dried in the oven at $45 \pm 1.0^\circ\text{C}$ for 24 hours. Moulinex Miller (Model dePOSE 00022, France) was applied to flour the seeds, which was filtered through a 60-mesh sieve (US standard sieve), packed in polyethylene bags followed by storing at 5°C (James, 2009).

Preparation of Quinoa Protein Isolated (QPI)

For his purpose, chloroform: methanol (2:1), 1:10 w/v shaking for 2 hours was used to eliminate lipids from the quinoa flour. The procedure was repeated in triplicate. Briefly, 50 g of fat-free quinoa flour was dissolved in 1,000 mL of Milli-Q water (1:20 w/v). The pH was then adjusted to 11 using 0.1N NaOH. The maximum degree of solubilization was obtained by holding the sample in a fixed position after stirring the suspension for 24 hours. The mixture was centrifuged at $6,000 \times g$ for 30 minutes at 20°C in a refrigerated high-speed centrifuge (Sigma 3-16pk, Sigma, Osterode, Germany). Furthermore, the pH of the supernatant was adjusted to 4.5 using 0.1N HCl. The

suspension was centrifuged at $10,000 \times g$ for 45 minutes at 4°C , followed by washing three times with deionized water. The precipitate was then lyophilized, and stored at -20°C for further use (Elsouhaimy *et al.*, 2015)

Proximate Analysis of Quinoa Seeds and QPI

The crude ash, total solids, crude protein, and crude fat content of QPI and seeds were determined by using the methods of Sekhavatizadeh *et al.* (2021). The Kjeldahl method with a conversion factor of 5.85 was used to determine the crude protein content of the seeds and QPIs. Crude fat was determined by extracting a known sample aliquot with hexane using a Soxhlet apparatus. The difference in the values was used to calculate the total carbohydrates, which were presented as a percentage (Marmouzi *et al.*, 2015). The ash content of each sample was determined at $550 \pm 15^\circ\text{C}$. Energetic values and total carbohydrates were evaluated based on the following equations:

$$\text{Energy (kcal } 100 \text{ g}^{-1}) = 9 \times (m_{\text{fat}}) + 4 \times (m_{\text{carbohydrates}} + m_{\text{proteins}}) \quad (1)$$

$$\text{Total carbohydrates (g } 100 \text{ g}^{-1}) = 100 - (m_{\text{ash}} + m_{\text{proteins}} + m_{\text{fat}}), \text{ (Sekhavatizadeh } et al., 2021) \quad (2)$$

All unites are w/w.



Figure 1. Quinoa Seeds (QS) included in this study: (A) T-QS, (B) Black-QS, (C) Q12-QS.



Amino Acid Analysis of Quinoa Seed

The amino acid analysis was performed after hydrolysis of seed samples with 6 mol L⁻¹ HCl and 0.5 g L⁻¹ of β-mercaptoethanol in vacuum-sealed tubes based on Sekhavatizadeh *et al.* (2021 and 2023) methods. For lysine analysis, HPLC system an auto-sampler system (Perkin Elmer, Australia) was used. The following reagents were used: 0.01 M sodium acetate in water (mobile phase A) and methanol (mobile phase B). The content of amino acid was recorded in mg 100 g⁻¹ d⁻¹ m⁻¹. For tryptophan determination, samples were decolourised with half-saturated n-butanol solution and digested in 75 mmol L⁻¹ KOH containing 0.5 g L⁻¹ β-mercaptoethanol at 110°C for 24 hours, in screw-capped test tubes. After centrifugation at 6,000×g for 30 minutes, the resulting supernatants were used for colorimetric tryptophan determination. The concentration of amino acids was expressed as g 100 g⁻¹ protein (Gonzalez *et al.*, 2012).

Foaming Capacity and Stability

The foam properties of protein isolates were determined as was described by Panozzo *et al.* (2014). For this purpose, foams were obtained by whipping 5 mL of QPIs for 3 min at 20°C in a 50 mL cylinder by a high speed mixer (ultra-turrax (IKA, T25, Staufen, Germany) operating at 9500 rpm. The volume of the foam and of the drained liquid was assessed just after whipping and during holding up to 30 minutes at 20°C. Percentage Foam Capacity (FC) (foam ability) and Stability (FS) were calculated as follows:

$$FC\% = (V_f - V_0) / V_0 \times 100 \quad (3)$$

$$FS\% = V_{f30} / V_f \times 100 \quad (4)$$

Where, V_f is the foam Volume, V_0 is the initial Volume of the QPIs and V_{f30} is the foam Volume after 30 minutes observation.

Viscosity

The viscosity of QPI samples (10%, w/v) was assessed, using a rheometer (MCR 302,

Anton Paar, Austria) (MCR 302, Anton Paar, Austria). The sample was left before the measurement of viscosity for 12 hours. The sample volume of QPI in concentric cylinder geometry was 5 mL at a temperature of 23°C and a shear rate from 10 to 100 s⁻¹ (Shaviklo *et al.*, 2012).

Oil and Water Absorption of QPIs

One gram of QPI samples was thoroughly mixed with distilled water (10 mL) for 30 seconds with a homogenizer (UltraTurrax IKA, T25, Werke, Germany). To settle the protein suspension, it was left at 25±1°C for 0.5 hour. It was centrifuged at 7,000×g for 0.5 hour and kept in a 10 mL measuring cylinder. To work out the oil absorption of the protein, the same procedure was employed (Elsohaimy *et al.*, 2015).

Statistical Analysis

To analyze the data, one-way Analysis Of Variance (ANOVA) was used with a confidence level of 0.05 (SPSS version 21.0). Duncan's multiple ranges at a significance level of 0.05 were used to compare the mean values. All experiments were carried out in triplicate.

RESULTS

Proximate Value of QPI and QS

The proximate value of QPIs and quinoa seed flour is shown in Table 1. The three quinoa flours had a significant difference in protein, carbohydrate, ash, and energy ($P \leq 0.05$). However, no significant differences in dry matter and fat content were observed ($P > 0.05$). The protein content of Black-QS flour (16.02±0.33 g 100 g⁻¹) and T-QS (16.40±0.22 g 100 g⁻¹) did not reveal any significant differences, while, a lower protein content (14.93±0.21 g 100 g⁻¹) of Q12-QS was shown. Q12-QS flour was

higher in carbohydrates than T-QS and Black-QS. The energy values in this study were (401.21±0.81 to 410.7±0.3 kcal 100 g⁻¹ dw), higher than the average value of quinoa (331-381 kcal 100 g⁻¹) (Nowak *et al.*, 2016).

The highest ash content (2.97±0.12 g 100 g⁻¹) was detected in Black-QPI, whereas the lowest value (2.13±0.6 g 100 g⁻¹) was observed in T-QPI. The highest level of pH in the Black-QPI was (5.61±0.04). The fat content of T-QPI was (0.63±0.01 g 100 g⁻¹), which was 40% higher than that of Black-QPI. The highest carbohydrate content was reported in Q12-QPI (21.42±0.96 g 100 g⁻¹), which was 44% higher than Black-QPI. The highest level of energy was (405.0±5.4 kcal 100 g⁻¹), which was allocated to the T-QPI.

Amino Acid Analysis

The composition of amino acids and chromatograms of Black-QS, Q12-QS, and T-QS are shown in Table 2 and Figure 2, respectively. The concentration of amino acids in quinoa varieties varied: with tryptophan (6.55-8.23%), glutamic acid (0.77-1.07%), and glycine (0.25-0.46%) the predominant amino acids in all varieties. T-QS was higher in amino acids than the others. Lysine (0.3%) and threonine (0.14%) were the most important essential amino

acids of T-QS, which were found as a limited amino acid in conventional grains, for example in wheat.

Foaming Capacity and Stability

The potential of QPI as a whipping agent depends on its foaming ability and stability.

Foams improve the texture, consistency, and appearance of food. The Black-QPI showed a higher foaming capacity (65.26%±11.76) than T-QPI and Q12-QPI (Table 3). However, no significant difference was found between Black-QPI and T-QPI in foam stability.

Viscosity of QPIs

The oscillatory rheology of QPIs is shown in Figure 3. As expected, all QPI samples were characterized as Newtonian liquids. As a result, the association between shear rate and resultant stress is linear, as with Newtonian fluid. There were no significant differences among the QPI samples. In addition, the shear rate versus viscosity relationships of QPIs are shown in Figure 3-B.

Table 1. Proximate value (Mean±SD, n= 3) of three Quinoa Seeds (QSs) and Quinoa Protein Isolated (QPI) of three genera (Black, Q12, and Titicaca).^a

Parameters	Black-QS	Q12-QS	T-QS	Black-QPI	Q12-QPI	T-QPI
pH	6.74±0.04a	6.45±0.05b	6.04±0.01c	5.61±0.04C	4.48±0.03A	4.84±0.03B
Dry matter (g 100 g ⁻¹ as fed)	95.10±1.47a	95.78±1.96a	95.29±1.24a	98.20±0.15A	98.35±0.22A	98.22±0.09A
Protein (g 100 g ⁻¹ dw)	16.02±0.33a	14.93±0.21b	16.40±0.22a	81.72±1.83A	75.42±0.87B	82.18±1.51A
Fat (g 100 g ⁻¹ dw)	3.62±0.16a	3.73±0.06a	3.90±0.20a	0.45±0.5B	0.56±0.03A	0.63±0.01A
Ash (g 100 g ⁻¹ dw)	5.43±0.15a	4.36±0.22b	3.67±0.2c	2.97±0.12A	2.60±0.1B	2.13±0.6C
†Carbohydrates (g 100 g ⁻¹ dw)	74.93±0.14c	76.98±0.19a	76.03±0.34b	14.86±1.74B	21.42±0.96A	15.06±1.43B
‡Energy (kcal 100 g ⁻¹ dw)	409.6±0.7c	401.21±0.81 b	410.7±0.3a	404.4±6.6B	404.1±3.5A	405.0±5.4C

^a Values are expressed as mean±SD. Means in the same row with different lowercase letters (A–C) among quinoa protein isolate (QPI) (Black, Q12 and Titicaca) averages differ significantly (P≤ 0.05). Means in the same row with different lowercase letters (a–c) among quinoa seeds (Black, Q12, and Titicaca) averages differ significantly (P≤ 0.05). dw: Dry weight, Quinoa Protein Isolate (QPI); Quinoa Seed (QS); Titicaca (T).

† Total carbohydrate (g 100 g⁻¹)= 100-(m_{fat}+m_{ash}+m_{proteins}); ‡ Energy= 4× (%Protein+%Carbohydrates)+9×(%Fat).

Table 2. Amino acid profile in three quinoa generations (Black-QS, Q12-QS, T-QS).^a

Amino acid (g 100 g ⁻¹)	Black-QS	Q12-QS	Titicaca-QS
Aspartic acid	0.44	0.45	0.57
Glutamic acid	0.77	0.86	1.07
Serine	0.14	0.17	0.25
Tyrosine	< 0.06	< 0.06	< 0.06
Arginine	< 0.06	0.16	0.18
Methionine	< 0.06	< 0.06	0.07
Tryptophan	6.55	7.99	8.23
Valine	0.17	0.20	0.29
Isoleucine	0.03	0.09	0.17
Lysin	< 0.06	< 0.06	0.30
Phenylalanin	< 0.06	< 0.06	0.08
Leucin	0.18	0.22	0.31
Histidin	< 0.06	< 0.06	< 0.06
Glycin	0.25	0.37	0.46
Teronin	0.06	0.09	0.14
Alanin	0.14	0.17	0.3

^a Quinoa Seed (QS); Titicaca (T).

Table 3. Foaming capacity and stability (Mean±SD, n= 3) of Quinoa Protein Isolated (QPI) of the three genera (Black, Q12, and Titicaca).^a

QPI genera	Protein conc.% (w/v)	Foaming capacity (%)	Foaming stability % at time interval (min)				
			0.5	5	10	40	60
Black-QPI	0.1	50.01±1.77 ^E	75.38±3.86 ^{aE}	70.77±2.72 ^{aC}	50.77±5.44 ^{bDE}	38.46±2.68 ^{cF}	33.85±2.69 ^{cG}
	0.5	60.03±2.32 ^C	78.22±0.44 ^{aCDE}	77.02±3.82 ^{aAB}	57.63±4.12 ^{bBC}	42.23±3.03 ^{cEF}	37.20±1.33 ^{cFG}
	1	72.53±2.08 ^B	80.60±0.92 ^{aBCD}	77.49±0.91 ^{bAB}	57.51±1.06 ^{cEF}	46.26±2.17 ^{dCDE}	42.49±0.99 ^{eDE}
	3	76.93±3.36 ^A	84.50±1.64 ^{aAB}	78.49±1.87 ^{bAB}	65.68±0.36 ^{cA}	53.00±2.34 ^{dB}	49.02±0.47 ^{eAB}
	Average	65.26±11.76 ^b	79.77±3.90 ^a	76.04±3.89 ^a	55.91±7.73 ^a	45.00±6.04 ^c	40.54±6.12 ^a
Q12-QPI	0.1	43.09±3.09 ^F	67.92±1.62 ^{aF}	48.43±4.95 ^{bE}	39.38±2.75 ^{cH}	33.89±1.20 ^{dG}	28.67±2.85 ^{dH}
	0.5	49.24±2.34 ^E	70.38±2.97 ^{aF}	53.17±4.50 ^{bDE}	43.74±3.66 ^{cGH}	38.99±2.47 ^{cdF}	34.43±3.75 ^{dFG}
	1	53.09±3.49 ^{DE}	76.87±1.56 ^{aDE}	49.41±3.73 ^{bE}	41.98±1.72 ^{cFG}	39.13±1.25 ^{cdF}	36.17±1.91 ^{dFG}
	3	55.39±0.43 ^D	83.33±5.01 ^{aBCD}	58.33±1.39 ^{bD}	54.17±2.41 ^{bcCDE}	50.00±2.41 ^{cdCD}	41.67±3.67 ^{dCD}
	Average	50.20±5.35 ^a	74.28±6.30 ^b	51.99±4.84 ^b	44.77±5.76 ^b	39.81±5.25 ^b	35.58±5.99 ^b
T-QPI	0.1	50.00±0.35 ^E	78.46±1.66 ^{aCDE}	73.85±1.42 ^{bBc}	55.38±2.74 ^{cBCD}	44.62±1.47 ^{dDE}	38.46±4.13 ^{eEF}
	0.5	53.08±1.7 ^{ED}	81.11±4.16 ^{aBCD}	76.76±4.24 ^{Aab}	57.95±1.8 ^{bBC}	49.23±3.33 ^{cBC}	42.05±1.04 ^{dDE}
	1	56.17±2.74 ^D	83.60±1.78 ^{aABC}	78.11±1.62 ^{Aab}	58.86±2.85 ^{cB}	52.12±3.27 ^{dB}	46.53±1.88 ^{eBC}
	3	74.62±0.69 ^B	88.68±4.43 ^{aA}	82.49±2.63 ^{bA}	65.99±2.39 ^{cA}	58.77±1.64 ^{dA}	52.57±1.85 ^{eA}
	Average	58.47±10.10 ^a	82.96±4.83 ^a	77.80±3.99 ^a	59.55±4.62 ^a	51.18±5.8 ^a	44.91±5.90 ^a

^a Data (mean ± standard deviation) are from three replications. Quinoa protein isolate (QPI); Titicaca (T); Means in the same column with different uppercase letters (A-H) and rows with different lowercase letters (a-e) among (Q12-QPI, Black-QPI, and T-QPI) differ significantly ($P \leq 0.05$); Means in the same column and rows with different bold underline lowercase letters (a–b) among (Q12-QPI, Black-QPI, and T-QPI) averages differ significantly ($P \leq 0.05$).

that of Gómez *et al.* (2021) results that reported a protein content range of 15.59–18.73% with The observed protein content of quinoa (14.93±0.21 to 16.40±0.22% w/w) is consistent with the findings of Gómez *et al.*, who reported a protein content range of 15.59–18.73% w/w.

According to the FAO/WHO/UNU (2007) standards for protein quality, quinoa protein can provide substantial excesses of several

essential amino acids relative to the recommended levels for adult nutrition. Specifically, it supplies approximately 180% of the histidine requirement, 274% of isoleucine, 338% of lysine, 212% of methionine plus cysteine, 320% of phenylalanine plus tyrosine, 331% of threonine, 228% of tryptophan, and 323% of valine. Moreover, quinoa contains unusually high concentrations of sulfur-containing

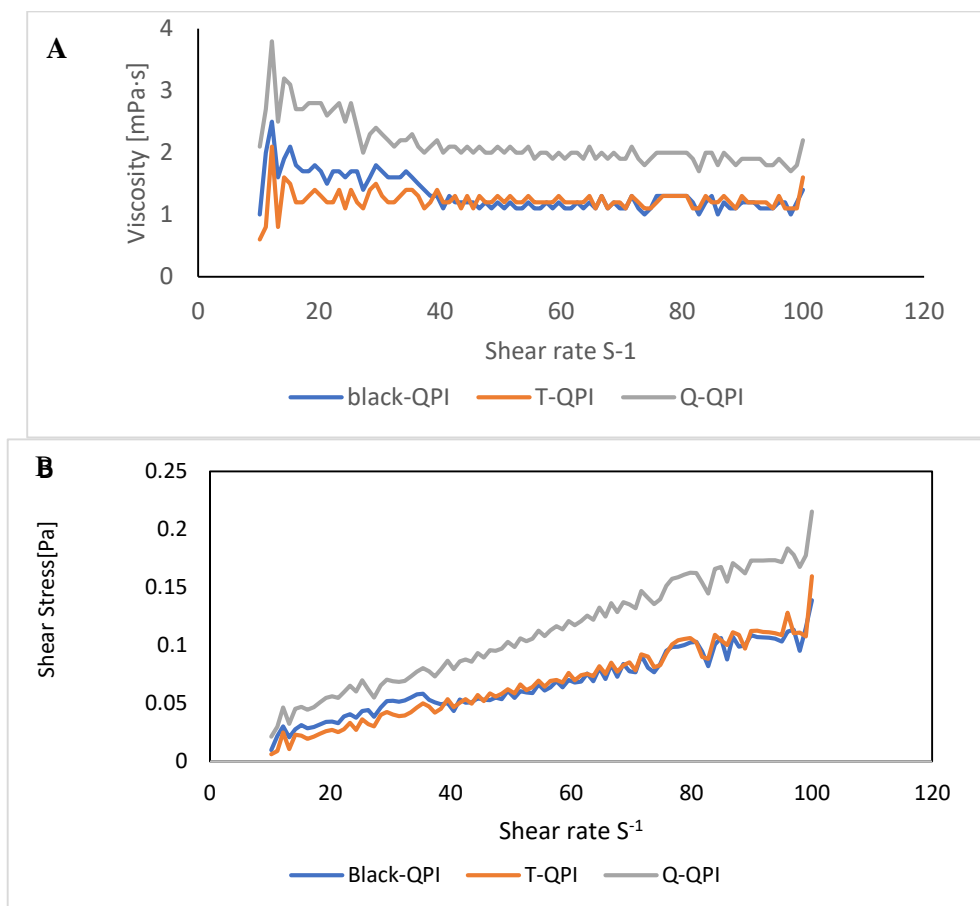


Figure 3. (A) The apparent viscosity, and (B) Shear stress versus shear rate curves of QPIs samples (Black-QPI, Q12-QPI, T-QPI). Quinoa Protein Isolate (QPI), Titicaca (T).

Table 4. Oil, and water absorption parameters (Mean±SD, n= 3) of Quinoa Protein Isolated (QPI) of the three genera (Black, Q12, and Titicaca).^a

Parameters		Black-QPI	Q12-QPI	T-QPI
Water and oil absorption	WA (mL g ⁻¹)	1.9±0.01b	1.0±0.06c	2.02±0.02a
	OA (mL g ⁻¹)	3.02±0.03a	2.0±0.02c	2.42±0.03b

^a WA: Water Absorption; OA: Oil Absorption; Quinoa protein isolate (QPI), Titicaca (T).

amino acids—methionine and cysteine—compared to most other plant sources. The overall profile of essential amino acids in quinoa surpasses that of the conventional cereal grains (Vega-Gálvez *et al.*, 2010). Histidine, isoleucine, lysine, sulfur amino acids, aromatic amino acids, threonine, tryptophan, and valine content met the daily requirements for these amino acids for all age groups (Craine *et al.*, 2020). Similarly, Dini *et al.* (1992) found that decorticated quinoa exhibited nutritional properties equal

to or better than those of commonly consumed cereals. Additionally, quinoa is recognized as an exceptional source of leaf protein concentrate, indicating its potential use as a protein substitute in both human food and animal feed, as well as in pharmaceutical applications (Vega-Gálvez *et al.*, 2010).

The carbohydrate content of quinoa (74.93±0.14 to 76.03±0.34%) was comparable to the results of Saavedra and Carmen Valdez-Arana (2021) who observed

a carbohydrate content of $70.81 \pm 0.11\%$ (Saavedra and Carmen Valdez-Arana, 2021).

Starch is the primary carbohydrate component in quinoa, accounting for 52 to 69% of its total composition. The total dietary fiber content of quinoa is comparable to that found in other cereal grains, ranging from 7 to 9.7%, with soluble fiber making up 1.3 to 6.1%. Quinoa also contains approximately 3% sugars, primarily in the form of maltose, D-galactose, and D-ribose, along with smaller amounts of fructose and glucose (James, 2009).

Due to its functional properties, quinoa serves as an effective thickening agent for sauces, soups, and flours. Its resistance to retrogradation further expands its culinary applications, enabling the creation of creamy smooth textures that mimic those of fats (Vega-Gálvez *et al.*, 2010; James, 2009).

The amylose content of quinoa starch ranges from 3 to 22%, which is lower than that of wheat and corn, higher than certain barley varieties, and comparable to common rice types. Compared to starches from wheat and barley, quinoa starch demonstrates greater maximum viscosity, enhanced water absorption capacity, and superior swelling power. Moreover, it exhibits notable stability during freezing and retrogradation processes (Tang *et al.*, 2002).

After all, the T-QS meal contained more energy than the others. The differences might be due to the interaction of various factors, including cultivars, analytical methods, and environmental conditions (Nowak *et al.*, 2016). The variations found among genera were supported by others (Alvarez-Jubete *et al.*, 2009; Nascimento *et al.*, 2014; Palombini *et al.*, 2013). These remarkable variations in the content of QPIS nutrients were noticed among different genera. The possible explanations for these variations are associated with the interaction of numerous factors including crop genetics, analytical methods, and multiple environmental circumstances (Razzeto *et al.*, 2019).

Cereals are a fundamental component of the human diet, providing approximately half of the dietary energy and protein intake for many populations. When we compare the nutritional composition of commonly consumed cereals with that of quinoa, it exhibits higher levels of protein, fat, and ash content compared to traditional cereals (Filho *et al.*, 2017).

The percentage of protein, in the current work, has been considerably improved when it was compared to the data reported by Abugoch *et al.* (2008) (77.2 and 83.5% in Q9 and Q11 QPIs, respectively) while, this was lower than the report of Ruiz *et al.* (2016) (90~93%) in sweet variety of Atlas quinoa. Such differences in the of the proteins' percentage were related to the varieties of quinoa (mentioned before), extraction, and post-extraction processes. For example, Wang *et al.* (2021) reported that QPI (Qingli 2 cultivar) and samples treated with microwave heating, steaming, boiling, and baking showed protein's contents of 89.8, 87.9, 89.1, 88.6, and 88.1%, respectively (Abugoch *et al.*, 2008; Ruiz *et al.*, 2016; Wang *et al.*, 2021). Quinoa protein isolate represents a promising nutritional ingredient with strong potential for use as a food supplement or functional food component. Beyond its high nutritional value—including a complete amino acid profile—it exhibits functional properties that make it well-suited for incorporation into cereal-based and other food products. These functional attributes, which are linked to the protein's physicochemical characteristics, play a key role in food processing and product development. As a nutrient-dense source of protein, fibre, healthy fats, and carbohydrates, quinoa can contribute meaningfully to balanced diets when consumed alongside a variety of other foods (Elsouhaimy *et al.*, 2015). Quinoa protein has gained attention as a high-quality plant-based protein due to its balanced amino acid profile, particularly its high lysine content. It exhibits good functional properties such as solubility, emulsification, and gelation,



which can be enhanced through processing techniques like fermentation and enzymatic hydrolysis. These proteins also possess antioxidant activity, contributing to food stability and health benefits. With the support of emerging green technologies, quinoa protein shows strong potential as a sustainable alternative to dairy proteins in food formulations (Alrosan *et al.*, 2022).

Amino Acid Analysis of Quinoa Seed

A wonderful amino acid profile was discovered in the quinoa seed, with acceptable amounts of Essential Amino Acids (EAAs) which are playing a crucial role in the growth and maintenance of metabolic activities and a desirable bioavailability. The QPIs are predominantly rich in histidine, methionine, and lysine that are generally observed in limited amounts in other common grains (Dakhili *et al.*, 2019).

In amino acid measurement, different findings are shown by Gómez *et al.* (2021). Different genotypes and years of growth of the plant can potentially influence these variables both in the calibration and the external validation set. This was ultimately important for developing calibration equations for future predictions (Escuredo *et al.*, 2014).

High amounts of all the essential amino acids, except methionine (0.33-0.41%), were recorded in the amino acid profiles of two pigeon pea varieties and two chickpea selections. In this work, the methionine value was lower than pigeon pea and chickpea. Lysine content was also higher (7.45–7.90%) in pigeon pea and chickpea varieties compared with QPIs (\leq 0.06-0.3%). The values of tryptophan were higher in QPIS (6.55-8.23%) than the mentioned legumes (0.46-0.96) (Anitha *et al.*, 2020).

Quinoa stands out as a highly nutritious plant-based protein source, with amino acid content closely aligned with FAO recommendations. It provides all essential amino acids, particularly rich in lysine and sulfur-containing amino acids, making its

protein quality superior to many cereal grains. Research indicates that the bioavailability of quinoa proteins improves significantly after cooking, varying depending on the variety consumed. Quinoa has high protein content and notable levels of tryptophan, often a limiting amino acid in other plants, which plays a key role in serotonin production. Additionally, quinoa contains non-protein tryptophan forms that are more readily absorbed, potentially enhancing brain function through improved neurotransmitter synthesis (Navruz-Varli *et al.*, 2016).

Foaming Capacity and Stability

The foaming properties of Quinoa Protein Isolates (QPIs) was evaluated as critical functional characteristics, particularly for their potential application in food systems requiring aeration, such as baked goods. Foaming ability generally increased with rising QPI concentration, ranging from 50.01±1.77 to 76.93±3.36% for Black-QPI, 43.09±3.09 to 55.39±0.43% for Q12-QPI, and a constant value of 50.00±0.35% for T-QPI. Among all samples, Black-QPI exhibited the highest average foaming ability (65.26±11.76%). Similarly, foaming stability improved with increasing concentration but declined over time. At 0.5 minutes of storage, foaming stability ranged from 75.38±3.86 to 84.50±1.64% for Black-QPI, 67.92±1.62 to 83.33±5.01% for Q12-QPI, and 78.46±1.66 to 88.68±4.43% for T-QPI, with T-QPI showing the highest average (82.96±4.83%). These results highlight the strong capacity of quinoa proteins to form stable foams, indicating their promising applicability in food formulations. Compared to egg albumin — a well-known excellent foaming agent with reported foaming ability values of 156–200% and foaming capacity of 33–54% (Lomakina and Mikova, 2006). Quinoa protein demonstrated relatively lower foaming ability, but comparable foam stability (35–44%). The foam stability of

QPI was found to be significantly higher than that of soybean protein, and slightly lower than that of egg white protein (Abugoch *et al.*, 2008). This behavior may be attributed to protein unfolding at low pH, which exposes hydrophobic regions and enhances interfacial activity.

Additionally, molecular configuration and solubility play crucial roles in determining foaming performance, with more flexible proteins typically exhibiting superior foaming properties (Jan *et al.*, 2018). Since foaming capacity and stability are influenced by factors such as interfacial film properties, moisture retention, and surface hydrophobicity, higher net charge can enhance solubility by reducing hydrophobic interactions and facilitating rapid spreading at the air–water interface (Ghumman *et al.*, 2021). The observed differences among QPI variants may also be related to variations in protein content and structural characteristics. For instance, Q12-QPI had the lowest protein content (Table 1), which corresponded with its inferior foaming properties. Moreover, Steffolani *et al.* (2016) emphasized that different quinoa genotypes exhibit variable foaming behaviors, underscoring the importance of genetic and compositional factors in determining functionality. Overall, these results suggest that certain QPI varieties, particularly T-QPI and Black-QPI, hold significant potential for use in aerated food products like cakes and meringues (Ogungbenle *et al.*, 2009).

Viscosity

Proteins are highly functional molecules in food systems that facilitate processing and affect the final product performance. Functional properties denote the physicochemical properties that govern protein behavior in foods with regards to their distinct amino acid sequences, molecular weight and other factors. Viscosity plays an important role that affects protein stability in food processing and

product application. Highly concentrated proteins are considered highly viscous; thus, its viscosity is considered as the most important factors to control in food processing (Yolandani *et al.*, 2023). The viscosity of plant protein dispersions is affected by factors such as pH, temperature, protein concentration, and ionic strength, making it essential to optimize these parameters for the desired consistency. One advantage of plant proteins is their ability to provide thickening and structural stability, enhancing product quality without the use of animal-derived ingredients. Their application in food formulations allows for the development of sustainable, nutritious, and texturally desirable plant-based alternatives to traditional dairy and meat products (Roy *et al.*, 2025).

Oil and Water Absorption of QPIs

The water and oil absorption of food materials is an important functional property that improves the sustainability of texture and flavour. In similar research, water and oil absorption capacities of quinoa seed were 147% and 46%, respectively (Abugoch *et al.*, 2008). Previous studies on the water and oil absorption capacity of QPIs by Ashraf *et al.* (2012) and Elsohaimy *et al.* (2015) showed that these mentioned factors had 3.94 ± 0.06 and 1.88 ± 0.02 mL g⁻¹ protein, respectively. Recently, Reséndiz *et al.* (2019) studied the oil absorption capacity of QPIs and discovered that QPIs had a 2.66 mL g⁻¹ value, which supported the results of the present work (Ashraf *et al.*, 2012; Elsohaimy *et al.*, 2015; Reséndiz *et al.*, 2019).

Oil intake is of utmost importance as oil acts as a flavor reservoir, it enhances the mouth feel of food. This indicates that Black-QPI may have stronger flavor retention than the other types. The oil and water absorption capacities were different among the genera. This can be explained by the difference between the varieties of quinoa and the areas where the quinoa



germinated (El Sohaimy *et al.*, 2018). The oil absorption capacity depends on the amount of exposed hydrophobic amino acid residues in the protein and the hydrophobic amino acid content. The water absorption rate of quinoa protein depended on the method of drying the protein and the pH level. Furthermore, this can be attributed to the particle size and larger specific surface area of QPI. We specifically highlight significant differences in protein content, carbohydrate composition, and key functional properties such as foaming capacity, water and oil absorption, and rheological behavior. Additionally, we provide detailed proximate composition data for both native seeds and isolated proteins, which can serve as a valuable reference for future studies aimed at optimizing food formulation and developing novel plant-based protein products. Overall, this study increases the scientific value of quinoa by providing a framework for selecting varieties based on specific nutritional and functional criteria for food applications.

CONCLUSIONS

In conclusion, significant differences in the chemical composition, structure, and rheological properties of Quinoa Protein Isolates (QPIs) from three varieties were identified. These were Titicaca (T-QPI), Q12 (Q12-QPI), and Black (Black-QPI), likely due to inherent seed composition differences. Q12-QPI showed the best rheological performance and suitability for texture-demanding food applications, while Black-QPI excelled in protein content, foaming, and oil absorption, making it ideal for emulsification and aeration. T-QPI demonstrated superior water absorption, beneficial for moisture retention, and showed similar protein content to Black-QPI, indicating comparable nutritional value. Titicaca quinoa seeds also exhibited the most balanced essential amino acid profile, emphasizing their potential as a high-quality plant protein. These results

highlight the importance of variety selection in optimizing quinoa proteins for specific food functions. Future research is needed to enhance processing methods that maintain protein quality across genotypes.

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خواص تغذیه ای و فیزیکیوشیمیایی ایزوله پروتئین های کینوا در ارقام سیاه، Q12 و تیتیکاکا

سید سعید سخاوتی زاده، و سعید حسین زاده

چکیده

کینوا یک شبه غله است که اخیراً در ایران کشت می شود. هدف از این تحقیق بررسی خواص پروتئین ایزوله آن برای استفاده در غذا می باشد. ایزوله های پروتئین کینوا از واریته های دانه کینوا سیاه، Q12 و تیتیکاکا استخراج شدند. محتوای پروتئین کینوای سیاه و تیتیکاکا به ترتیب $(87/30 \pm 1/96)$ ، $(87/80 \pm 1/161)$ وزنی/وزنی بوده است. نتایج نشان داد در پروتئین کینوای سیاه ظرفیت کف کردن $(40/54)$ درصد، پایداری کف $(65/26)$ در 60 دقیقه و جذب روغن $(3/02)$ میلی لیتر بر گرم) به طور معنی داری $(P \leq 0.05)$ بیشتر از سایر نمونه ها بود. پارامترهای بافتی نشان داد که ویسکوزیته و تنش برشی در Q12 بیشتر از سایرین بود. پروفایل اسید آمینه نشان داد که رقم تیتیکاکا دارای پروفایل متعادل با بالاترین محتوای تریپتوفان $(8/23)$ درصد بوده است. در نتیجه، ارزش غذایی و عملکردی مناسب پروتئین کینوای تیتیکاکا، آن را به عنوان گزینه مناسبی عنوان افزودنی در مواد غذایی تبدیل می کند.

Enhancing the Shelf Life and Sensory Properties of Rainbow Trout Fillets through Sodium Alginate Coating Containing *Eryngium campestre* Extract at 4°C

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ABSTRACT

Fresh fish is a highly perishable food item and spoils easily. In this research, after investigating the antioxidant properties of the *Eryngium Campestre* extract (Ece), its effect along with the sodium alginate coating was evaluated on the shelf-life of rainbow trout under refrigerated conditions (4°C) for 12 days. To assess the antioxidant properties of the extract, tests such as DPPH, total phenolics, reducing power, and ABTS were performed. Subsequently, samples treated with an Ece containing alginate coating applied via spray method were analyzed for chemical parameters (TBA, TVN, and pH), microbiological parameters (total psychrotrophic and mesophilic bacteria), and sensory evaluations at four day intervals up to 12 days. The results indicated that Ece possessed significant antioxidant properties. Furthermore, treatments that included the extract combined with the sodium alginate coating significantly reduced pH, TVN, and TBA levels compared to the control sample ($P < 0.05$). Microbial tests indicated that all treated samples inhibited bacterial growth, with a reduction of approximately 3 log CFU g⁻¹ compared to the control group. In the sensory evaluation, treatments containing Ece and sodium alginate yielded more favorable results than those of the control group. Accordingly, coating the samples with sodium alginate and Ece improved the microbial, chemical, and sensory properties and shelf life of rainbow trout in refrigerator conditions by about four days.

Keywords: DPPH, Fresh fish, Microbiological parameters, Sensory properties, TBA, TVN.

INTRODUCTION

Fish is a vital source of protein and omega-3 fatty acids (ω -3 PUFAs), which are important for a healthy diet. Since the body cannot produce omega-3s, they must be obtained from food. These fatty acids offer numerous health benefits, including improved heart health and reduced inflammation (Zarandi *et al.*, 2022). Rainbow trout (*Oncorhynchus mykiss*) is one of the most extensively farmed freshwater fish species worldwide and is preferred by consumers for its high nutritional value. The chemical composition of trout fillets can differ based on various factors such as age,

gender, season, water temperature, and dietary conditions (Foromandi and Khani, 2023). Typically, trout fillets contain approximately 73% water, 20% protein, 5% fat, and 1.5% minerals. This nutritional composition, along with significant levels of essential amino acids and polyunsaturated fatty acids, categorizes rainbow trout as highly perishable food (Popelka *et al.*, 2014).

The shelf life and overall quality of fish can be affected by enzymatic and microbial processes, along with the oxidation of fats (Mazandrani *et al.*, 2016). Additionally, there are safety concerns related to lipid oxidation and microbial growth (Shakour *et*

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al., 2021). Various strategies have been investigated to preserve fish products and extend their shelf life, including the incorporation of plant extracts as natural additives (Charoenphun *et al.*, 2023).

Plant extracts have antibacterial and antioxidant properties (Rathod *et al.*, 2021). To reduce the negative effects of chemical preservatives and meet consumer demand for natural products, plant extracts and edible coatings are used to extend the shelf life and prevent spoilage of fresh fish (Fadiloğlu and Emir Çoban, 2018). These properties are essential as they can control the growth of spoilage microorganisms and shield the fish from oxidative damage. Nevertheless, their application is often restricted due to costs and potential toxicity.

Edible coatings derived from alginate, a biodegradable hydrocolloid, have been implemented to preserve fish fillets. Alginate coatings provide a physical barrier while also improving the overall quality and acceptability of the fish by preserving moisture and minimizing lipid oxidation (Urbonavičiūtė *et al.*, 2023).

Eryngium campestre L., part of the *Apiaceae* family, is a perennial plant that grows in Asia, Europe, and Africa. This plant is widely used in traditional medicine to treat various conditions, including coughs, urinary infections, increased urination, kidney dysfunction, and the removal of kidney and bladder stones (Azizkhani and Sodanlo, 2021). *E. campestre* is abundant in phenolic compounds, which contribute to its antioxidant and antimicrobial properties (Kartal *et al.*, 2006). This study examines the impact of *E. campestre* extract in edible and biodegradable coatings, made from sodium alginate, on the quality and shelf life of rainbow trout fillets stored at 4°C. The incorporation of *E. campestre* extract is intended to enhance the preservative properties of these coatings, thereby maintaining the fish's sensory characteristics and nutritional value during extended storage. The research aimed to support sustainable practices in the seafood industry

by promoting natural preservation methods over synthetic chemicals.

MATERIALS AND METHODS

Preparation of Ethanolic Extract of *E. campestre*

The maceration method was used to prepare the ethanolic extract from the *E. campestre*. This plant was picked from the forest areas of Amol City. The leafy part and stem were dried in the shade and ground into a powder, passed through a sieve with a 60 µm mesh size. Then, 200 g of powder were mixed with 1 L of pure ethanol and placed in a shaker incubator at 150 rpm for 24 hours at 42°C. To remove most of the solvent, the mixture was placed in a rotary evaporator (Heidolph, Laborota 400 efficient, Germany) at 50°C and 100 rpm under vacuum conditions. To determine the concentration, it was placed in an oven at 45°C and, finally, lyophilized at -50°C for 24 hours. Then, it was refrigerated in a closed container (Alizadeh Amoli *et al.*, 2019).

GC-MS Analysis of *E. campestre* Extract

GC-MS analysis was performed using an Agilent 7890A GC device equipped with an HP-5MS column and a 5975 mass spectrometer (Agilent Technologies, USA) (Mishra and Patnaik, 2020).

Antioxidant Activity Analyses

The following four methods were used to evaluate and check the antioxidant power of the plant extract.

DPPH Test (1,1-Diphenyl-2-Picrylhydrazyl)

The study tested the antioxidant activity of an extract by diluting it to 0.25 mg mL⁻¹ and adding it to a DPPH solution. Absorbance was measured at 517 nm with spectrophotometer (Pharmacia Biotech, Sweden) after 30 minutes in the dark. The free radical inhibition was calculated using the formula: $AC \times 100 / (AC - AS)$, where AC is the Absorbance of the Control, and AS is the Absorbance of the Sample. BHT was used as a positive control at 1 mg mL⁻¹ (Ebrahimi and Larypoor, 2022).

Determination of the Total Phenolic Content

The total phenolic content of the plant was measured using the Folin-Ciocalteu method. Extract dilutions were mixed with Folin's reagent, gallic acid, and sodium carbonate, then, kept in the dark for specified times. Absorbance was measured at 760 nm, and the total phenolic content was expressed as mg of gallic acid per gram of material (Gharedaghi *et al.*, 2020)

Determination of the Reducing Power

Extracts were mixed with sodium phosphate, potassium ferricyanide, and incubated at 50°C for 20 minutes. After adding trichloroacetic acid and centrifuging, distilled water and iron chloride were added to the supernatant. Absorbance was measured at 700 nm to assess the results (Merghache *et al.*, 2014).

ABTS Radical Cation Method

The antioxidant capacity was assessed using a modified ABTS method. ABTS•+ radical was generated by mixing ABTS and potassium persulfate, and incubated for 16 hours. The solution was diluted to achieve

an absorbance of 0.7 at 734 nm. Extracts or BHT were added to the ABTS•+ solution and, after 6 minutes, absorbance was measured. Inhibition percentage was calculated using the following formula:

$$(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}} \times 100$$

The results were expressed as antioxidant capacity equivalent to ascorbic acid (Kikowska and Thiem, 2021).

Preparation of Fish Samples

Rainbow trout, weighing approximately 600±50 g, was procured from a fish sales center in Urmia City. The fish was properly examined, and its internal organs were removed. The body of the fish was thoroughly rinsed with water to eliminate any residual blood. The dimensions of the fish fillet were measured at approximately 15 cm in length, 10 cm in width, and 3 cm in height, and its weight was approximately 100 g. Subsequently, the fish was taken to the Food Hygiene Laboratory at the Faculty of Veterinary Medicine, Urmia University, to ensure sample integrity and minimize the risk of microbial contamination.

Preparation of Treatments

The sodium alginate solution was prepared by dissolving 1.5 g of sodium alginate in 100 mL of warm distilled water, and the extract of *E. campestre* was added to this solution. The resulting mixture was thoroughly combined to achieve a uniform consistency, transferred to a spray bottle, and shaken well before use. Fish fillets were then coated with sodium alginate solution using a spraying method. Subsequently, the fillets were treated with 2% calcium chloride solution and air-dried at room temperature. Then, the fish fillets were prepared for coating in 3 groups and 1 control sample as follows:

1. Without coating and extract
2. With alginate spray coating and without extract



3. With alginate 1.5% spray coating containing 0.5% *E. campestre* extract
4. With alginate 1.5% spray coating containing 1% *E. campestre* extract.

The control sample and coated fillets were stored in resealable plastic bags in a refrigerator at 4°C. Microbiological, chemical, and sensory evaluations were performed on days 1, 4, 8, and 12 to assess the quality and stability.

Chemical Analyses of Treated Fish Fillets

pH Values

For this purpose, 5 g of each fish fillet sample was placed in 10 mL of distilled water and homogenized for 30 seconds at a speed of 13,500 rpm. Subsequently, the pH of the homogenized sample was measured using a calibrated pH meter. The pH meter had been calibrated prior to measurement using standard buffer solutions with pH values of 4 and 7 to ensure accuracy and reliability in the results (Ojagh *et al.*, 2010).

Total Volatile Base Nitrogen (TVB-N)

To determine TVB-N, 10 g of fish fillet was homogenized with distilled water, then, mixed with magnesium oxide or NaOH and heated in a Kjeldahl flask. The distillation vapors were collected in a boric acid solution, and after reaching 50 mL, the solution was titrated with sulfuric acid. The TVB-N value was calculated based on the amount of sulfuric acid consumed, expressed as mg of TVB-N per 100 g of fish fillets (Ghasemi *et al.*, 2023).

Thiobarbituric Acid Reactive Substance

To measure malonaldehyde content (TBARS), 10 g of fish fillet was homogenized with 5% TCA (Trichloroacetic

Acid) and BHT, filtered, and the filtrate adjusted to 50 mL. TBA reagent was added to the filtrate, heated at 100°C for 1 hour, and absorbance was measured at 532 nm. The TBARS value, indicating malonaldehyde, was calculated and expressed as mg k⁻¹ of fish meat (Ghasemi *et al.*, 2023).

Microbiological Analyses

Total Mesophilic Bacterial Count

After diluting the samples from each dilution tube, 100 microliters of each sample were inoculated onto plates containing PCA culture medium and spread uniformly using a Pasteur pipette. Subsequently, the plates were incubated upside down at 37°C for 48 hours. Then, the colonies were counted and reported as CFU g⁻¹ (Muñoz-Tebar *et al.*, 2023).

Total Psychrotrophic Count

After diluting the samples from each dilution tube, 100 µL of each sample was inoculated onto plates containing PCA culture medium and spread uniformly across the medium using a Pasteur pipette. The plates were then incubated in an inverted position at 10°C for 7 days. Following incubation, the colonies were counted and reported as CFU g⁻¹ (Muñoz-Tebar *et al.*, 2023).

Sensory Evaluation

A trained panel of 10 assessors evaluated the organoleptic properties of the treated fish fillets in two stages. In the first stage, they assessed the taste of cooked fillets, in the second stage, they evaluated refrigerated fillets for texture, aroma, and color on days 1, 4, 8, and 12. Fresh fillets at 4°C were used as the reference for maximum scores, and a 5-point hedonic scale was used for

evaluation (Bazargani-Gilani and Pajohi-Alamoti, 2020).

Statistical Analysis

After obtaining the data from the tested factors, SPSS version 26 software was used for statistical analysis, including one-way ANOVA for data analysis. Excell version 2022 was used to draw the graphs. Duncan's test was used to classify the samples according to the statistical difference between them and to measure their average. Normality tests (e.g., Shapiro-Wilk and Kolmogorov-Smirnov) were conducted prior to performing ANOVA to ensure the suitability of the parametric test. The results were considered significant at a P-value of < 0.05.

RESULTS AND DISCUSSION

GC-MS Results of *E. campestre* Extract

According to Table 1, The GC-MS analysis of *Eryngium campestre* extract identified 32 compounds, with limonene, δ -3-carene, β -sesquiphellandrene, and cyclobuta having the highest peak areas. This study's findings align with Fernandes (2013), who identified compounds like germacrene D and α -cadinol, although there are differences. Variations in chemical profiles are attributed to differences in plant samples, extraction methods, and environmental conditions, underscoring the chemical diversity of *Eryngium campestre* and its potential applications in the pharmaceutical and food industries. (Fernandes, 2013).

DPPH Antioxidant Test Results

In Figure 1, The DPPH assay results show that, as the concentration of *E. campestre* extract increases, the inhibition of free radicals also increases, indicating a strong

concentration-dependent antioxidant effect. This trend was evident across all concentrations, with the extract demonstrating high antioxidant activity in every dilution. These findings align with the work of Charoenphun *et al.* (2023) and Gharedaghi *et al.* (2020), who observed similar antioxidant effects in plant extracts. Furthermore, Kremer *et al.* (2021) reported comparable results in *E. amethystinum* and *E. alpinum*, confirming the potential of *E. campestre* as a natural antioxidant for applications in food preservation and oxidative stress reduction.

Total Phenolic Content

Based on the data from Table 2 and Figure 2, the total phenolic content in the *E. campestre* extract was determined using a calibration curve for gallic acid. The study revealed a strong correlation between the high phenolic content and antioxidant activity of the extract. This finding is consistent with Al-Askar *et al.* (2023), who also highlighted significant polyphenolic compounds in *E. campestre* extracts, which are directly linked to antioxidant and antimicrobial properties. These phenolic compounds play a crucial role in scavenging free radicals, supporting their potential in food preservation (Al-Askar *et al.*, 2023)

The Reduction Power Results

According to Figure 3, the reduction and absorption power at 700 nm for spectrophotometric measurements were observed with 2 mg of *E. campestre* extract (absorbance value of 1.8) and Butylated Hydroxytoluene (BHT) (absorbance value of 3). These results suggest that *E. campestre* extract exhibits significant antioxidant potential, comparable to BHT. The strong reducing power observed in this extract, particularly in its ability to reduce ferric ions, is vital for antioxidant applications. This finding is consistent with Merghache *et*

**Table 1.** GC-MS analysis of *E. campestre* extract.

No.	Compounds	RT	percentage
1	Heptanal	11.23	0.15
2	α -Pinene	11.47	1.87
3	n-Heptanol	12.31	0.42
4	Verbenene	13.57	0.84
5	Myrcene	14.54	1.95
6	n-Octanal	14.89	1.53
7	δ -3-Carene	15.56	6.79
8	p-Cymene	16.55	0.37
9	Limonene	16.69	26.71
10	Benzene acetaldehyde	17.15	0.16
11	n-Octanol	18.47	0.54
12	p-Mentha-2,4(8)-diene	19.65	0.27
13	Linalool	20.08	0.42
14	cis-p-Mentha-2,8-dien-1-ol	21.91	0.18
15	Z-4-Decenal	24.13	0.13
16	trans-Carveol	25.92	0.53
17	Citronellol	27.12	0.38
18	Thymol	29.97	0.23
19	Carvacrol	30.36	0.35
20	β -Elemene	33.74	0.21
21	α -cis-Bergamotene	35.84	0.72
22	α -Acoradiene	37.17	0.46
23	E- β -Ionone	38.02	1.23
24	Z- α -Bisabolene	38.65	2.57
25	β -Bisabolene	38.94	1.84
26	Myristicin	39.45	0.17
27	β -Sesquiphellandrene	39.61	15.25
28	Widdrol	41.13	0.83
29	trans-Longipinocarveol	45.87	5.28
30	Cyclobuta	46.16	24.19
31	n-Octadecane	50.87	0.28
32	n-Hexadecenoic acid	59.65	2.69
Total identified			99.54

al. (2014), who noted similar reducing power in *E. tricuspidatum* essential oil, further supporting the potential of *E. campestre* as a natural antioxidant and a promising alternative to synthetic antioxidants in food preservation (Merghache et al., 2014).

ABTS Radical Inhibitory Test Results

In Table 3, *E. campestre* extract showed inhibitory activity at all concentrations, with its performance lower than BHT, except at the highest concentration where it was

comparable. Increasing the extract concentration enhanced its free radical scavenging ability, as confirmed by the ABTS test. While its antioxidant activity was somewhat lower than BHT, the improved efficacy with higher concentrations suggests its potential for food preservation, supporting its use as a natural antioxidant to extend shelf life and improve food quality, consistent with previous studies (Kikowska and Thiem, 2021).

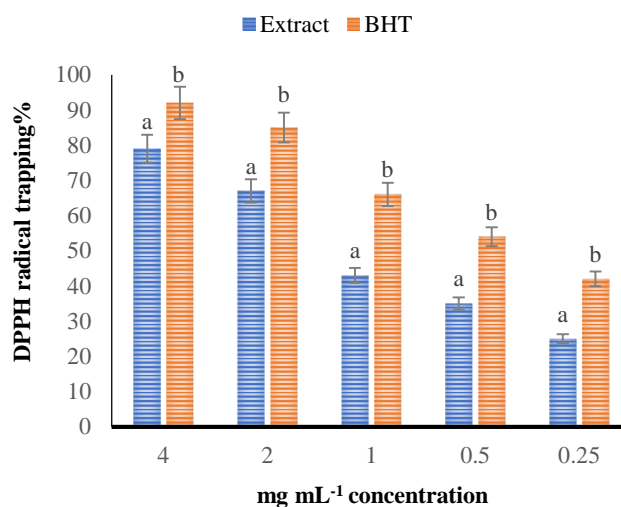


Figure 1. DPPH radical scavenging rate of different concentrations of *E. campestre* ethanol extract compared to BHT. a-b: Different letters in each concentration indicate a significant difference ($P < 0.05$).

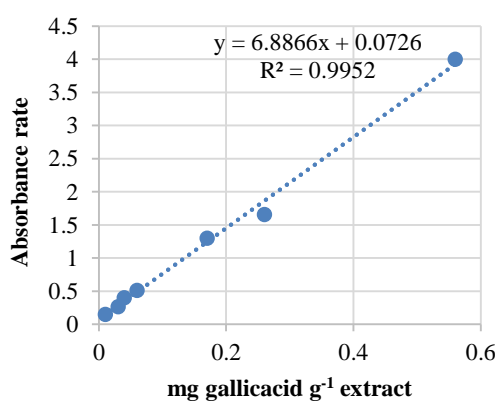


Figure 2. Gallic acid standard curve.

Table 2. Comparison of total phenol content of the ethanolic extract of *E. campestre* and the correlation between total phenol and antioxidant activity values.

Antioxidant assay by DPPH method			
R	(P) Sig.	mg gallic acid g ⁻¹ extract	Total phenol in the Alcoholic Extract
0.950	0.005**	125±9.80	

** Correlation is significant at the 0.01 level.

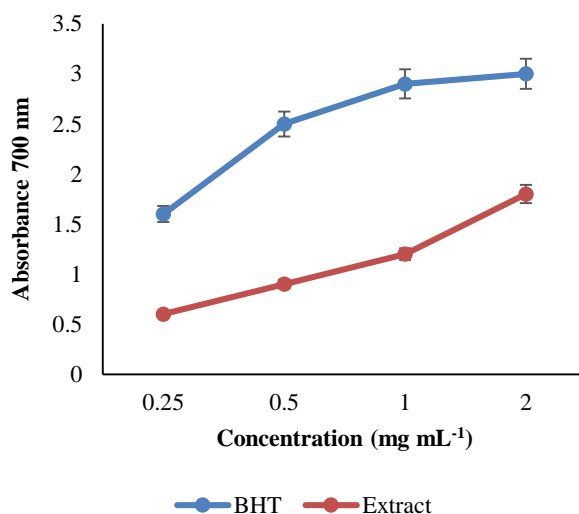


Figure 3. The rejuvenating potency of *E. campestre* extracts compared to BHT.

Table 3. Inhibition percentage and antioxidant capacity equivalent to ascorbic acid of different concentrations of alcoholic extract and BHT.^a

Concentration (mg mL ⁻¹)		Antioxidant capacity eq. ascorbic acid (mg mL ⁻¹)	Inhibition %
0.125	Extract	0.00 ± 0.001 ^{aA}	9.61 ± 6.51 ^{aA}
	BHT	0.01 ± 0.001 ^{bA}	64.85 ± 11.01 ^{bA}
0.25	Extract	0.006 ± 0.00 ^{aA}	46.37 ± 12.59 ^{aB}
	BHT	0.12 ± 0.01 ^{bB}	91.51 ± 1.67 ^{bB}
0.5	Extract	0.013 ± 0.02 ^{aB}	84.21 ± 0.68 ^{aC}
	BHT	0.18 ± 0.01 ^{bC}	92.46 ± 4.30 ^{bC}
1	Extract	0.09 ± 0.01 ^{aC}	85.26 ± 8.01 ^{aC}
	BHT	0.18 ± 0.01 ^{bC}	94.64 ± 4.21 ^{bD}
2	Extract	0.14 ± 0.01 ^{aD}	92.29 ± 2.21 ^{aD}
	BHT	0.18 ± 0.01 ^{aC}	96.86 ± 1.21 ^{bE}

^a In each column, non-identical lowercase letters indicate a significant difference at the P < 0.05 level; between the extract and BHT at the same concentration. Non-identical uppercase letters also indicate a significant difference between different concentrations of the same compound at the P < 0.05 level.

Chemical Composition of Fish Fillet

According to the analysis carried out on the rainbow trout sample, the approximate

amount of ash, fat, protein, and moisture was presented in Table 4, the obtained results are consistent with the findings of Torabi Delshad *et al.* (2012).

Table 4. Chemical composition of rainbow trout.

Composition	Percentage
Moisture	71.5 ± 0.23
Protein	22.16 ± 0.33
Fat	3 ± 0.63
Ash	1.6 ± 0.23

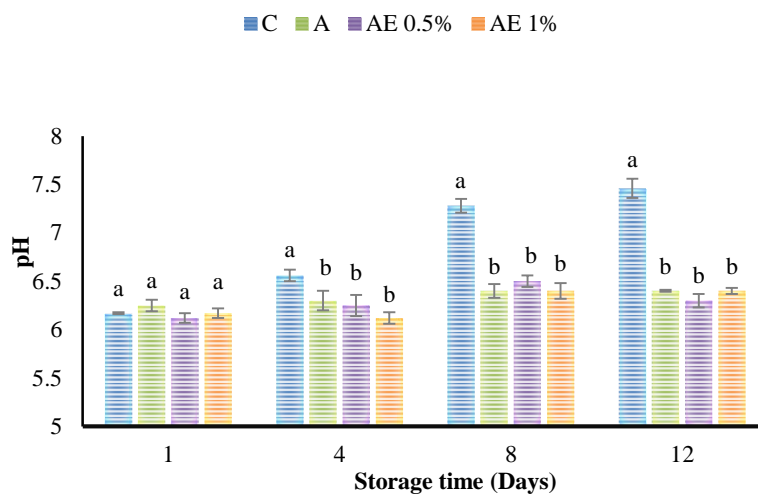


Figure 4. Comparison of pH changes in rainbow trout fillet samples during the storage at 4°C. Each day, non-identical lowercase letters indicate a significant difference at the $P < 0.05$ level. (C: Control, A: Alginate coating, AE 0.5%: Alginate containing 0.5% extract, AE 1%: Alginate containing 1% extract).

pH Measurement Results

During refrigerated storage, all rainbow trout samples showed an increase in pH, with the control sample exhibiting a much higher increase, especially on days 8 and 12. As shown in Figure 4, coated fillet samples with the extract maintained a stable pH within the permissible range (6-7), showing no significant increase from day 4 onwards. This aligns with Alizadeh *et al.* (2020), who found that treated samples displayed better pH stability, highlighting the antimicrobial and preservative efficacy of plant-derived bioactive compounds in delaying spoilage and maintaining food quality (Alizadeh Amoli *et al.*, 2019).

TVB-N Results

According to Figure 5, our study revealed an upward trend in TVB-N levels in all samples during storage. However, the

bioactive coating effectively kept these levels below the permissible limit ($25 \text{ mg } 100 \text{ g}^{-1}$) throughout the storage period. In contrast, the control sample exceeded this limit after 4 days, and the alginate-coated sample did so after 12 days. These results align with previous studies (Ojagh *et al.*, 2010), which found that chitosan-based coating combined with plant compounds significantly reduced TVB-N levels in fish samples and maintained them below the acceptable limit for 16 days. Similarly, Öz (2018) demonstrated that garlic supplementation inhibited microbial growth, and lowering TVB-N levels during frozen storage. Ozogul *et al.* (2017) reported that nanoemulsions with essential oils like rosemary and thyme effectively reduced spoilage, while Öz *et al.* (2017) highlighted the role of black cumin oil in slowing TVB-N increases in fish fillets at 2°C. These findings are inconsistent with ours, and underscore the potential of bioactive coatings in preserving fish quality.

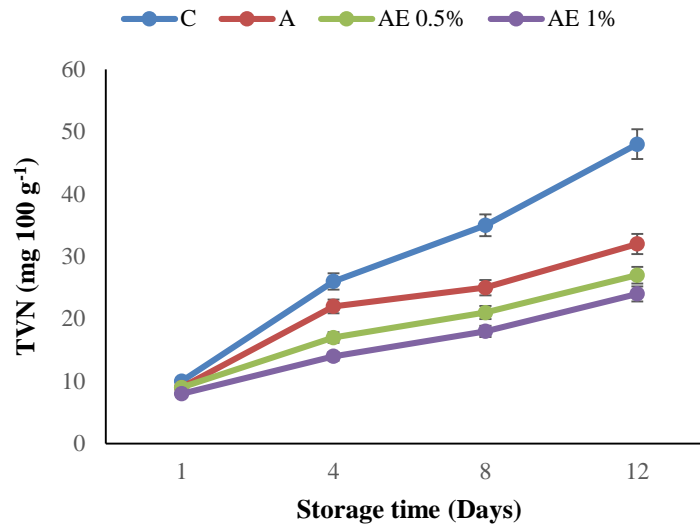


Figure 5. Changes of TVB-N in rainbow trout fillet during the storage period at 4°C. (C: Control, A: Alginate coating, AE 0.5%: Alginate containing 0.5% extract, AE 1%: Alginate containing 1% extract).

Table 5. Changes in the index of thiobarbituric acid (mg MDA kg⁻¹) in rainbow trout fillets during storage at 4 °C).

Treatments	Storage days			
	1	4	8	12
C	0.42 ± 0.01 ^{Aa}	2.02 ± 0.11 ^{Ba}	2.72 ± 0.04 ^{Ca}	3.12 ± 0.02 ^{Da}
A	0.40 ± 0.00 ^{Aa}	1.7 ± 0.06 ^{Bb}	2.42 ± 0.01 ^{Cb}	2.62 ± 0.02 ^{Db}
AE 0.5%	0.41 ± 0.01 ^{Aa}	0.89 ± 0.04 ^{Bc}	1.62 ± 0.02 ^{Cc}	2.40 ± 0.06 ^{Dc}
AE 1%	0.32 ± 0.01 ^{Aa}	0.78 ± 0.06 ^{Bd}	1.48 ± 0.01 ^{Cd}	2.22 ± 0.06 ^{Dd}

(C: Control, A: Alginate coating, AE 0.5%: Alginate containing 0.5% extract, AE 1%: Alginate containing 1% extract). Small letters in each column and non-identical capital letters in each row indicate a significant difference in the level ($p < 0.05$).

TBARS Value Results

In agreement with Mehdizadeh *et al.* (2019), our data indicated that all treatments, except the control group, maintained TBA levels within the revised permissible range of 1-2 mg MDA kg⁻¹ throughout the 12-day storage period, as delineated 0 in Table 5. A more granular analysis revealed a markedly attenuated rate of TBA increase in the coated samples, particularly evident from day 4 onwards. During this interval, coated samples consistently exhibited demonstrably lower TBA values than the control, a trend that persisted until day 8. Subsequently, a gradual elevation in TBA levels was observed from day 12 onwards, primarily attributed to the

progressive nature of lipid oxidation. This body of evidence underscores the efficacy of the coatings in providing a robust protective barrier against lipid peroxidation, effectively retarding the formation of malonaldehyde, a critical marker of lipid degradation and concomitant quality decline in the fish products. This refined permissible range accentuates the subtle yet significant protective influence of our coatings. Alginate and extract coatings slowed TBA increases more effectively than the control or alginate alone samples, demonstrating a statistically significant impact on oxidation. Similarly, Gharehdaghi *et al.* (2020) noted alginate coatings delayed lipid oxidation by acting as oxygen barriers, with enriched coatings further

reducing oxidation and microbial growth in fish.

Microbiological Results

Total Mesophilic Bacterial Count

Table 6 shows the data related to the total microbial load of fish samples stored in the refrigerator during 12 days. According to it, the microbial load increased with increasing storage time in all samples. In this study, the total microbial load in the control sample on

threshold was surpassed on day 8. Consistent with the cited study, extract-treated samples in our research exhibited lower microbial loads, remaining below the 7 log CFU g⁻¹ limit, highlighting the effective antimicrobial properties of the extracts.

Total Psychrotrophic Bacterial Count

According to Table 7, the total count of psychrotrophic bacteria in rainbow trout fillets exceeded the established 7 log CFU g⁻¹ limit by day 4 in the control group, whereas

Table 6. Total mesophilic bacterial counts (log CFU g⁻¹) of rainbow trout fillets during storage at 4°C.^a

Treatments	Storage days			
	1	4	8	12
C	4.42 ± 0.12 ^a	6.02 ± 0.11 ^a	7.8 ± 0.04 ^a	9.12 ± 0.02 ^a
A	3.40 ± 0.10 ^b	5.7 ± 0.06 ^b	7.42 ± 0.11 ^a	7.95 ± 0.02 ^b
AE 0.5%	2.41 ± 0.11 ^c	3.89 ± 0.04 ^c	5.62 ± 0.02 ^b	7.40 ± 0.06 ^b
AE 1%	2.32 ± 0.16 ^d	3.78 ± 0.19 ^d	5.48 ± 0.26 ^b	7.22 ± 0.06 ^c

^a Different letters in each day indicate significant differences (P < 0.05). C: Control, A: Alginate coating, AE 0.5%: Alginate containing 0.5% extract, AE 1%: Alginate containing 1% extract.

Table 7. Total psychrotrophic bacterial counts (log CFU g⁻¹) of rainbow trout fillets during storage at 4°C.^a

Treatments	Storage days			
	1	4	8	12
C	5.32 ± 0.25 ^a	7.02 ± 0.11 ^a	7.72 ± 0.04 ^a	9.12 ± 0.02 ^a
A	4.40 ± 0.10 ^b	5.40 ± 0.16 ^b	7.32 ± 0.11 ^b	7.52 ± 0.12 ^b
AE 0.5%	2.48 ± 0.01 ^c	4.88 ± 0.14 ^c	6.85 ± 0.02 ^c	7.40 ± 0.05 ^c
AE 1%	2.32 ± 0.01 ^c	4.58 ± 0.19 ^c	6.38 ± 0.26 ^d	7.22 ± 0.06 ^d

^a Different letters in each day indicate significant differences (P < 0.05). C: Control, A: Alginate coating, AE 0.5%: Alginate containing 0.5% extract, AE 1%: Alginate containing 1% extract.

day 8, and other treatments on day 12, exceeded the permissible limit (7 log CFU g⁻¹). The highest total microbial load was for the control sample, which exceeded the limit on all days of study days 1 and 4. The results are consistent with the research of Bazargani-Gilani (2018). The initial TVC in both studies was approximately 4 log CFU g⁻¹. In the control group of the cited study, TVC reached 7.46 log CFU g⁻¹ by day 6, exceeding the permissible limit, whereas in our study, this

the alginate-coated and extract-coated samples reached this limit on days 8 and 12, respectively. These results are consistent with the findings of Raeisi *et al.* (2020), who also observed a significant antimicrobial effect of plant extracts in extending the shelf life of fish during cold storage. On day 12, the psychrotrophic bacterial load reached 7.3 log CFU g⁻¹, aligning with our findings and indicating a progressive increase in microbial load as storage time increased. In



contrast, Sallam (2007) reported a similar trend, with the control group exceeding the 7 log CFU g⁻¹ limit by day 12. However, our study underscores the effectiveness of the *E. campestre* extract, which inhibited psychrotrophic bacterial growth earlier, by day 4, highlighting its potential in preserving fish quality during refrigerated storage. The antimicrobial effects of *E. campestre* extract were significant, especially against

mesophilic and psychrotrophic bacteria, delaying microbial spoilage during refrigeration. This finding aligns with Ebrahimi and Larypoor (2022), who observed similar effects of plant extracts on refrigerated fish. Extract-treated samples showed lower microbial load than the controls, with levels remaining below permissible limits, highlighting its potential in delaying spoilage.

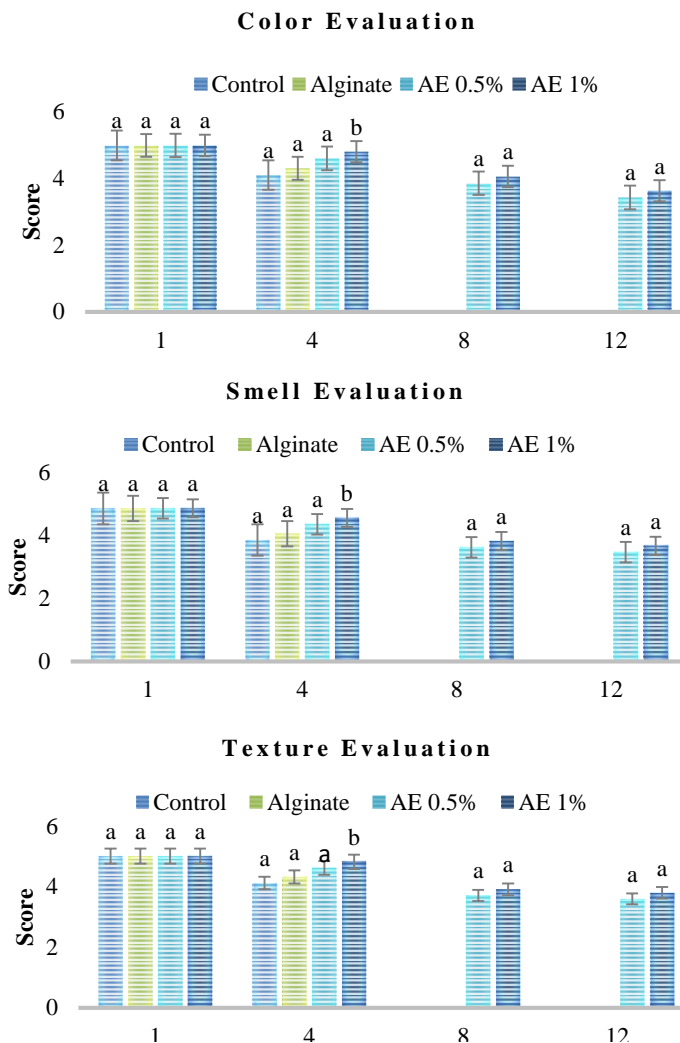


Figure 6. The results of the three parameters (color, smell, texture) of evaluating the sensory properties of different treatments during storage in the refrigerator (temperature 4±1°C). Different letters in each day indicate significant differences (P < 0.05). *Note: The control and alginate treatments were removed on the 8th day as they did not receive any scores, while the extract and alginate treatments continued to be evaluated and remained in the scoring table until the 12th day.

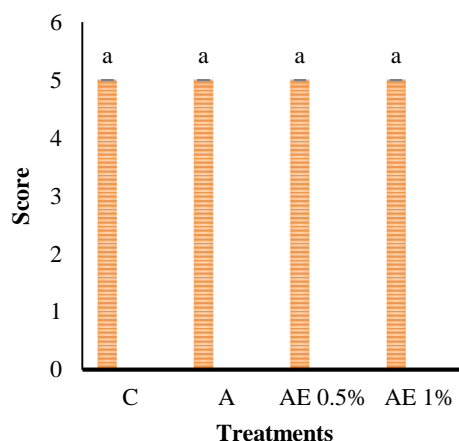


Figure 7. The sensory evaluation of taste of different treatments after cooking at first day of storage. (C: Control, A: Alginate coating, AE 0.5%: Alginate containing 0.5% extract, AE 1%: Alginate containing 1% extract).

Sensory Evaluation Results

Figure 6 presents the sensory evaluation of texture, color, and smell. The results of all three parameters were measured and presented for each treatment. The results demonstrating that fish fillets treated with *E. campestre* extract maintained a superior overall quality compared to the untreated samples throughout storage. As these were the only sensory attributes assessed in this study, all relevant findings were included. The observed delay in sensory deterioration aligns with the findings of Foromandi and Khani (2023), who reported that chitosan coatings enriched with garlic extract and coriander essential oil enhance the quality of fish fillets. This highlights the dual advantage of the *E. campestre* extract in preserving both antioxidant and sensory properties, underscoring its potential as a valuable option for the food industry.

According to Figure 7, no significant difference was observed in the scoring of the taste parameter after cooking the samples. The results of this section are consistent with the findings of Alizadeh Amoli *et al.* (2019) who did not observe any significant differences between the treatments after cooking.

CONCLUSIONS

This research showed that adding *E. campestre* extract to alginate coating enhanced antimicrobial and antioxidant properties, and effectively preserved the sensory qualities of the fish fillets. This coating improved smell, texture, color and delayed spoilage, leading to the shelf life extension of rainbow trout by approximately four days, reaching 8 days compared to the control. However, further studies are needed to better understand the efficacy and mechanisms of *E. campestre*'s antimicrobial and antioxidant effects for food preservation.

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افزایش ماندگاری و خواص حسی فیله ماهی قزل آلاهی رنگین کمان از طریق پوشش سدیم آلژینات حاوی عصاره *Eryngium campestre* در دمای 4 درجه سانتی گراد

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چکیده

ماهی تازه یک ماده غذایی بسیار فاسد شذنی است و به راحتی فاسد می شود. در این تحقیق، پس از بررسی خواص آنتی اکسیدانی عصاره *Eryngium campestre* (Ece)، اثر آن به همراه پوشش آلژینات سدیم بر ماندگاری ماهی قزل آلاهی رنگین کمان در شرایط سردخانه (4 درجه سانتیگراد) به مدت 12 روز بررسی شد. برای ارزیابی خواص آنتی اکسیدانی عصاره، آزمایشاتی مانند DPPH، فنول کل، قدرت کاهشی و ABTS انجام شد. پس از آن، نمونه های تیمار شده با پوشش آلژینات حاوی Ece که به روش اسپری اعمال شده بود، از نظر پارامترهای شیمیایی (TBA، TVN و pH) پارامترهای میکروبیولوژیکی (کل باکتری های روان گردان و مزوفیل)، و ارزیابی های حسی در فواصل چهار روزه تا ۱۲ روز آنالیز شدند. نتایج نشان داد که Ece دارای خواص آنتی اکسیدانی قابل توجهی است. علاوه بر این، تیمارهایی که شامل عصاره همراه با پوشش آلژینات سدیم بودند به طور قابل توجهی سطوح pH، TVN و TBA را نسبت به نمونه شاهد ($P < 0/05$) کاهش دادند. آزمایش های میکروبی نشان داد که تمام نمونه های تیمار شده رشد باکتری را در مقایسه با نمونه شاهد، با کاهش تقریباً $3 \log \text{CFU/g}$ نسبت به گروه کنترل، مهار کردند. در ارزیابی حسی، تیمارهای حاوی Ece و آلژینات سدیم نتایج مطلوب تری نسبت به گروه کنترل به همراه داشت. با توجه به نتایج به دست آمده، پوشش نمونه ها با آلژینات سدیم و Ece باعث بهبود خواص میکروبی، شیمیایی، حسی و ماندگاری ماهی قزل آلاهی رنگین کمان در شرایط یخچال تا حدود چهار روز شد.

ABA Accumulation and *PsAO* Gene Expression in Field Pea under Water Deficit

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ABSTRACT

The plant hormone Abscisic Acid (ABA) plays a crucial role in plant responses to drought and other abiotic stresses, facilitating adaptation mechanisms under water-deficit conditions. This study aimed to investigate the response of field pea (*Pisum sativum*) varieties to drought stress by evaluating ABA concentrations, stomatal conductance, and the expression of *PsAO* genes during the third leaf pair stage. Drought stress was simulated by withholding irrigation to impose moderate and severe levels of water deficit. A statistically significant increase in ABA concentration was observed in all tested pea varieties under stress conditions. Under moderate drought, stomatal responses varied among genotypes; however, severe drought triggered accelerated stomatal closure across all varieties. The cultivar Dukat exhibited the highest stomatal sensitivity, which corresponded with a tenfold increase in ABA concentration, suggesting a strong reliance on chemical (ABA-mediated) drought signalling. In contrast, Javor cultivar showed only a modest (2.5-fold) increase in ABA, despite reduced stomatal conductance, indicating a likely reliance on hydraulic signals for drought response. Gene expression analysis revealed that *PsAO2* and *PsAO3* genes were upregulated under drought, while *PsAO1* expression remained relatively unchanged compared to the control plants. Notably, *PsAO3* expression was consistently elevated under both moderate and severe drought, suggesting that this gene may play a central role in conferring drought tolerance in field pea. These findings highlight the importance of ABA biosynthesis and signalling, particularly via *PsAO3*, in the adaptation of pea plants to water-deficit conditions.

Keywords: ABA biosynthesis, Adaptation mechanisms, Drought tolerance, *Pisum sativum*.

INTRODUCTION

Pea (*Pisum sativum* L.) is the second most important food legume worldwide that, together with other legumes, provides one-third of the entire amount of protein for human and animal consumption. Field pea serves as a significant source of fodder and forage for livestock, as well as a key component in the production of edible and industrial oils (Petrović *et al.*, 2016). Like other pulses, field pea exhibits moderate

sensitivity to various abiotic stressors, particularly drought (Petrović *et al.*, 2021). Drought is defined as the prolonged absence of sufficient moisture, which is essential for normal plant growth and completion of its life cycle (Latif, 2014). It is a major abiotic factor that adversely affects crop plant growth and development, primarily reducing vegetative growth, shoot and root length, and leaf area (Petrović *et al.*, 2021). Water deficit reduces plant growth through the inhibition of various physiological and biochemical processes, such as reduction of

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the rate of photosynthesis, respiration, translocation, ion uptake, carbohydrates, nutrient metabolism, and hormones (Latif, 2014).

One of the initial responses of plants to water deficit is stomatal closure, a process regulated by the plant hormone Abscisic Acid (ABA), which plays a crucial role in the plant's response to drought stress by mediating cellular signalling that regulates water movement from the root to the leaf (Alves and Setter, 2004). Under drought conditions, accumulation of ABA helps plants adapt to drought and other abiotic stress factors (Tuteja, 2007; Sah *et al.*, 2016). The constant presence of ABA in low concentrations is necessary for the normal growth and differentiation of plant cells. Increased ABA concentration is necessary for stomatal closure, seed dormancy, and aging (Sah *et al.*, 2016; Wu *et al.*, 2022). ABA is very intensively studied worldwide because it has been established that it represents a universal "stress hormone" whose accumulation, especially in roots, helps plants adapt to abiotic stress factors, such as drought, high and low temperatures, salt or ion stress (Nakashima and Yamaguchi-Shinozaki, 2013; Liu *et al.*, 2017). It is one of the most important signalling molecules in plants, which plays multiple roles in regulating many developmental processes and adaptive stress processes (Santner *et al.*, 2009; Cutler *et al.*, 2010; Wu *et al.*, 2022).

In different plant tissues and organs, the intensity of biosynthesis and ABA concentrations can vary dramatically during different stages of development, depending on the changing environmental conditions (Xiong and Zhu, 2003). ABA biosynthesis primarily occurs in chloroplasts and other plastids. The concentration of free ABA in the cytosol is regulated through processes such as degradation, conjugation, and transport. For example, cytosolic ABA increases during drought as a result of synthesis in the leaf, redistribution within the mesophilic cells, transport from the shoots, and recirculation from other leaves.

The concentration of ABA decreases after water rehydration due to degradation and reduction in the rate of synthesis, but also due to translocation from leaves to other organs (Zdunek and Lips, 2001).

The primary objective of studying plant responses to abiotic stresses, such as drought, salinity, and cold, is to develop crops with enhanced tolerance to these stress factors. Plants usually respond to water deficit in the soil or in the growth substrate by closing the stoma and reducing leaf growth in order to reduce its transpiration surface (Wilkinson and Davies, 2008; Goodger *et al.*, 2005). These stress responses can be initiated by the so-called chemical (Davies and Zhang, 1991) or hydraulic signals (Gowing *et al.*, 1990). ABA has the main role in sending chemical signals related to drought stress.

A lot of research has been done to clarify molecular mechanisms behind plant adaptation to abiotic stresses. Now, most of the genes involved in ABA biosynthesis have been identified. Water deficit stimulates changes in the expression profiles of different gene classes, most of which are associated with ABA signalling pathways (Zdunek-Zastocka *et al.*, 2004; Zdunek-Zastocka, 2008). Metabolic pathways leading to free active ABA have been described (Wu *et al.*, 2022). Aldehyde Oxidase (AO; EC 1.2.3.1) plays a crucial role in the final step of ABA biosynthesis, and it is also involved in the biosynthesis of other important phyto-hormones, including Indole-3-Acetic Acid (IAA). The enzyme is localized in the cytosol and catalyses the oxidation of Indole-3-Acetaldehyde (IAAld) to IAA and Abscisic Aldehyde (ABAld) to ABA (Seo *et al.*, 2004; Wu *et al.*, 2022). AO is a molybdoenzyme, meaning it contains a Molybdenum cofactor (Moco) at its catalytic site, which is essential for its enzymatic function. Three AO isoforms were isolated from the leaves and roots of peas: PsAO1, PsAO2, and PsAO3 (Zdunek-Zastocka *et al.*, 2004; Zdunek-Zastocka, 2008). The activity of PsAO1 was dominant in the leaves of seedlings and young leaves of mature

plants, while PAO3 showed the highest band intensity in old leaves and roots. PsAO2 mRNA was not affected by salinity or ammonium treatment. PsAO3 could oxidize abscisic aldehyde, a precursor of ABA, indicating the possible involvement of this isoform in ABA synthesis in pea plants (Zdunek-Zastocka, 2008).

Due to the limited information available on the physiological responses of Serbian field pea (*Pisum sativum* L.) varieties to drought, this study aimed to evaluate the effects of water deficit on selected physiological and biochemical parameters. Specifically, it investigated differences in abscisic acid (ABA) concentration, stomatal conductance, and the expression of Ascorbate Oxidase (AO) isoforms among selected cultivars at both the early seedling stage and the stage of the third pair of leaves. In addition, the study sought to identify the most active AO isoform under drought conditions in field pea plants.

MATERIALS AND METHODS

Plant Material

This study was conducted on seven Genera of *Pisum* genotypes: Junior, Dukat, Partner, Trezor, Javor, Pionir, and Mraz selected based on morphological characteristics and whether they are spring or winter genotypes, taken from the collection of the Institute of Field and Vegetable Crops, Novi Sad, Serbia (five spring and two winter cultivars). Seeds were sterilized with 0.1% sodium hypochlorite (w/v) for 1 min and then thoroughly rinsed with distilled water (Asmat *et al.*, 2019).

Drought Stress Experiment

The drought stress experiment was conducted under controlled conditions (phytotron chamber), with a photoperiod of 14-hour, brightness (PAR) 300 $\mu\text{mol m}^{-2}$, temperature 25/18°C and 70% relative

humidity of air. The plants were grown in 1 L pots filled with commercial substrate (Potground H, Klasmann-Deilmann, Germany) and irrigated to maintain an optimal soil water content of 36% until the development of the third pair of leaves. Drought stress was then induced by withholding irrigation, reducing the substrate water content to 18% for moderate drought (treatment T1) and to 9% for severe drought (treatment T2). Control plants were maintained under optimal soil water conditions, with irrigation adjusted to achieve a substrate water content of 36%. The amount of water in the substrate was measured by the Theta Probe (ThetaProbe, type ML2X, Delta-T Devices Ltd., England).

Measurement of the Stomatal Conductance

Stomatal conductance (gs) was measured on the abaxial surface of the third leaf pair using a SC-1 Leaf Porometer (Decagon Devices Inc., USA). Five plants per treatment were used for measurements. Measurements were made for each variety in the control, as well as in two different drought treatments (T1 and T2). Values are expressed in $\text{mmol m}^{-2} \text{s}^{-1}$.

ABA Concentration Measurement

Before extraction, frozen plant leaves were ground to a fine powder in pre-chilled steel cylinders, with liquid nitrogen, by a mixer mill (A-11 basic, IKA). ABA extraction was done from 0.5 g of plant tissue mixed with 1.5 mL of water and 2% PVP (polyvinylpyrrolidone) without further purification (Asch, 2000). Cross-reaction of the antibody with other compounds in the extract was avoided by briefly boiling plant tissue in water before extraction, then, in a Thermomixer comfort (Eppendorf) at 4°C, in a dark room, during the night. Before the analysis, the cross-reaction test confirmed



that the isolated ABA extract did not contain other substances that react with the antibody without being antigenic, i.e. abscisic acid. ABA content in the leaves of the investigated plants was measured by the ELISA method using MAC 252 monoclonal antibody for ABA according to Asch (2000). Plate contents (Nunc: F96 Maxisorp immuno plate) were read at 405 nm by an ELISA reader (Sunrise, Tecan).

Gene Expression Analysis

RNA was extracted from 100 mg of frozen plant tissue, using RNeasy Plant Mini Kit (Qiagen, Germany), according to the manufacturer's manual. The quality and quantity of the extracted RNA were checked with an UV/VIS spectrophotometer (Evolution 100, Thermo Scientific, USA), by measuring the absorbance at 260 (A260) and 280 nm (A280). The A260/A280 ratio is a widely used method to assess the purity of RNA samples, specifically to check for protein contamination. It is based on UV absorbance measurements and provides useful information about the quality of the RNA. The ratio for the isolated RNA ranged between 1.8 and 2.0, indicating high purity. The use of Ambion 171 DNA-free DNase Treatment and Removal Reagents before cDNA synthesis is an essential step to ensure the removal of genomic DNA from the RNA sample. cDNA synthesis was

carried out with 100 ng of total RNA in a final reaction volume of 20 μ , using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) following the manufacturer's protocol. The synthesized cDNA, using it as a template in a PCR reaction is a key step for amplifying specific genes of interest.

The amplification was carried out on Mastercycler gradient (Eppendorf, Germany), using a premix of 2 \times PCR Master Mix, (Fermentas, Lithuania) containing 4 mM MgCl₂, 0.4 mM dNTP, 0.05 units μ L⁻¹ Taq DNA Polymerase (recombinant in a funnel volume of 25 μ L. The temperature program was as follows: denaturation at 95°C for 5 minutes, followed by 30 cycles of 95°C for 30 seconds, 53–57°C for 30 seconds, and 72°C for 1 minute, and final extension at 72°C for 10 minutes.

After performing PCR, amplification products were analyzed by electrophoresis on a 1% agarose gel with ethidium bromide (0.5 μ g mL⁻¹). The expected fragment sizes were estimated by comparison with the FastRuler™ Low Range DNA Ladder (Fermentas, Lithuania). Gels were visualized under UV light using a trans-illuminator, and images were captured with the BioDoc Analyze documentation system (Biometra, Germany).

Table 1. Sequences of oligonucleotide primers.

Gene	Sequence (5'–3')	Annealing temperature (°C)	Reference
<i>18S rRNK</i>	F: CCAGGTCCAGACATAGTAAG R: GTACAAAGGGCAGGGACGTA	55	
<i>PsAO1</i>	F: GACAGTTCTGCAAGAAAAACCAGTGG R: CAGCACCAGCTATAGATTTTCATGCTC	57	Zdunek-Zastocka, 2008
<i>PsAO2</i>	F: GTCAAGGCCCTGAGTTTGCACA R: GGAGTTGGTTTTAGATGCATCTCTTG	57	
<i>PsAO3</i>	F: CAGCTGGATGGAGTAAGAGATG R: GTGCTGTA ACTATGTTATGTGG	55	

Statistical Analysis

Percentage data were arcsine-transformed before statistical analysis. Analysis Of Variance (ANOVA) was performed for all investigated parameters using SigmaPlot 10.0 (Systat Software Inc., San Jose, CA, USA). We followed the statistical methodology described in Marchin *et al.* (2020). Significant differences among the mean values were compared by Student's *t*-test ($P < 0.05$). Figures were drawn using SigmaPlot. Genotypes and treatments were compared by means of the two-factor analysis of variance with significance levels of $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

RESULTS

Stomatal Conductance (g_s)

Stomatal conductance was measured for each variety on the third pair of leaves under

Table 2. Mean values of the stomatal conductivity by varieties in the control and drought treatment group.

Variety	Treatment	g_s ($\text{mmol m}^{-2} \text{s}^{-1}$)
Mraz	Control	124.13 \pm 2.04
	T1	60.86 ^{***} \pm 1.10
	T2	25.08 ^{***} \pm 0.57
Junior	Control	143.61 \pm 2.57
	T1	76.08 ^{***} \pm 0.94
	T2	16.08 ^{***} \pm 0.87
Javor	Control	166.57 \pm 6.74
	T1	64.70 ^{***} \pm 1.22
	T2	30.66 ^{***} \pm 0.74
Dukat	Control	194.00 \pm 14.96
	T1	64.06 ^{***} \pm 3.94
	T2	37.96 ^{***} \pm 1.31
Pionir	Control	147.17 \pm 5.46
	T1	50.04 ^{**} \pm 0.74
	T2	22.62 ^{***} \pm 0.86
Partner	Control	131.80 \pm 4.98
	T1	66.48 ^{**} \pm 1.26
	T2	36.64 ^{**} \pm 1.61
Trezor	Control	189.33 \pm 10.07
	T1	80.64 ^{**} \pm 1.44
	T2	28.54 ^{**} \pm 1.34

*** - $P < 0.001$, ** - $P < 0.01$.

control conditions and two drought treatments (T1 and T2) (Table 2). Notable differences were observed between T1 and T2, with all varieties exhibiting significantly reduced stomatal conductance under severe drought conditions (T2).

The conductivity of the stoma was the lowest in cultivar Pionir at the moderate drought (T1) ($50.04 \text{ mmol m}^{-2} \text{ s}^{-1}$), while cultivar Trezor had the highest conductivity ($80.64 \text{ mmol m}^{-2} \text{ s}^{-1}$). In T2, cultivar Dukat showed the highest stomatal conductance ($37.96 \text{ mmol m}^{-2} \text{ s}^{-1}$), while Junior had the lowest ($16.08 \text{ mmol m}^{-2} \text{ s}^{-1}$).

Regression analysis was conducted in order to interpret treatment effects correctly. A sigmoidal fourth-order regression pattern was made to draw the curve of the dependence of the stomatal conductance in relation to the volume of water content in the substrate (Figure 1). The regression model explains a significant portion of the variance in stomatal conductance under T1, with statistical significance ($P < 0.001$).

Dukat showed the highest tolerance in this treatment with faster closing of stomata and decreased conductance by 66.9% compared to conductance under optimal conditions. Similar results were found in the cultivar Pionir (reduction in conductivity by 63.3%). Under T1 treatment, the stomas of cultivar Junior had a significantly higher conductivity of stoma than other varieties (52.9% compared to control), so, they were more open. However, with a further decline of water in the substrate and stronger stress (T2), the stoma of this cultivar closed faster and the lowest conductance was measured (11.2% compared to the control). The slowest decrease in conductance was observed in Partner.

ABA Accumulation in Leaves of Field Peas

Measurements of ABA accumulation (Table 3) were made in plant leaves under control and in plants exposed to drought stress (moderate and severe).



In all cultivars, a statistically significant increase in ABA content, compared to the control, was observed at both drought treatments (Table 3). Compared to control, under the conditions of moderate and severe stress, the lowest increase in ABA concentration was found in cultivar Javor (moderate 95.62%, severe 147.26%), and the highest was in cultivar Dukat (moderate 637.2%, severe 917.0%). The lowest increase in ABA at T2 compared to T1 was observed in cultivar Mraz (13.08%) and the highest in Pionir (55.3 %).

Cultivars Dukat exhibited the highest accumulation of ABA stress hormone under both drought treatments, with the differences being statistically significant compared to other cultivars. Conversely, Javor consistently showed the lowest ABA content across both treatments. Furthermore, the highest increase in ABA concentration was observed under drought conditions compared to the optimal water regime, indicating a strong drought-responsive hormonal adjustment.

The dependence of ABA content in leaves and water content in the soil is shown in Figure 2. A statistically significant increase in the ABA concentration in leaves in reducing soil water content is evident at both drought treatments in all varieties ($P <$

Table 3. ABA concentrations in plant leaves (ng g^{-1} fresh weight) under the influence of drought treatment.

Variety	Treatment	ABA (ng g^{-1} FW)
Mraz	Control	187.00 ± 11.23
	T1	$486.20^{***} \pm 9.81$
	T2	$549.80^{***} \pm 15.23$
Junior	Control	171.00 ± 13.72
	T1	$389.00^{***} \pm 26.63$
	T2	$458.40^{***} \pm 15.24$
Javor	Control	146.00 ± 8.84
	T1	$285.60^{***} \pm 11.71$
	T2	$361.00^{***} \pm 10.16$
Dukat	Control	88.00 ± 7.44
	T1	$648.80^{***} \pm 19.80$
	T2	$895.00^{***} \pm 27.21$
Pionir	Control	164.00 ± 7.66
	T1	$454.00^{***} \pm 15.29$
	T2	$705.20^{***} \pm 46.19$
Partner	Control	161.00 ± 9.44
	T1	$456.00^{***} \pm 20.04$
	T2	$657.80^{***} \pm 26.89$
Trezor	Control	146.00 ± 10.78
	T1	$356.40^{***} \pm 8.12$
	T2	$506.00^{***} \pm 24.00$

*** - $P < 0.001$.

0.001). Variety Dukat showed the highest accumulation of ABA, while accumulation of ABA was the lowest in Javor (Table 3).

The results of stomata cell conductivity and changes in the conduction of stomata are

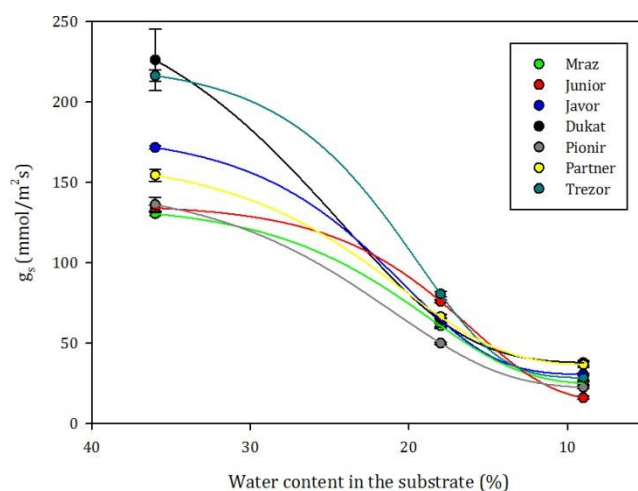


Figure 1. Changes in stomatal conductivity (g_s) depending on the content of water in the substrate.

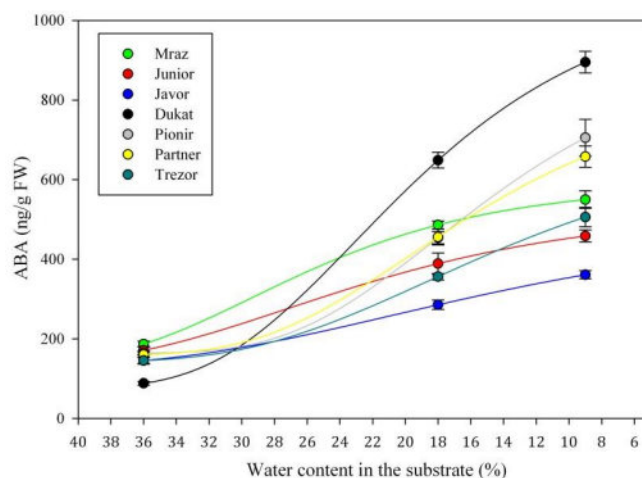


Figure 2. Changes in the concentration of ABA in leaves depending on the water content of the soil.

also shown in correlation with ABA content in leaves (Figure 3).

The results showed different sensitivity of the stomata of the tested cultivars to the changes in ABA concentration. In this case, the accumulation of ABA in leaves greatly affected Dukat's stomata and less than cultivar Javor's.

Dukat showed the highest accumulation of ABA in leaves, while the lowest accumulation was measured in Javor. Dukat also showed the highest sensitivity of stomata in drought treatments because the

stomatal conductivity decreased, compared to the conductivity under optimal conditions. Accordingly, Dukat was the most resistant cultivar.

Influence of Drought on Expression of Genes Involved in the Synthesis of ABA

The expression levels of three genes, namely, *PsAO1*, *PsAO2*, and *PsAO3* were analyzed in two field pea genotypes: Dukat, which showed the highest ABA accumulation, and Javor, which showed the

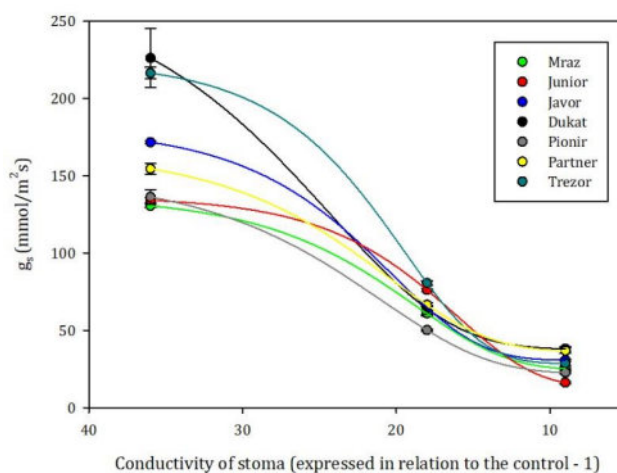


Figure 3. The ratio of ABA concentration in leaves and conductivity of stoma (Expressed in relation to the control= 1).



lowest, relative to the control. Gene expression was assessed in leaf tissue collected from plants grown under the control conditions and those subjected to severe drought stress. The *Arabidopsis thaliana* 18S rRNA gene was used as the internal reference for normalization.

Changes in the expression levels of the transcript of three genes for AO in the leaves of selected pea cultivars are presented in Figure 4. Compared to the control, the ABA content increased by 917% under drought conditions in Dukat and by 147% in the Javor (Table 3). For both cultivars, the increased expression of the *PsAO2* and *PsAO3* genes was observed under drought, but it was more expressed in Dukat than in Javor. The expression of the *PsAO1* gene in both cultivars was similar to the expression in the control group. It can be assumed that *PsAO1* and *PsAO3* play a greater role than *PsAO2* in the synthesis of ABA in leaves of the selected pea cultivars under drought stress.

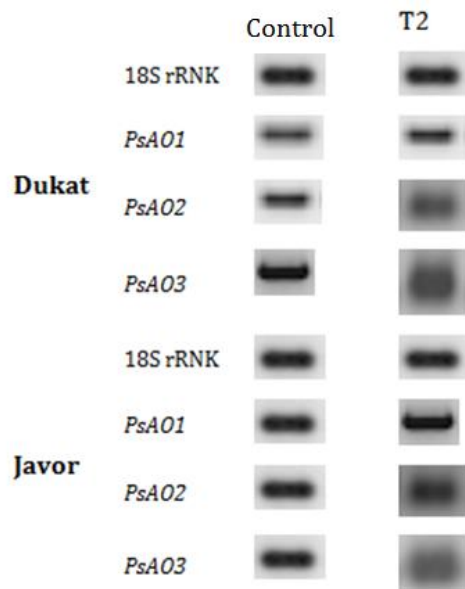


Figure 4. Effect of drought on the expression of the *PsAO1*, *PsAO2* and *PsAO3* genes in plant leaves of the selected field pea varieties.

DISCUSSION

Different environmental and internal signals including drought, salt stress, light, temperature, CO₂ concentration, relative humidity, phyto-hormones, and micro-organisms affect and regulate the function of the stomata on the plant leaves (Bawa *et al.*, 2020; Driesen *et al.*, 2020). The stomatal response is a crucial aspect of plant defence against abiotic stress. As one of the first reactions in response to stress, it emphasizes the essential role of stomatal regulation in maintaining water balance and minimizing water loss, particularly in drought and high-temperature conditions. Research continues to deepen our understanding of how ABA and other regulatory pathways control stomatal function and help plants survive in challenging environmental conditions. (Matkowski and Daszkowska-Golec, 2023; Ferguson, 2019; Kollist *et al.*, 2019). Stomatal conductance (g_s) is a key physiological parameter that estimates the rate of gas exchange and transpiration through the stomata, particularly under abiotic stress conditions such as drought or high temperatures. Early stomatal closure in response to mild drought can be positively correlated with drought tolerance in pea genotypes. However, the timing and extent of stomatal response must be finely tuned—too early or too severe closure can hurt productivity (Agurla *et al.*, 2018).

The rate of gas exchange and transpiration through the leaf stomata is estimated by stomatal conductance (g_s). Generally, stomatal conductance is higher when stomata are open wider, and lower when stomata are closed or narrower. Our study of the stomatal cell conductance in seven field pea cultivars under drought stress showed differences between cultivars, and also between treatments. All cultivars had significantly lower stomatal conductance ($P < 0.0001$) in conditions of severe drought. In order to explain the obtained results, regression analysis was conducted. The curve of the dependence of the stomatal

conductance in relation to the volume of water content in the substrate showed that the greatest differences in the reactions of the stoma of the investigated cultivars were expressed under moderate drought (T1). However, with further decline of water in the substrate, the stoma of all cultivars closed faster. R-squared values ranged from 0.96 to 0.99. This means that 85% of the variance in stomatal conductance can be explained by the independent variables in the model, suggesting a strong relationship between the predictors and stomatal conductance. Under drought, the fastest partial closure of the stomata in the cultivar Dukat may represent a significant adaptive response that allows this cultivar to maintain the highest stomatal conductance compared with other cultivars under severe drought conditions (T2). This enables the assimilation process to be continued, which results in the highest drought resistance index of both the aerial part and the roots (Petrović, 2021). The stomatal response observed in Dukat is an intriguing aspect of its drought tolerance strategy. However, to assess whether this trait is generalizable across other species, it is important to consider the genetic and physiological diversity within legumes. Similar results of changes in stoma conductivity were determined by Maksimović *et al.* (2010), by measuring stomatal conductance on the leaves of pea plants under the influence of salinity, as well as Sassi *et al.* (2010), who investigated the effect of osmotic stress on bean plants. Many legumes, such as common beans (Polania *et al.*, 2022) and chickpeas (Pang *et al.*, 2016), also exhibit adaptive stomatal responses to drought, suggesting that this trait may be a common mechanism for drought resilience in the family. However, the effectiveness of these responses can vary based on environmental conditions and specific genetic backgrounds. Therefore, while similar patterns may be observable in other legumes, it is crucial to conduct comparative studies to understand the nuances of stomatal behavior and its

adaptive significance in different cultivars and species.

Many signals induce stomatal closure, among these, ABA is the best-known signal that regulates water status and stomatal movement. The amount of ABA in the plant tissue is regulated in several metabolic steps, both in biosynthesis and inactivation steps (Brookbank *et al.*, 2021). Under drought conditions, plants produce and accumulate increased amounts of ABA in the guard cells, and this induces stomatal closure to conserve water (Bharath *et al.*, 2021). Measurement of ABA concentration in the leaves of the tested field pea cultivars exposed to drought in this work also confirmed these claims. In all cultivars, a statistically significant increase in ABA content, compared to the control, was observed, at both drought treatments. Dukat showed the highest accumulation of ABA stress hormone in both treatments compared to other cultivars, and this difference is statistically significant. In both treatments, the lowest content of ABA, compared to other cultivars, was found in Javor. In addition, the maximum increase in the concentration of ABA was determined under drought compared to the optimal water regime conditions. There is a clear relationship between ABA response and drought tolerance. In pea genotypes, more efficient ABA perception and signalling are often key traits in drought-tolerant lines. Breeding programs increasingly use these traits as selection criteria for improving drought resilience.

The results of stomatal conductance measurements, together with changes in ABA content in the leaves, revealed differential sensitivity of stomatal cells among the tested cultivars in response to ABA accumulation. In particular, ABA accumulation had a pronounced effect on stomatal closure in Dukat, while the stomata of Javor exhibited lower responsiveness to changes in ABA levels. Dukat displayed the highest stomatal sensitivity under drought stress, as indicated by a significant reduction



in stomatal conductance compared to well-watered conditions.

Taken together, these findings suggest that Dukat possesses enhanced drought resistance, likely mediated through more efficient ABA-regulated stomatal control. This response is clearly influenced by chemical signalling, particularly through the accumulation of ABA. In contrast, Javor exhibited only a minimal increase in ABA concentration under drought stress, while still showing a reduction in stomatal conductance. This suggests that, in Javor, stomatal regulation may be primarily governed by hydraulic signals rather than chemical cues. Hydraulic signalling involves changes in leaf water status, often resulting from reduced root water uptake and decreased turgor pressure in leaf cells (Goodger *et al.*, 2005). These changes can inhibit leaf growth and promote stomatal closure. According to the concept of chemical drought signalling (Gowing *et al.*, 1990), the root system acts as a critical "sensor" of soil conditions—detecting not only reductions in water availability but also changes in the soil's mechanical composition. Roots perceive environmental stress and initiate a complex chemical communication network with the shoot. This involves ABA, ethylene, cytokinins, sugars, and ions, each playing roles in: modulating stomatal behaviour, reprogramming growth and metabolism and activating systemic stress responses. The results of Davies and Zhang (1991) showed that ABA begins to accumulate at the root in the first stages of water deficiency. As a result of the changed pH values of xylem juice and under water deficit, the synthesized compounds, as chemical stress signals, are transported by the xylem to shoots, where they cause the closure of the stoma and reduce the growth of leaf cells (Bahrun *et al.*, 2002; Davies *et al.*, 2005; Schachtman and Goodger, 2008; Wilkinson and Davies, 2008). ABA-driven closure is a more direct, specific response, where the plant actively synthesizes a hormone (ABA) to signal closure under stress conditions. Hydraulic signals are more

of a passive, mechanical response to physical changes in water status, where the plant detects internal water loss and reacts accordingly by closing the stomata. In summary, while both mechanisms lead to stomatal closure in response to water stress, ABA-driven closure involves a hormonal signalling pathway that triggers biochemical changes in the guard cells, while hydraulic signals result from physical changes in the plant's water status, which lead to stomatal closure as a protective mechanism against excessive water loss (Wilkinson and Davies, 2008).

It should also be noted that ABA biosynthesis is related to carotenoid biosynthesis. Reduced ABA concentration in the leaves may therefore be the result of the metabolic pathway in drought conditions being oriented in the direction of the synthesis of carotenoids as antioxidant agents necessary for metabolism. Other authors suggest a potential trade-off between ABA production and antioxidant capacity. Specifically, during stress conditions (e.g., drought or high salinity), an upregulation of key genes involved in ABA biosynthesis is noted—*NCED3*, for example—which diverts carotenoid precursors like 9-cis-violaxanthin and 9'-cis-neoxanthin toward ABA synthesis. Interestingly, this increase in ABA biosynthesis coincided with a reduction in xanthophyll cycle activity and lower expression of *VDE* (Violaxanthin De-Epoxidase) and *ZEP* (zeaxanthin epoxidase), which are critical for non-photochemical quenching (NPQ)—a major photo protective, antioxidant mechanism. These changes were also accompanied by measurable decreases in total carotenoid content and antioxidant enzyme activities (like SOD and APX), suggesting that, as more carotenoids are diverted into ABA synthesis, they are less available for direct ROS quenching or photo protection. This supports the hypothesis that, under stress, plants may prioritize hormonal signalling (ABA) over maintaining maximum antioxidant capacity, possibly as a short-term adaptation to optimize water-use

efficiency and stomatal regulation (Cazzonelli, 2011; Ruiz-Sola *et al.*, 2014; Van Norman *et al.*, 2014; McAdam *et al.*, 2016, Mercado-Reyes *et al.*, 2024).

The cellular and molecular mechanisms underlying ABA-induced stomatal closure have been extensively studied and are now relatively well understood. Most of the genes involved in ABA biosynthesis have now been identified. Water deficit stimulates changes in the expression profiles of different gene classes, most of which are associated with ABA signalling pathways (Zdunek-Zastocka *et al.*, 2004; Zdunek-Zastocka, 2008). According to Yao *et al.* (2023), understanding the function of the AAO gene family is of great significance for insight into plants' response to abiotic stresses. Analysis of the expression of *PsAO1*, *PsAO2*, and *PsAO3* was performed in selected field pea genotypes, in which the highest and lowest ABA accumulation occurred in the leaves of pea plants exposed to severe drought treatment, compared to the control. It can be assumed that the genes *PsAO2* and *PsAO3* play a more important role in the synthesis of ABA in the leaves of plants that were in an older vegetative phase of development than *PsAO1*, while in the seedlings, the *PsAO2* gene had less importance in the synthesis of ABA, compared to other genes. This is especially expressed in Dukat where, under the influence of severe drought, an 90-fold increased concentration of ABA was measured in the leaves of the plants compared to the control. Accumulation of ABA in this cultivar also contributes to faster closing of stomata, which reduces transpiration, as well as activation of antioxidant mechanisms (Petrović *et al.*, 2023). An increase in ABA concentration was also measured in Javor, but slightly less than in Dukat, so, the stomata of this cultivar closed slower and the activation of antioxidant mechanisms was reduced.

The results of this study are consistent with the results of Zdunek-Zastocka (2010) and Zdunek-Zastocka and Sobczak (2013), who showed that the most important gene, whose

expression increases in pea plants exposed to drought, was *PsAO3*. The expression pattern of *PsAO1* parallels that of other ABA-related genes, such as *NCED3* and *ABI5*, indicating coordination with major components of the ABA pathway. This suggests a potential functional association or regulatory coordination between *PsAO2* and *PsAO3* and core ABA signalling elements (Barrero, 2006).

CONSLUSIONS

The findings of this study underscore the link between drought tolerance and specific physiological and molecular adaptations in the selected field pea (*Pisum sativum*) genotypes. The genotypes exhibited distinct responses to drought stress, with notable differences in both physiological and molecular mechanisms depending on the severity of drought. Under moderate drought, stomatal behaviour varied among genotypes, whereas all genotypes responded to severe drought with accelerated stomatal closure, a classical water-conserving defence mechanism. A clear correlation was observed between leaf ABA concentration and stomatal conductance. In Dukat, stomatal conductance was strongly affected by a tenfold increase in ABA content, suggesting a dominant role of ABA-mediated "chemical signalling" in this cultivar's drought response. In contrast, Javor showed minimal stomatal sensitivity despite a 2.5 -fold increase in ABA, indicating a response more reliant on "hydraulic signals", likely driven by changes in leaf water status and turgor pressure. At the molecular level, all three analyzed genes (*PsAO1*, *PsAO2*, and *PsAO3*), which are involved in the biosynthesis of the key ABA-producing enzyme aldehyde oxidase, were expressed under drought conditions. Notably, *PsAO3* expression consistently increased under drought, suggesting it plays a central role in ABA biosynthesis during stress and may be a critical component of the drought response pathway in pea. These



findings highlight the complexity of drought adaptation mechanisms in pea and point to *PsAO₃* as a potential target for future research and breeding programs. However, further studies are needed to elucidate the localization and regulation of ABA transporters, as well as the detailed dynamics of ABA biosynthesis and catabolism under drought conditions.

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تجمع ABA و بیان ژن *PsAO* در لوبیای صحرایی در شرایط کمبود آب

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چکیده

هورمون گیاهی ابسایسیک اسید (ABA) نقش مهمی در پاسخ گیاه به کم آبی و دیگر تنشهای ابیوتیک (Abiotic) و تسهیل مکانیزم سازگاری در شرایط کمبود آب بازی میکند. هدف این پژوهش بررسی پاسخ رقمهای لوبیای صحرایی

(*Pisum sativum*) به کم‌آبی با ارزیابی غلظت ABA، هدایت روزنه ای، و بیان ژن *PsAO* در طی مرحله سومین جفت برگ (third leaf pair) بود. تنش خشکی با استفاده از ندادن آب برای ایجاد سطوح متوسط و شدید کم‌آبی اعمال شد. در چنین تنشی، در همه تیمارها غلظت ABA در همه وارپته های لوبیا به گونه‌ای معنادار افزایش یافت. در شرایط خشکی متوسط، پاسخ روزنه ها در بین ژنوتیپها متغییر بود، ولی خشکی شدید منجر به تشدید بسته شدن روزنه ها در همه رقم ها گردید. رقم *Dukat* بیشترین حساسیت روزنه ها را نشان داد که به صورت 10 برابر شدن غلظت ABA بود و اشاره به وابستگی زیاد به علائم خشکی شیمیایی (به کمک ABA) داشت. درمقابل آن، کولتیوار *Javor* فقط افزایشی 2/5 برابری در ABA نشان داد هر چند که هدایت روزنه ای آن کاهش داشت و اشاره به وابستگی احتمالی به علائم هیدرولیکی پاسخ به خشکی بود. تحلیل بیان ژن آشکار ساخت که ژنهای *PsAO2* و *PsAO3* در شرایط خشکی تنظیم بالا (Upregulated) شده بود در حالیکه بیان *PsAO1* تقریباً بدون تغییر در مقایسه با گیاهان شاهد باقی مانده بود. به وضوح، بیان *PsAO3* در هر دو حالت خشکی متوسط و شدید به صورت مشخصی افزایش (Elevated) یافته بود و اشاره به این داشت که این ژن احتمالاً نقش مهمی در تحمل خشکی لوبیای صحرایی دارد. این یافته ها اهمیت بیوسنتز ABA و علامت دهی آن، به ویژه از طریق *PsAO3*، را در سازگاری گیاه لوبیا به شرایط کمبود آب نشان میدهد.

Effects of Starvation, Dietary Regimes, and Temperature Stress on Hemocyte Profiles and Phenoloxidase Activity in Larvae of *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae)

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ABSTRACT

T. absoluta-infected tomato fruits were collected from fields and larvae were extracted from fruits after two generations of rearing. Hemolymph was extracted with a capillary tube and placed on a slide. Hemocytes were identified through Giemsa staining and observed under light microscopy at 40× magnification. Starvation stress was induced for 12 and 24 hours while the control group remained unstressed. Hemocyte counts were determined using a hemocytometer under light microscopy at 40× magnification. Starved larvae exhibited significantly reduced total hemocyte counts, plasmatocytes, and granulocytes compared to the control group. Larvae reared on eight tomato varieties (Superchef, Basimo, Hartiva, Berantta, Breivio, Gs15, 1012, and 8320) displayed variable hemocyte densities, with the highest counts observed in those fed on Superchef and Gs15 cultivars. For thermal stress experiments, third- and fourth-instar larvae were exposed to 28 and 4°C for 12 and 24 hours. Control groups for the third and fourth instar larvae were maintained at 25±1°C. In total, hemocyte and granulocyte densities were significantly reduced across all thermal treatments compared to the controls. Plasmatocyte counts in the third-instar larvae subjected to 12 hours of heat stress (327.5±18 cell mm³ hemolymph) and cold stress (320±34.3 cell mm³) were higher than those in the control (294.3±23.3 cell mm³). Phenoloxidase activity exhibited a direct correlation with hemocyte alterations across all experimental conditions. This study provides a foundation for further investigations into the pest's physiological defense mechanisms.

Keywords: Food deprivation, Tomato leaf miner, Thermal stress.

INTRODUCTION

The tomato leaf miner, *Tuta absoluta* (Meyrick, 1994) (Lepidoptera: Gelechiidae), is a highly destructive and a global pest of tomato and solanaceous plants (Ferracini *et al.*, 2012). In Iran, *T. absoluta* was classified as a quarantine pest until its initial detection in West Azarbaijan Province in 2010, after which it rapidly dispersed throughout the country (Gharekhani and Salek, 2014). The larvae invade stems, leaves, terminal buds, and fruits, mining mesophyll tissues between the epidermal layers. This feeding behavior significantly reduces

photosynthetic surface area, impairing plant growth and yield (Desneux *et al.*, 2011).

Immunity in insects includes cellular and humoral immunities (Beckage, 2007; Vengateswari *et al.*, 2020). Hemocytes constitute the primary components of cellular immunity, exhibiting changes in morphology, type, number, phagocytic activity, and nodulation in response to foreign agents (Strand, 2008). In contrast, humoral immune responses emerge several hours post-infection (Zhong *et al.*, 2017). Phenol oxidase and antimicrobial peptides play crucial roles in humoral immunity. Phenol oxidase, secreted by malpighian

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tubules and epidermal cells, becomes activated during defense responses, leading to the melanization of foreign agents and their sequestration through quinone secretion. Melanin also binds to pathogens, immobilizing them and enhancing their susceptibility to host defense mechanisms, including phagocytosis and encapsulation (Cerenius and Söderhäll, 2004; Castillo *et al.*, 2006). Multiple factors influence insect immune responses, including diet, food deprivation, environmental changes, and hemolymph contamination (Mowlds *et al.*, 2008; Strand, 2008). The impact of diet on immunological responses relates to the quality and quantity of macromolecules. Energy derived from macromolecules plays a vital role in insect growth, metabolism, reproduction, survival, and immunity (Triggs and Knell, 2012; Kang *et al.*, 2011). Insects subjected to low-quality diets or short- and long-term starvation often exhibit prolonged development, reduced reproductive rates, and altered longevity. Dietary deprivation also diminishes hemocyte density, weakens immune responses, and decreased resistance mechanisms to pathogens invasion (Stączek *et al.*, 2020; Siva-Jothy and Thompson, 2002). For instance, low-protein diets adversely affected immunity in bumblebees (Roger *et al.*, 2017). In damselfly larvae *Coenagrion puella* (L.), Odonata: Coenagriidae), one week of starvation led to a 10% reduction in weight compared to the controls, lower male emergence rates, and significantly reduced hemocyte density and phenoloxidase activity in both sexes (Campero *et al.*, 2008).

Temperature fluctuations also significantly impact insect physiology, as insects typically develop and reproduce within narrow temperature ranges (Chown and Nicolson, 2004). Environmental temperature changes affect body water content, osmolality, hemolymph volume, hemocyte density, and morphology (Lubawy and Słocińska, 2020). For instance, in *Dacus ciliatus* Loew (Diptera: Tephritidae) larvae, heat (30°C) and cold (4°C) stress increased

Total Hemocyte Counts (THC), but cold stress reduced granulocyte and plasmatocyte (Ajamhassani *et al.*, 2025). Similarly, in *Danaus chrysippus* (L.) (Lepidoptera: Danaidae), cold stress reduced hemocyte counts, while heat stress increased them (Pandey *et al.*, 2008). Temperature stress has caused hemocyte morphological changes, nuclear division anomalies, and cell wall ruptures, further highlighting its profound effects on insect immunity (Ghasemi *et al.*, 2013).

Understanding hemocyte morphology is a foundational step in insect immunology research (Zibae and Malagoli, 2014). Investigating the effects of food deprivation and temperature stress on hemocyte dynamics provide a basis for understanding interactions between insect immune systems and biological, microbial, and chemical control agents (Lubawy and Sticinska, 2020). The tomato leaf miner is a significant pest of tomatoes across various climatic regions of Iran, affecting both greenhouse and field-grown crops. Different tomato varieties exhibit varying degrees of resistance or susceptibility to this pest, influenced by trichome morphology, plant volatiles, structural traits, and nitrogen content in leaves and fruits. These factors and temperature fluctuations have been shown to impact the pest's development, fecundity, and survival (Ghaderi *et al.*, 2017; Rostami *et al.*, 2017; Coqueret *et al.*, 2017). Accordingly, this study aimed to identify hemocyte types and evaluate the effects of food deprivation and temperature stress on hemocyte density and phenoloxidase activity in *T. absoluta* larvae.

MATERIALS AND METHODS

Insect Rearing

Infected tomato fruits (Gs15 cultivar) were collected from tomato fields in Miami County (36° 24' 54" North, 55° 39' 42" East), Semnan Province, Iran. The fruits were transferred to a laboratory growth

chamber maintained under controlled conditions: temperature $25\pm 1^\circ\text{C}$, relative humidity 50%, and a photoperiod of 14:10 (light: dark) hours. Larvae were identified at different instars based on Dyar's rule (Dyar, 1980). Developmental stages of *T. absoluta* are shown in Figure 1. Using forceps, larvae were carefully extracted from the infected fruits and placed on fresh, healthy tomato fruits. After two generations of rearing, the third and fourth instar larvae were selected for our experiments. Rearing was conducted in plastic containers (30 cm length \times 30 cm width \times 25 cm height) covered with white organza mesh to ensure ventilation. As the fruits began to decay, larvae were transferred to fresh, healthy tomatoes to support continuous development (Krechemer and Foerster, 2015).

Hemocyte Identification and Determination of Hemocyte Frequency

Hemocytes were identified using a procedure described by Gupta, 1991 and Jones, 1962. The ventral surface of the larval body was punctured with a sterile needle,

and hemolymph was collected using a capillary tube and placed onto a microscope slide. Hemocytes were stained with Giemsa solution (Merck KGaA, Germany) for 10 minutes. The stain was then rinsed off, and the slides were observed under a BH2 light microscope at $40\times$ magnification (Yeager, 1945; Gupta, 1991). After staining, the abundance of hemocytes was quantified in the second, third, and fourth instar larvae and pupae. One hundred hemocytes were randomly selected at $40\times$ magnification and differentially counted using an Olympus BH2 microscope. For each developmental stage, 25 samples were examined.

Effects of Starvation on Total Hemocytes, Plasmatocytes and Granulocytes

The fourth instar larvae of *T. absoluta* were subjected to starvation stress for 12 and 24 hours. The experiment consisted of three treatments: A control group (larvae feeding within fruits) and two starved groups (12- and 24-hour starvation). Each treatment included six replicates, with hemolymph

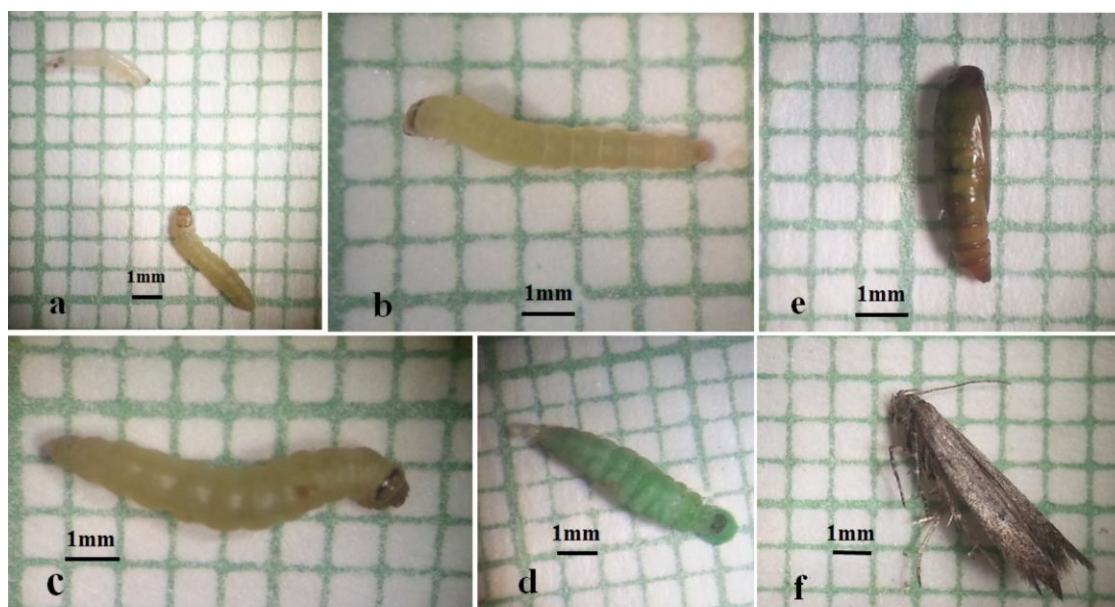


Figure 1. Developmental stages of *Tuta absoluta*: (a) First and second instar larva, (b) Third instar larva, (c) Fourth instar larva, (d) Pre-pupa, (e) Pupa, (f) Adult (original photo).



extracted from four larvae per replicate (4 μL). The extracted hemolymph was mixed with 24 μL of Tyson buffer as an anticoagulant solution containing methyl violet (0.06 mM), glycerol (43 mM), sodium chloride (NaCl , 72 mM), sodium sulfate (Na_2SO_4 , 9 mM), and distilled water (250 mL). (The Chemical compounds were obtained from Merck, Germany). The hemolymph-Tyson buffer mixture was loaded onto a Neubauer hemocytometer (HBG, Germany) for analysis. THC, plasmacyte count, and granulocyte count were determined under a light microscope at 40 \times magnification using Jones' formula (Jones, 1967).

Dilution= 10 times, Depth factor of the chamber= 10, No. of squares counted= 5.

Effect of Tomato Cultivars on Total Hemocytes, Plasmacytes and Granulocytes

Eight tomato cultivars, namely, Superchef, Basimo, Hartiva, Berantta, Breivio (prepared from Yekan Bazr Company, Iran) Gs15, 1012, and 8320 (obtained from Golsam Company, Iran) were used in this experiment. Newly emerged adults of *T. absoluta* were allowed to mate and oviposit on each cultivar. The fourth-instar larvae reared on these cultivars were subsequently used for immunological assessments. As in previous experiments, each treatment included six replicates, with hemolymph collected from four larvae per replicate (4 μL). The hemolymph was mixed with 24 μL of Tyson buffer solution. Total Hemocyte Count (THC), granulocyte density, and plasmacyte density were recorded.

Effect of Temperature Stress on Total Hemocytes, Plasmacytes and Granulocytes

The third and fourth instar larvae of *T. absoluta* were used in this experiment,

which included ten treatments: control groups for the third and fourth instar larvae maintained at $25\pm 1^\circ\text{C}$, larvae exposed to heat stress at 28°C for 12 and 24 hours (both instars), and larvae exposed to cold stress at 4°C for 12 and 24 hours (both instars). Each treatment consisted of six replicates, with hemolymph collected from four larvae per replicate (4 μL). The hemolymph was mixed with 24 μL of Tyson buffer solution. Hemocyte counts, including THC, were performed using a Neubauer hemocytometer.

Effect of Temperature Stress on Hemocyte Morphology

$$\frac{\text{Hemocyte count} \times 1 \text{ mm}^2 \times \text{Dilution} \times \text{Depth factor}}{\text{No. of squares counted}}$$

No. of squares counted

For this experiment, hemolymph samples from larvae subjected to heat and cold stress were analyzed, with 40 larvae included in each temperature stress treatment. Hemocytes were examined under a microscope to assess plasmacytes and granulocytes for signs of cellular wall wrinkling, ruptures, or nuclear divisions. After the analysis, the percentage of the damaged cells was calculated for each type of temperature stress.

Effect of Starvation and Temperature Stress on Phenoloxidase Activity

The hemocyte lysate method assessed the effects of starvation periods and temperature stress on phenoloxidase activity in *T. absoluta* larvae (Leonard *et al.*, 1985). Larval rearing conditions in this experiment were consistent with previous studies. Fourth-instar larvae were used to assess the effects of starvation. The experimental treatments included three groups: larvae subjected to starvation for 12 and 24 hours and a control group. Each treatment

consisted of 40 replicates (larvae), with the hemolymph from each replicate pooled together. In the experiment examining temperature stress, the fourth-instar larvae were similarly used. The control group was maintained at $25\pm 1^\circ\text{C}$, while treatment groups were exposed to heat stress at 28°C for 12 and 24 hours or cold stress at 4°C for the same duration. Each treatment included 40 replicates.

Hemolymph from each treatment group was collected separately and centrifuged at 10,000 rpm for 5 minutes. After removing the supernatant, 100 μL of phosphate buffer (pH 7) was added to the pellet homogenized. The homogenized solution was centrifuged again at 12,000 rpm for 15 minutes, and the resulting supernatant was used for enzymatic analysis. To estimate enzyme activity, 25 μL of each sample was mixed with 50 μL of a 10 mM solution of L-DOPA (L-Dihydroxyphenylalanine) and 50 μL of phosphate buffer. The mixture was incubated at 30°C for 5 minutes and analyzed using an ELISA reader (Model

490 nm. Unit of phenoloxidase activity is $\mu\text{mol min}^{-1} \text{mg protein}^{-1}$.

Statistical Analysis

All data obtained from a complete randomized design were compared by one-way Analysis Of Variance (ANOVA) followed by Tukey's test when significant differences were found at $p \leq 0.05$ (SAS, 9.4). Differences between samplings ($n=3$) were considered statistically significant at a probability less than 5% and marked in figures and tables.

RESULTS

Identification of Hemocytes

Figure 2 illustrates the types of hemocytes identified in the fourth instar larvae of *T. absoluta*. The hemocyte types and their morphological characteristics are

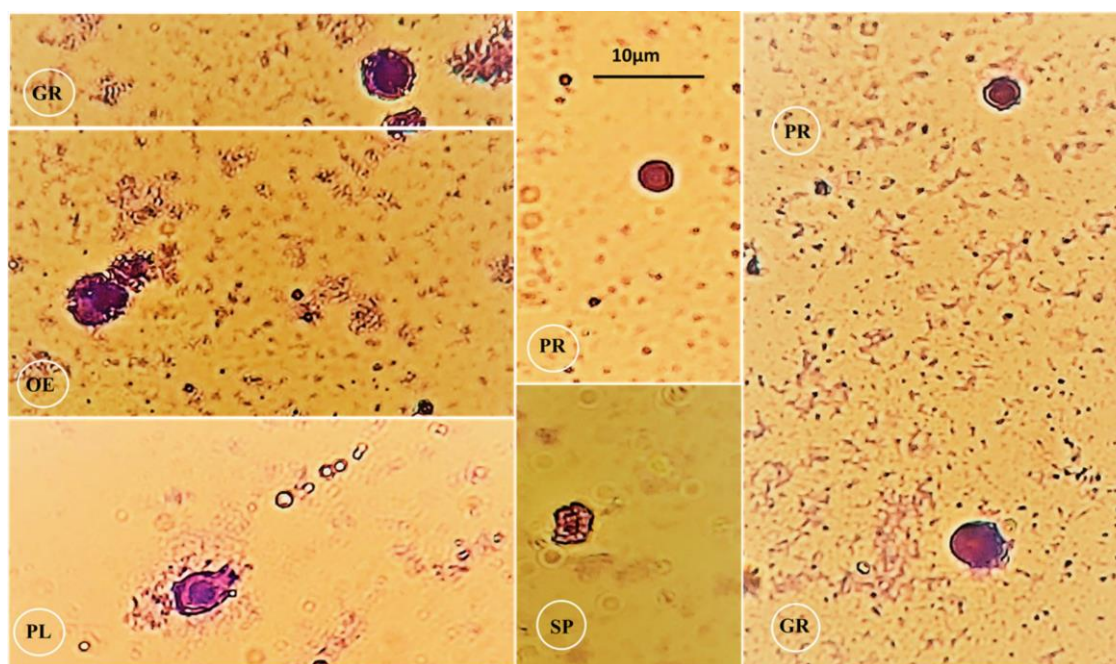


Figure 2. Light microscopy pictures of *Tuta absoluta* hemocytes stained with Giemsa. PR (Prohemocyte), PL (Plasmatocyte), OE (Oneocytoid), GR (Granulocyte), SP (Spherulocyte), Scale bar= 10 μm .

ELX800, BioTek, USA) at a wavelength of

summarized in Table 1.



Prohemocytes were the smallest hemocytes, round in shape, with prominent nuclei. The cytoplasmic area was minimal, extending along the cell wall margin. The highest abundance of prohemocytes was observed in the first instar larvae ($26.5 \pm 2\%$), with numbers decreasing in subsequent developmental stages (Table 2).

Plasmatocytes were medium-sized cells, often with one or two projections, and occasionally oval in appearance. The nuclei were typically centrally located and stained darker with Giemsa than the cytoplasm. Plasmatocyte frequency was highest in the third ($23.2 \pm 0.7\%$) and fourth instar larvae ($22.1 \pm 1.5\%$).

Granulocytes varied in size, ranging from small to medium, and contained numerous granules in their cytoplasm. These hemocytes were the most abundant cell type across all larval instars, with their population peaking in the fourth instar ($50.2 \pm 2.5\%$) (Table 2).

Oenocytoids were circular cells with large peripheral nuclei. They were larger than prohemocytes and the same size as granulocytes and plasmatocytes. The frequency of oenocytoids was lower than

that of plasmatocytes and granulocytes across the developmental stages.

Spherulocytes were rarely observed. These cells had central nuclei with visible spherules on their cytoplasmic surface (Figure 2).

Effect of Starvation Stress on THC, Plasmatocytes, Granulocytes and Phenoloxidase Activity

The effect of starvation stress on THC ($F=171.5$, $df_{t,e}=2,15$, $P \leq 0.0001$), plasmatocyte count ($F=94.5$, $df_{t,e}=2,15$, $P \leq 0.0001$), and granulocyte count ($F=75.2$, $df_{t,e}=2,15$, $P \leq 0.0001$) was significant (Table 3). THC decreased progressively with starvation, reducing to nearly half of the control group count (442 ± 32.2 cells mm^{-3} of hemolymph) after 12 hours of starvation. By 24 hours, the count declined to 118.16 ± 15 cells mm^{-3} (Table 3).

Plasmatocyte and granulocyte counts followed a similar pattern, with significant reductions observed after 12 hours of starvation, reaching 135.32 ± 12.6 and 134 ± 15.5 cells mm^3 , respectively. These

Table 1. Morphometric measurements of hemocytes larvae of *Tuta absoluta* (n= 20).

Hemocyte type	Size (μm)	
	Length (Mean \pm SE)	Width (Mean \pm SE)
Prohemocyte	2.8 \pm 0.3	2.4 \pm 0.2
Plasmatocyte	6.7 \pm 2.5	2.8 \pm 0.5
Granulocyte	6 \pm 3.2	4.5 \pm 2.1
Oenocytoid	6.6 \pm 2.4	5.8 \pm 1.3
Spherulocyte	3.1 \pm 0.3	2 \pm 1.2

Table 2. Frequency of hemocytes in developmental stages of *Tuta absoluta* (n=25).^a

Developmental stages	Hemocyte frequency (%)				
	Prohemocyte	Plasmatocyte	Granulocyte	Oenocytoid	Spherulocyte
Second instar larvae	26.5 \pm 2a	18.7 \pm 0.4c	40 \pm 2.7c	15.5 \pm 0.35b	-
Third instar larvae	24.8 \pm 2.4a	23.2 \pm 0.7a	45.4 \pm 2.2b	6.4 \pm 0.2d	1.2 \pm 0.2
Fourth instar larvae	17.2 \pm 1b	22.1 \pm 1.5a	50.2 \pm 2.5a	10.2 \pm 1.4c	-
Pupa	15.2 \pm 1b	20 \pm 2.5b	46 \pm 1.7b	18.2 \pm 1.1a	-

^a Different letters in each column show significance using Tukey's test at $P < 0.05$.

Table 3. Effect of starvation period on hemocyte number of the fourth instar larvae of *Tuta absoluta*.^a

Hemocyte number (cell mm ³)	Starvation period		
	Control	12 h	24 h
Total hemocyte	961.33±35.5a	442±32.2b	118.16±15c
Granulocyte	407.66±42.6a	134±15.5b	80±12.23c
Plasmatocyte	328.84±24.4a	135.32±12.6b	61.88±10.5c

^a Different letters in each column show significance using Tukey's test at P < 0.05

counts continued to decline with prolonged starvation, showing further reductions by 24 hours (Table 3).

In addition to the decrease in hemocyte density, phenoloxidase activity in the fourth instar larvae of *T. absoluta* also declined under starvation stress. After 12 hours of starvation, phenoloxidase activity dropped to 0.073±0.004 µmol min⁻¹ mg protein⁻¹, and after 24 hours, it decreased further to 0.055±0.008 µmol min⁻¹ mg protein⁻¹. Both values were significantly lower than the control group (Table 4).

df_{t,e} = 7,40, P ≤ 0.000) (Table 5). The highest THC was recorded in larvae reared on the Superchef cultivar (1188±64.5 cells mm³ of hemolymph), while the lowest THC was observed in larvae fed on the Breivio cultivar (735±34.7 cells mm³ of hemolymph). Larvae fed on Superchef and Gs15 cultivars exhibited the highest plasmatocyte and granulocyte count values. In contrast, larvae reared on the Breivio cultivar showed the lowest frequency of these hemocyte types compared to the larvae fed on other cultivars (Table 5).

Effect of Tomato Cultivars on THC, Plasmatocytes and Granulocytes

Feeding *T. absoluta* larvae on different tomato cultivars significantly influenced THC (F= 614.3, df_{t,e}=7,40, P ≤ 0.002),

Effect of Temperature Stress on THC, Plasmatocytes, Granulocytes, and Phenoloxidase Activity

Temperature stress, including heat and cold, significantly influenced THC (F= 90.4,

Table 4. Effect of starvation period on phenoloxidase activity in the fourth instar larvae of *Tuta absoluta*.

Phenoloxidase activity (µmol min ⁻¹ mg protein ⁻¹)	Starvation period		
	Control	12 h	24 h
	0.11±0.02a	0.073±0.004b	0.055±0.008c

^a Different letters in the row show significance using Tukey's test at P < 0.05

Table 5. Effect of tomato cultivar on hemocyte number of the fourth instar larvae of *Tuta absoluta*.

Cultivar	Hemocyte number (cell mm ³)		
	Total hemocyte	Granulocyte	Plasmatocyte
Gs15	948±24.7b	415±18.6b	338±13.67b
Superchef	1188±64.5a	603±42.2a	482±34.6a
Brevio	735±34.7d	312±14.4d	221±24.4d
Basimo	880±17.7c	388±15.55b	277±12.2c
Berantta	910±32bc	355±20.3bc	273±30c
Hartiva	861±28cd	390±21.2b	290±27.5c
1012	921±21.6bc	344±28.4c	287±24.5c
8320	870±25cd	350±18.8bc	267±10c

^a Different letters in each column show significance using Tukey's test at P < 0.05.

plasmatocyte count (F= 225.3, df_{t,e}=7,40, P ≤ 0.03), and granulocyte count (F= 277.3,

df_{t,e} = 9,157, P ≤ 0.002) and plasmatocyte and granulocyte counts in *T. absoluta*. All

**Table 6.** Effect of thermal stress on hemocyte number of the third and fourth instar larvae of *Tuta absoluta*.^a

Treatment	Hemocyte number (cell mm ³)		
	Total hemocyte	Granulocyte	Plasmatocyte
Third instar larvae (Control)	782±31.5b	358.3±42b	294.3±23.3ab
Fourth instar larvae (Control)	959±55.3a	468.8±33.6a	327.3±31.5a
Third instar larvae (Heat stress 12 h)	712±21.4c	264.5±25.3c	327.5±18a
Third instar larvae (Heat stress 24 h)	243.1±15g	115.8±22.3f	103±17.7e
Fourth instar larvae (Heat stress 12 h)	404.5±37.8e	201.8±31.4d	175.5±32.5c
Fourth instar larvae (Heat stress 24 h)	192.3±32.2	95±16.7g	73.2±7.4f
Third instar larvae (Cold stress 12 h)	702.8±34.4cd	297.4±15.5bc	320±34.3a
Third instar larvae (Cold stress 24 h)	331±23.7f	104±12.2g	141.3±14.3d
Fourth instar larvae (Cold stress 12 h)	648±43.3d	308.6±25.5bc	281.5±33b
Fourth instar larvae (Cold stress 24 h)	360.8±27.3ef	174.5±34.5e	141.6±10.5d

^a Different letters in each column show significance using Tukey's test at $P < 0.05$.

Table 7. Effect of thermal stress on phenoloxidase activity in third and the fourth instar larvae of *Tuta absoluta*.^a

Larval stages	Phenoloxidase activity ($\mu\text{mol min}^{-1} \text{mg protein}^{-1}$) in different temperature ($^{\circ}\text{C}$)		
	Control (25±1)	4	28
Third instar larvae	0.101±0.002a	0.022±0.001c	0.053±0.003b
Fourth instar larvae	0.134±0.003a	0.066±0.005b	0.058±0.003b

^a Different letters in each row show significance using Tukey's test at $P < 0.05$

treatments showed a reduction in THC and granulocyte counts compared to the control groups. The most significant decreases in THC were observed in the fourth- and third-instar larvae subjected to 24 hours of heat stress, with counts of 192.6 ± 4.5 and 243 ± 8.8 cells mm^{-3} , respectively, indicating that heat stress had a more pronounced impact on hemocyte reduction than cold stress (Table 6).

The lowest granulocyte counts were recorded in the third- and fourth-instar larvae exposed to 24 hours of heat stress, as well as in the third-instar larvae subjected to 24 hours of cold stress ($F = 78.5$, $df_{t,e} = 9,157$, $P \leq 0.0001$). Plasmatocyte counts, however, displayed a slightly different trend. While the fourth-instar larvae exposed to 24 hours of temperature stress exhibited the lowest plasmatocyte counts across all treatments, the third-instar larvae subjected to 12 hours of heat or cold stress showed higher plasmatocyte counts than their respective control groups. This suggests that

plasmatocyte numbers temporarily increase under short-term (12-hour) temperature stress, but decline with prolonged exposure (24 hours), aligning with the trends observed in other treatments (Table 6) ($F = 121.4$, $df_{t,e} = 9,157$, $P \leq 0.0001$).

Temperature stress also significantly reduced phenoloxidase activity in the fourth-instar larvae. After 24 hours of heat stress, phenoloxidase activity decreased to $0.058 \pm 0.003 \mu\text{mol min}^{-1} \text{mg}^{-1} \text{protein}$, while cold stress reduced activity to $0.066 \pm 0.005 \mu\text{mol min}^{-1} \text{mg}^{-1} \text{protein}$. These levels represented approximately half the enzymatic activity observed in the control group (Table 7). In the third-instar larvae, phenoloxidase activity was similarly reduced under temperature stress, with cold stress causing a more pronounced inhibitory effect on enzyme activity than heat stress (Table 7).

Effect of Temperature Stress on Hemocyte Morphology

Temperature stress-induced significant morphological changes in the hemocytes of *T. absoluta*, particularly in granulocytes and plasmatocytes (Figure 3). Under heat stress, approximately 27% of granulocytes and 18% of plasmatocytes exhibited cell wall wrinkling (Figure 4). In contrast, cold stress

had a more pronounced effect on granulocyte morphology, with approximately 70% of granulocytes displaying severe wrinkling, the most notable morphological alteration observed under cold conditions (Figure 4). Cold stress also caused approximately 10% wrinkling in plasmatocytes and induced granulocyte nuclear divisions.

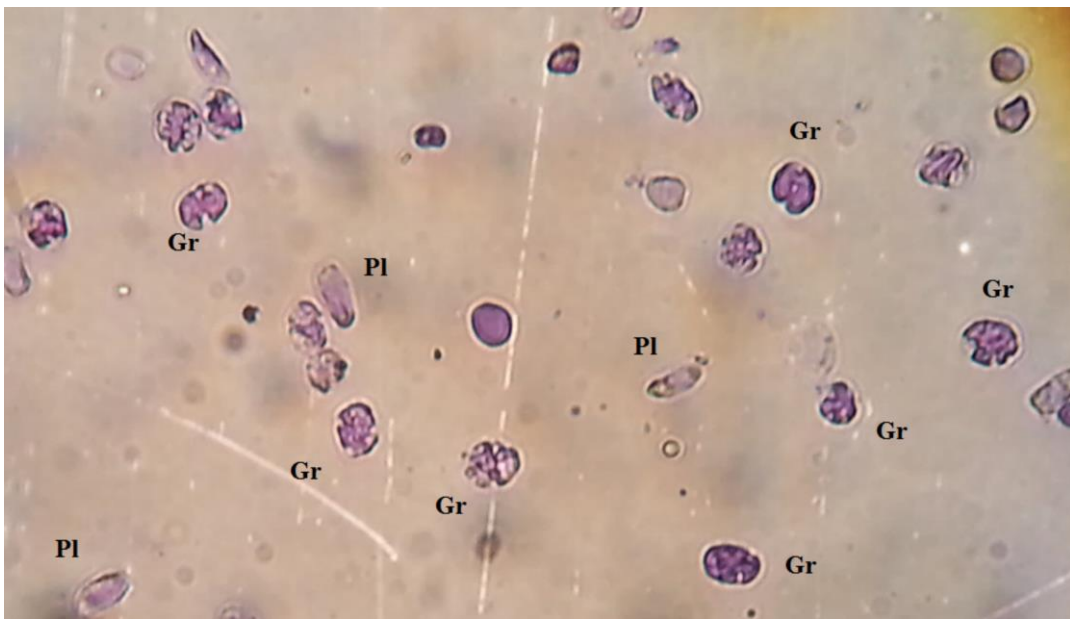


Figure 3. Morphological changes of granulocytes and plasmatocytes of *Tuta absoluta* affected by cold stress.

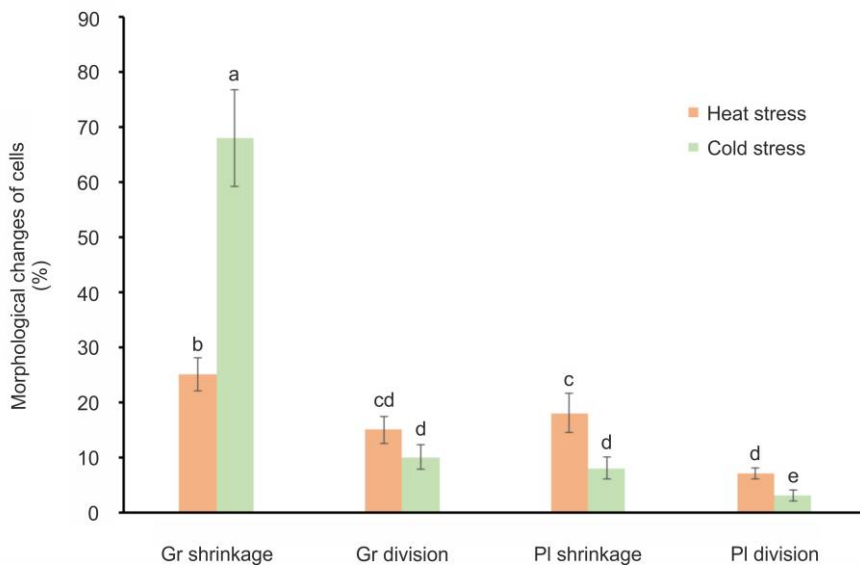


Figure 4. Hemocyte deformation percentage of *Tuta absoluta* affected by heat (28°C) and cold (4°C) stress.



DISCUSSION

The insect circulatory system is vital in transporting nutrients, metabolites, hormones, water, and ions. Hemolymph is a medium for carrying waste products and toxins to the Malpighian tubules, acting as a final defense barrier against stresses and infections (Sinclair *et al.*, 2015). Hemocytes are the primary cellular components of the insect's physiological defense system. These cells are synthesized continuously in hematopoietic organs, replacing aged or damaged cells, a process critical for maintaining hemostasis (Nakahara *et al.*, 2003).

In the hemolymph of *T. absoluta* larvae, five types of hemocytes were identified: prohemocytes, plasmatocytes, granulocytes, oenocytoids, and spherulocytes. Another form of hemocyte, adipohemocytes, has been observed in the hemolymph of *T. absoluta* adult (Maingi *et al.*, 2023), but it was absent in the hemolymph of larvae. Similar hemocyte classifications have been reported in various insects, particularly in Lepidoptera (Liu *et al.*, 2013; Blanco *et al.*, 2017; Ajamhassani, 2021; Gogoi *et al.*, 2023). Our findings indicated that the size and frequency of hemocytes in the larval hemolymph of the tomato leaf miner were lower than those in adult hemolymph regarding Maingi *et al.* (2023), potentially due to genetic factors, nutritional regimes, temperature variations, and climate differences. (Mason *et al.*, 2014). On the other hand, the abundance of hemocytes in insects is diverse and even these differences were documented depending on the developmental stage and gender in one species. It seems that nutrition, hormonal changes and antimicrobial peptides during growth can also affect the variation of hemocyte density (Shapiro, 1979). This variability suggests that there is no the same hemocyte pattern within this order (Bruno *et al.*, 2022). Usually, the abundance of granulocytes and plasmatocytes as the main cells participating in the immune processes

in the late instar larvae of Lepidoptera are more than the other hemocytes (Kholghahmadi *et al.*, 2025). Our finding also confirmed that the granulocytes and plasmatocyte counts were the highest in hemolymph of the third and fourth instar larvae of *T. absoluta*.

Hemocytes morphology and abundance changed in response to food deprivation, dietary modifications, and temperature stress similar to the finding of Carper *et al.* (2019) and Ayres (2024). Starvation and dehydration affect insect growth, survival, longevity, reproduction, movement, and adaptability, depleting the energy required for these processes. Our findings indicate that the circulatory system of *T. absoluta* is susceptible to food deprivation, even over short periods. Starvation for 12 and 24 hours significantly reduced plasmatocytes, granulocytes, and phenoloxidase activity in the hemolymph of *T. absoluta* larvae. This reduction may be explained by hemocytes exiting circulation and adhering to the body wall, decreasing their numbers in the hemolymph. In fact, reduced digestion and nutrient absorption due to malnutrition likely affect circulatory system physiology, causing hemocytes to migrate from the bloodstream to the body wall until refeeding occurs. Similar observations have been reported in *Galleria mellonella* Fabricius (Lepidoptera: Pyralidae) and *Malacosoma pluvial* Dyar (Lepidoptera: Lsiaoampidae) larvae, where food deprivation reduced hemocyte density and phenoloxidase activity (Banville *et al.*, 2012; Myers *et al.*, 2011). Siva-Jothy and Thompson (2002) reported that starvation significantly reduces the hemocyte population in the hemolymph of both male and female *Plodia interpunctella* despite the presence and maintenance of relatively large fat reserves. The study found that phenoloxidase activity increased soon after food became available. This finding suggests that maintaining high phenoloxidase activity is metabolically costly, explaining its lower levels during periods of food limitation. Based on some reports, starvation weakens insect immunity,

potentially increasing the pest's susceptibility to microbial and chemical control methods (Lord, 2010; Zhu *et al.*, 2012).

In examining the effects of dietary regimes on *T. absoluta* immune responses, our results showed that larvae fed on the Superchef and Gs15 cultivars exhibited significantly higher hemocyte counts and phenoloxidase activity compared to larvae fed on other cultivars. This underscores the influence of diet on hemocyte dynamics and immune function. However, the specific quantities of macromolecules (e.g., carbohydrates, proteins, and lipids) in the mentioned varieties remain unknown and warrant further investigation (Littlefair and Knell, 2016). Nutritionally richer diets, significantly those rich in carbohydrates and proteins, enhance insect immune responses and physiological functions (Vogelweith *et al.*, 2016). Insects feeding on nutrient-dense resources display higher hemocyte densities and phenoloxidase activity, while poor-quality diets reduce immune capacity and increase pathogen susceptibility (Manjula *et al.*, 2020). Our findings suggest that Superchef and Gs15 are more palatable cultivars for *T. absoluta* larvae, likely due to their nutritional composition. Additionally, fruit size, physical structure, firmness and plant volatile may influence feeding efficiency, hemolymph volume, and hemocyte density. Based on our observation, the fruits of the Superchef variety have thin skin, whereas Gs15 fruits are larger and juicier, making Superchef more susceptible to larval penetration than other varieties. In contrast, the superior nutritional quality of larger fruits may significantly influence larval weight and, consequently, the density of circulating hemocytes (Kholghahmadi *et al.*, 2025). Mirhosseini *et al.*, (2022) reported that tomato cultivars vary in their suitability for the survival and development of the tomato leaf miner. The role of secondary metabolites in pest feeding, such as alkaloids, should not be overlooked, as many of these compounds contribute to the host plant resistance against pests (Veyrat *et*

al., 2016). Additionally, the resistance of certain tomato varieties to leaf miners, such as *T. absoluta*, is likely influenced by nutritional availability and larval physiological characteristics, including immune factors. Supporting this, Venjateswari *et al.* (2020) and Ajamhassani *et al.* (2023) highlighted the critical role of diet in the immunological and physiological responses of insects.

Temperature is another critical factor influencing insect growth, fecundity, dispersal, and survival (Klepsatel *et al.*, 2019). Polyols and lipids in hemolymph increase during cold exposure, preventing freezing (Goodhead and MacMillan, 2017), while specific genes are expressed under high temperatures to maintain protein structure and prevent denaturation (Nyamukondiwa *et al.*, 2010). Temperature stress also affects hemocyte structure, morphology, and abundance, the central components of insect immunity. For example, hemocytes of *Gromphadorhina coquereliana* exposed to 4°C were smaller than those in the control insects (Lubawy and Stocinska, 2020). Heat stress in *Antheraea mylitta* resulted in compacted cytoplasmic projections in plasmatocytes, vacuolization in plasmatocytes and granulocytes, nuclear fragmentation in prohemocytes, and, in some cases, cell death at 42°C (Pandey *et al.*, 2010).

Our findings revealed that temperature stress similarly impacted *T. absoluta* hemocyte profiles. THCs and granulocyte numbers significantly decreased in all stress treatments compared to the controls. Interestingly, plasmatocyte counts in the third-instar larvae exposed to 12 hours of heat or cold stress were higher than in the control groups. However, prolonged exposure (24 hours) led to a decline, aligning with the trends observed in other hemocyte types. The high proportion of disintegrated granulocytes and plasmatocytes under temperature stress suggests that these cells became compacted, leading to cell wall rupture and eventual death. Figures 3 and 4 show that many



immunocytes, particularly granulocytes, shrank and disintegrated under cold and heat stress. Consequently, these hemocytes were no longer detectable in circulating hemolymph. Similarly, Maingi *et al.* (2023) revealed that when *T. absoluta* moths were treated with *Metarhizium anisopliae* and exposed to temperatures of 15–25°C, a significant reduction in Total Hemocyte Counts (THCs) occurred. This effect may be attributed to the ability of *M. anisopliae* isolates to produce toxins that impair hemocyte viability or function (Maingi *et al.*, 2023).

Temperature-induced variations in hemocyte abundance differ across insect species. Some studies have documented enhanced immune responses with increasing temperature (Laughton *et al.*, 2017), whereas others have reported weakening certain immune functions, such as melanization (Ehrlich and Zuk, 2019). Generally, thermal effects on the immune system remain complex and unpredictable (Chau-Berlinck *et al.*, 2004). For instance, hemocytes of *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae) increased significantly under heat stress at 35 °C, while cold stress at 4 °C reduced hemocyte counts in *Nicrophorus vespilloides* Herbst (Coleoptera: Silphidae) (Pourali and Ajamhassani, 2018; Urbanski *et al.*, 2017). Conversely, hemocyte counts in *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) decreased under short-term heat stress (Herren *et al.*, 2024).

Phenol oxidase activity and melanization responses also vary with temperature fluctuations. Our findings demonstrated that in *T. absoluta* larvae, phenol oxidase activity declined under both heat and cold stress, whereas in different populations of *Sepsis thoracica* (Robineau-Desvoidy) (Diptera: Sepsidae), phenol oxidase activity positively correlated with developmental temperature (Gourgoulianni *et al.*, 2023). Similarly, *T. molitor* larvae maintained at 30°C exhibited increased phenol oxidase activity and antibacterial responses compared to those kept at 10 or 20°C (Catalán *et al.*, 2012).

These findings underscore the complexity and diversity of cellular and humeral immune to thermal stress, highlighting the intricate relationship between temperature fluctuations and insect immunity.

CONCLUSIONS

This study demonstrated that starvation, dietary composition, and temperature fluctuations significantly affected the hemocyte profile and phenoloxidase activity of *T. absoluta*. The findings highlight the high sensitivity of *T. absoluta* to food deprivation, diet quality, and temperature stress. Stress conditions induced notable changes in the shape and abundance of hemocytes, emphasizing the variability in immune responses of *T. absoluta* larvae. To deepen our understanding of the immunological mechanisms of this pest, future research should focus on field-level studies and investigate the effects of prolonged starvation and extended exposure to temperature stress on hemocyte activity and detoxifying enzymes. Further research on addressing questions such as “Can temperature and climate fluctuations weaken an insect's immune system?” or “Does feeding on resistant plant varieties influence an insect's immune responses to natural enemies or chemical compounds?” will provide valuable insights into effective control measures and management strategies for *T. absoluta*.

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تأثیر گرسنگی، رژیمهای غذایی و تنش دما بر پروفایل هموسیت ها و فعالیت
فنل اکسیداز در لارو مینوز گوجه فرنگی *Tuta absoluta* Meyrick
(Lepidoptera: Gelechiidae)

جمانه کریمیان، و مریم عجم حسنی*

چکیده

میوه های آلوده گوجه فرنگی از مزارع جمع آوری و به آزمایشگاه منتقل شدند. بعد از دو نسل پرورش، لاروها از میوه ها خارج شده و همولف آنها با استفاده از لوله موپین استخراج شده و روی لام قرار داده شد. هموسیت ها پس از رنگ آمیزی توسط گیمسا، و با استفاده از لام هماسیتومتر و بزرگنمایی 40 میکروسکوپ نوری، شناسایی شدند. در لاروهایی که تنش گرسنگی را تحمل

کرده بودند، تعداد کل هموسیت ها، پلاسماتوسیت ها و گرانولوسیت ها به طور معنی داری در مقایسه با شاهد کاهش یافت. تراکم هموسیت ها در لاروهای پرورش یافته روی هشت رقم گوجه فرنگی (Superchef, Basimo,) (Hartiva, Berantta, Breivio, Gs15, 1012, and 8320) با هم تفاوت داشتند و بیشترین فراوانی هموسیت ها در لاروهای پرورش یافته روی ارقام Superchef و Gs15 بود. در آزمایش بررسی تنش دما، لاروها در دمای 28 و 4 سانتیگراد به مدت 12 و 24 ساعت قرار گرفتند. گروه شاهد لاروهای سنین سوم و چهارمی بودند که در دمای سانتیگراد 25 ± 1 قرار داشتند. تعداد کل هموسیت ها و گرانولوسیت ها به طور معنی داری در همه تیمارهای دمایی در مقایسه با شاهد کاسته شد. فراوانی پلاسماتوسیت ها در لاروهای سن سوم که 12 ساعت تنش گرما ($327/5 \pm 18$) سلول در میلیمتر مکعب همولنف و تنش سرما ($320 \pm 34/3$) سلول در میلیمتر مکعب همولنف را تحمل کرده بودند به طور معنی داری بیشتر از شاهد ($23 \pm 294/3$) سلول در میلیمتر مکعب همولنف به دست آمد. فعالیت فنل اکسیداز در همه آزمایشها ارتباط مستقیمی با تغییرات هموسیت ها نشان داد. این مطالعه، پایه ای برای تحقیقات بیشتر در مورد مکانیسم های دفاعی فیزیولوژیکی بید گوجه فرنگی فراهم می کند.

Foliar Nutrient Concentrations and Antioxidant Activity of Tea (*Camellia sinensis* L. (O) Kuntze) Planted in Peninsular Malaysia and its Relation to Soil Edaphic Factors

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ABSTRACT

This study aimed to (1) Compare the nutritional characteristics and AOA (antioxidant activity) of clonal tea grown in lowland and highland plantations and (2) Investigate the influence of soil edaphic factors on tea foliar nutrition and AOA. Tea leaves and soils of fourteen tea clones were sampled between October 2021 to March 2022 from lowland and highland plantations in Peninsular Malaysia. Leaves were analysed for nutritional content and antioxidant activity, while soil samples underwent physical and nutritional analysis. Results showed significant variations in most foliar nutrients, except for Ca in the lowlands and Fe in the highlands. While the highland-grown tea exhibited higher nutrient concentration, lowland-grown tea demonstrated superior AOA. AT53 and 1248 clones were identified as promising among the clones, characterized by the highest nutrients and AOA levels, respectively. Soil nutrient availability significantly influenced foliar nutrient uptake, while soil pH was associated with AOA. Thus, the critical role of soil edaphic factors in shaping tea quality, providing valuable insight for tea growers to optimize soil and maintain tea yield and quality in the future. We found that soil nutrients have a significant association with nutrient uptake, while soil pH is associated with the agronomic characteristics of tea.

Keywords: AT53 and 1248 clones, Soil nutrients, Tea quality.

INTRODUCTION

Tea (*Camellia sinensis* L. (O) Kuntze) is a popular caffeinated non-alcoholic beverage around the globe and is currently consumed by 3 billion people (Pan *et al.*, 2022). It is

attributed to several health benefits in addition to its unique taste and aroma. The health-promoting properties of tea are due to the presence of polyphenols, particularly the flavan-3-ols, widely known as catechins, which are the main polyphenols in tea and have been reported to have a few derivatives

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(Lee *et al.* 2014). Epigallocatechin Gallate (EGCG) is the catechin derivative with the highest prevalence and pharmacological activity. EGCG is responsible for up to 70% of total catechins and has been proven to have chemo-preventive/chemotherapeutic actions against several malignancies (Steinmann *et al.*, 2013) and other disorders such as obesity, diabetes, neurological and cardiovascular diseases (Khan and Mukhtar, 2019). In addition to polyphenols, tea also contains minerals and trace element that play an important role in human metabolism. Regular consumption of tea may contribute to the daily dietary requirement of certain elements, such as manganese. Furthermore, having more potassium than sodium may benefit hypertensive patients (Fernandez *et al.*, 2002). Hence, both nutrient elements and polyphenols (particularly catechin, which has a strong antioxidant effect) are the main compounds that have great contributions to human health.

Variation in tea nutritional characteristics and AOA is associated with either internal (genetic make-up and stage of leaf development) or external factors, including season, altitude as well as soil physicochemical properties. For instance, earlier research revealed that certain nutrients, such as N, P, K and Mg (Xiang *et al.*, 2021) increase proportionally with altitude. Other researchers addressed this phenomenon as a result of growth reduction experienced by the plants grown in higher altitudes, which is highly associated with the highland's lower average temperature. This result is quite the opposite of tea AOA. Previous studies conducted by Owuor *et al.* (2011) and Martono *et al.* (2016) found that tea grown at lower altitudes tends to have better antioxidant performance. This indicates that higher temperature is one of the factors affecting catechin accumulation in plants grown at lower altitudes. Other environmental factors influencing the accumulation of tea catechin are blue light, water stress, shading treatment as well as soil physicochemicals, which act by modulating the expression of biosynthetic genes (Samynathan *et al.*, 2021). An earlier study by Zhao *et al.* (2017) discovered that the

contents of Na, Mg, Ca, Cr, Fe, Ni, Sr and Cd in tea leaves were significantly and positively correlated with those in topsoil or subsoil. It suggested a considerable rise of these elements in tea leaves when soil contents increased. Another study by Tseng and Lai (2022) in Taiwan revealed that soil characteristics, such as soil pH, exchangeable calcium and magnesium, significantly impact tea's free amino acid content.

Previous studies on the influence of soil edaphic factors on nutritional characteristics as well as AOA of tea have been conducted in Malaysia (Chan, *et al.*, 2007; Izzreen and Fadzelly, 2013; Amirah *et al.*, 2023), mostly in lowland plantations. In Malaysia, tea has been planted in two different altitudes for almost a century by BOH Plantation Sdn. Bhd., a Malaysian leading tea company. However, research on the influence of soil physicochemical factors, as well as altitude, on nutritional characteristics and the AOA of tea was less reported. Therefore, it is essential to determine the characteristics of distinct altitudes and each soil edaphic factor on the capabilities of tea clones in uptaking nutrients and accumulating catechin, as this may benefit growers in designing future strategies for sustaining tea yield and quality.

We hypothesized that soil physicochemical properties from different altitudes might play a major role in influencing the variation of foliar nutrient concentrations and the quality of tea. Therefore, in this study, we aimed to: (1) Compare the nutritional characteristics and AOA among tea clones from lowland and highland plantations; and (2) Investigate the association between soil physicochemical properties with nutritional characteristics and quality of clonal tea from both plantations.

MATERIALS AND METHODS

Study Sites

Highland tea clones were planted in Cameron Highland, Pahang, while lowland clones were grown in Bukit Cheeding, Selangor. The Cameron Highland is located

at approximately 1,400 m above sea level (asl), with an average temperature ranging from 18 – 25°C with an average humidity of 79–92% and 152–1,077 mm rainfall. The Bukit Cheeding Plantation is at a lower elevation, approximately 20 m asl, having a higher average temperature ranging from 28–31°C, lower humidity (74–86%), and rainfall (53.6– 596.3 mm) compared to the highland location.

Sample Collection and Analysis

Fourteen clones were sampled between October 2021–March 2022, from lowland and highland tea plantations. Leaf sampling sites were randomly selected (Table 1) and triplicated for young tea leaves (bud and the first three fully expanded leaves). Fresh tea leaves were cleaned with tap water and distilled water to remove the adhering materials before oven-drying at 60 °C for 4 days. The samples were then ground to get the fine powdered texture for further antioxidant and foliar nutrient analysis. Total N was analyzed using a CNS analyzer (CNSTruMax Determinator version 1.1x). For other foliar nutrients (total P, K, Ca, Mg, Al, and Fe), tea leaves were digested using the dry-ashing method following Alarefee *et al.* (2021) and, subsequently, analyzed by ICP–OES (Optima 8300, PerkinElmer,

USA).

Tea leaves were extracted using 80% aqueous methanol by digital ultrasonic bath following Afroz Bakht *et al.* (2019) with minor modifications using 40°C for 30 minutes before AOA analysis. The extract produced was evaluated for its Total Phenolic Content (TPC), DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay and FRAP (Ferric Reducing Antioxidant Power) assay according to Amirah *et al.* (2023). The TPC was evaluated employing the Folin-Ciocalteu solution with gallic acid, which served as a reference. Ten-fold diluted tea extract (15 µL) was combined with distilled water (240 µL) and 0.25N Folin-Ciocalteu reagent (15 µL), and then mixed thoroughly. After 3 minutes of dark incubation at room temperature, 30 µL of 1N Na₂CO₃ was added. Following 2 hours of dark incubation at room temperature, absorbance was measured at 765 nm. In the DPPH assay, 20 µL tea extract was added to 180 µL of DPPH solution (150 µmol L⁻¹) and incubated for 40 minutes in darkness. Absorbance was measured at 517 nm, with 80% methanol used as blank. L-ascorbic acid served as the positive control. The antioxidant activity was expressed as an IC₅₀ value, calculated using GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA). In the FRAP assay, the FRAP

Table 1. Geographical locations of four clones sampled from both plantations.

Clones	Lowland plantation		Highland plantation	
	North latitude (°)	East longitude (°)	North latitude (°)	East longitude (°)
AT53	2.92450	101.57789	4.52340	101.39976
TV9	2.91870	101.57658	4.52110	101.40415
1248	2.92172	101.58327	4.52091	101.40018
2024	2.92813	101.58134	4.52322	101.39959
663	2.92231	101.58356	–	–
1294	2.92201	101.58292	–	–
2026	2.92368	101.58454	–	–
TBR2020	–	–	4.51790	101.41153
196	–	–	4.52321	101.39954
664	–	–	4.52281	101.39865



reagent needs to be freshly prepared. The reagent was made by mixing acetate buffer (0.3M, pH 3.6), 10 mM TPTZ in 40 mM HCl and 20 mM FeCl₃ in a 10:1:1 ratio, then, warmed at 37°C. A mixture of FRAP reagent (280 µL) and tea extract (20 µL) was incubated at 37°C for 30 minutes and measured the absorbance at 593 nm.

The soils were sampled at 0–20 cm depth using an auger within 1–2 meters from each clone for soil physicochemical analyses. The soil samples were air-dried for several days and filtered through a 2 mm sieve before chemical and physical analysis (Alarefee *et al.*, 2021; Khairil and Burslem, 2018). Soil pH was measured at a soil: water ratio of 1:2.5. Soil total N was analyzed using CNS analyzer (CNSTruMax Determinator version 1.1x), while other soil nutrients (total P, K, Ca, Mg, Fe and Al) were analyzed using an ICP–OES (Optima 8300, PerkinElmer, USA) after the aqua regia extraction method (Alarefee *et al.*, 2021).

Statistical Analysis

All analytical results were performed as the average of three replicates using R-studio version 4.1 (Team R Core, 2021). Data were subjected to one-way ANOVA continued by Tukey's HSD to examine the variation of nutritional characteristics, AOA, and soil physicochemical properties. The significance level was set at a 95% confidence level ($\alpha=0.05$). In combination with Pearson's correlation, Principal Component Analysis (PCA) was performed to determine the influence of soil edaphic factors (nutrient concentrations and pH) on nutritional characteristics and the AOA of tea from both plantations. These were conducted using R-studio version 4.1 (Team R Core, 2021). PCA helps to identify patterns and relationships between variables by transforming the data into Principal Components (PCs) that summarize the dataset. Principal components were selected based on eigenvalues >1 and cumulative variance explained.

RESULTS

Nutritional Characteristics and AOA of Tea

The concentration of foliar nutrients differed significantly ($P < 0.05$) between lowland tea clones, except for Ca (Table 2). Among seven clones evaluated, AT53 had the highest foliar K ($1.84 \pm 0.38 \text{ mg g}^{-1}$), Mg ($0.80 \pm 0.16 \text{ mg g}^{-1}$), Fe ($13.00 \pm 0.79 \text{ mg g}^{-1}$) and Al concentration ($16.60 \pm 0.78 \text{ mg g}^{-1}$). Meanwhile, clones 2026 and 663 had the highest foliar N ($4.39 \pm 0.2\%$) and P concentration ($13.76 \pm 1.06 \text{ mg g}^{-1}$), respectively. In highland tea plantations, the majority of the foliar nutrition concentrations varied significantly ($P < 0.05$) among clones, except for Fe (Table 3). Of the seven clones evaluated, TBR2020 had the highest foliar Ca (3.89 mg g^{-1}), Mg (1.78 mg g^{-1}) and Al content (0.69 mg g^{-1}). Meanwhile, clones AT53, 1248 and 196 had the highest foliar N (5.18 %), P (8.35 mg g^{-1}) and K content (13.4 mg g^{-1}), respectively.

In terms of the AOA of tea, DPPH assay from lowland (Table 2) and highland (Table 3) plantations varied significantly ($P < 0.05$) among the clones. Clone 1248 ($50.66 \mu\text{g mL}^{-1}$) and TBR2020 ($127 \mu\text{g mL}^{-1}$) had the highest value for lowland and highland plantations, respectively. In addition, FRAP (Ferric Reducing Antioxidant Power) value significantly varied among the highland population only, with clone 1248 ($1.19 \text{ mM Fe (II) g}^{-1}$) having the highest value. However, Total Phenolic Content (TPC) displayed insignificant variations among tea clones from both plantations.

A PCA based on population mean values of seven nutrient elements and three antioxidative assays for tea grown in both plantations displayed the first axis explaining 76.97% of the variation in the data (Figure 1). This axis reflected a positive correlation with most of the variables (loadings 0.83 – 1.02), except N. The second PC axis that explained 8.45% variations, captured variation in N concentration

Table 2. Foliar nutrient concentration and antioxidant activity of lowland tea plantation.

Clone	Nutrient concentration							Antioxidant activity		
	N %	P	K	Ca	Mg	Fe	Al	TPC mg GAE g ⁻¹	DPPH µg mL ⁻¹	FRAP mM Fe(II) g ⁻¹
1248	4.22 ± 0.7ab	11.11 ± 0.4bc	0.26 ± 0.06b	0.61 ± 0.11	0.22 ± 0.07b	12.77 ± 0.8a	4.53 ± 1.2b	19.64 ± 0.15	50.66 ± 1.86c	2.10 ± 0.2
2024	3.79 ± 0.2ab	9.45 ± 0.5c	0.31 ± 0.12b	0.38 ± 0.04	0.14 ± 0.06b	6.01 ± 1.2b	16.19 ± 0.2a	19.29 ± 0.30	74.25 ± 2.79a	1.99 ± 0.1
AT53	3.55 ± 0.4ab	10.22 ± 1.0bc	1.84 ± 0.38a	0.70 ± 0.08	0.80 ± 0.16a	12.97 ± 1.4a	16.61 ± 1.4a	19.29 ± 0.26	74.36 ± 4.99a	1.55 ± 0.3
TV9	3.99 ± 0.6ab	11.85 ± 0.9ab	0.15 ± 0.04b	0.68 ± 0.09	0.11 ± 0.03b	10.26 ± 0.6a	3.66 ± 0.7b	19.30 ± 0.04	73.89 ± 4.31a	1.86 ± 0.3
663	2.97 ± 0.2b	13.76 ± 1.06a	0.55 ± 0.09b	0.58 ± 0.13	0.27 ± 0.06b	3.32 ± 0.8bc	4.43 ± 0.9b	19.03 ± 0.21	62.90 ± 4.93abc	1.90 ± 0.5
2026	4.39 ± 0.2a	9.19 ± 1.2c	0.47 ± 0.11b	0.64 ± 0.12	0.22 ± 0.003b	3.43 ± 1.3bc	3.72 ± 0.8b	19.23 ± 0.20	72.49 ± 1.97ab	1.65 ± 0.3
1294	4.57 ± 0.7ab	11.06 ± 0.5bc	0.24 ± 0.03b	0.38 ± 0.03	0.23 ± 0.04b	2.67 ± 0.4c	3.91 ± 1.7b	19.04 ± 0.10	54.61 ± 3.64b	1.76 ± 0.5
Mean	3.93 ± 0.2	10.95 ± 1.6	0.54 ± 0.13	0.57 ± 0.04	0.29 ± 0.05	7.35 ± 4.4	7.58 ± 5.8	19.26 ± 0.08	66.17 ± 2.40	1.83 ± 0.4
p-value	0.018*	0.000***	0.000***	0.116	0.000***	0.000***	0.000***	0.428	0.000***	0.551

The significance of the values is indicated as follows: * P < 0.05; ** P < 0.01; *** P < 0.001.

Table 3. Foliar nutrient concentration and antioxidant activity of highland tea plantation.

Clone	Nutrient concentration							Antioxidant activity		
	N %	P	K	Ca	Mg	Fe	Al	TPC mg GAE g ⁻¹	DPPH µg mL ⁻¹	FRAP mM Fe(II) g ⁻¹
1248	4.46 ± 0.1ab	8.35 ± 0.8a	11.1 ± 1.0ab	2.20 ± 0.1b	1.28 ± 0.1b	0.057 ± 0.003	0.38 ± 0.1ab	18.80 ± 0.94	140 ± 1.15a	1.190 ± 0.04a
2024	4.47 ± 0.3ab	7.98 ± 0.2ab	12.2 ± 0.5ab	2.42 ± 0.1b	1.59 ± 0.1ab	0.057 ± 0.01	0.30 ± 0.1b	17.34 ± 0.16	141 ± 2.38a	0.975 ± 0.05ab
AT53	5.18 ± 0.1a	6.74 ± 1.0ab	10.3 ± 1.4ab	2.63 ± 0.2b	1.74 ± 0.2ab	0.057 ± 0.01	0.36 ± 0.1ab	18.80 ± 0.49	133 ± 3.53ab	0.851 ± 0.05b
TV9	4.31 ± 0.6ab	5.78 ± 0.5b	10.8 ± 0.5ab	2.52 ± 0.3b	1.46 ± 0.02	0.050 ± 0.004	0.44 ± 0.1ab	18.35 ± 0.56	132 ± 2.34ab	0.989 ± 0.11ab
TBR2020	3.84 ± 0.1b	6.22 ± 0.3ab	10.9 ± 1.0ab	3.89 ± 0.3a	1.78 ± 0.1a	0.053 ± 0.01	0.69 ± 0.1a	16.97 ± 0.16	127 ± 2.99b	0.932 ± 0.05ab
196	4.59 ± 0.1ab	6.71 ± 0.3ab	13.4 ± 0.3a	2.62 ± 0.2b	1.91 ± 0.1a	0.060 ± 0.01	0.51 ± 0.1ab	17.80 ± 0.50	131 ± 0.58ab	1.180 ± 0.10a
664	4.74 ± 0.1ab	6.73 ± 0.1ab	8.55 ± 0.6b	2.25 ± 0.1b	1.47 ± 0.1ab	0.047 ± 0.003	0.22 ± 0.02b	16.58 ± 0.03	129 ± 3.43ab	0.801 ± 0.01b
Mean	4.51 ± 0.1	6.93 ± 0.3	11.03 ± 0.4	2.65 ± 0.1	1.60 ± 0.1	0.054 ± 0.003	0.41 ± 0.04	16.81 ± 0.31	133 ± 1.5	1.12 ± 0.07
p-value	0.049*	0.0454*	0.0347*	0.000***	0.009**	0.741	0.022*	0.051	0.014*	0.006**

The significance of the values is indicated as follows: * P < 0.05; ** P < 0.01; *** P < 0.001.



(loadings 0.83) of tea. Following the PCA, tea clones were clustered into two distinct groups. Clones with numbers 1–7 were from the lowland plantation and had a significant association with all the anti-oxidative assays, as well as foliar P, Fe and Al concentrations. Another cluster consisted of clones with numbers 8 – 14 originating from the highland plantation, and found to be highly associated with foliar K, Ca and Mg.

Four clones out of the ten clones evaluated were planted in both locations, namely AT53, TV9, 1248 and 2024. Even though they had similar genetic make-up, different geographical areas (altitude) and their microclimate influenced their physiology and metabolism. Based on our foliar analysis and PCA result, we found that four clones grown in both locations had similar clustered patterns as shown in Figure 1. Tea clones planted in the highlands tend to have higher foliar nutrients, particularly K, Ca,

and Mg. On the other hand, lowland-grown clones were associated with better antioxidant performance, as well as foliar P, Fe, and Al.

Soil Edaphic Factor

Soil P, K, Mg, Fe, and Al varied significantly among the seven lowland clones evaluated (Table 4). Soil obtained around clone 663 had the highest P (0.37 mg g^{-1}), Mg (0.39 mg g^{-1}) and Fe (10.20 mg g^{-1}) concentration. The highest soil K (1.17 mg g^{-1}) and Al (23.8 mg g^{-1}) concentrations were found from soil-derived near clones TV9 and 2026, respectively. In highland plantations, most of the soil nutrient content varied significantly, except for Ca (Table 5). Soil derived near clone 196 had the highest soil N (0.49%) and P (7.22 mg g^{-1}) concentration. The highest soil K (2.83 mg g^{-1}), Mg (3.10 mg g^{-1}), Fe (29.9 mg g^{-1}) and

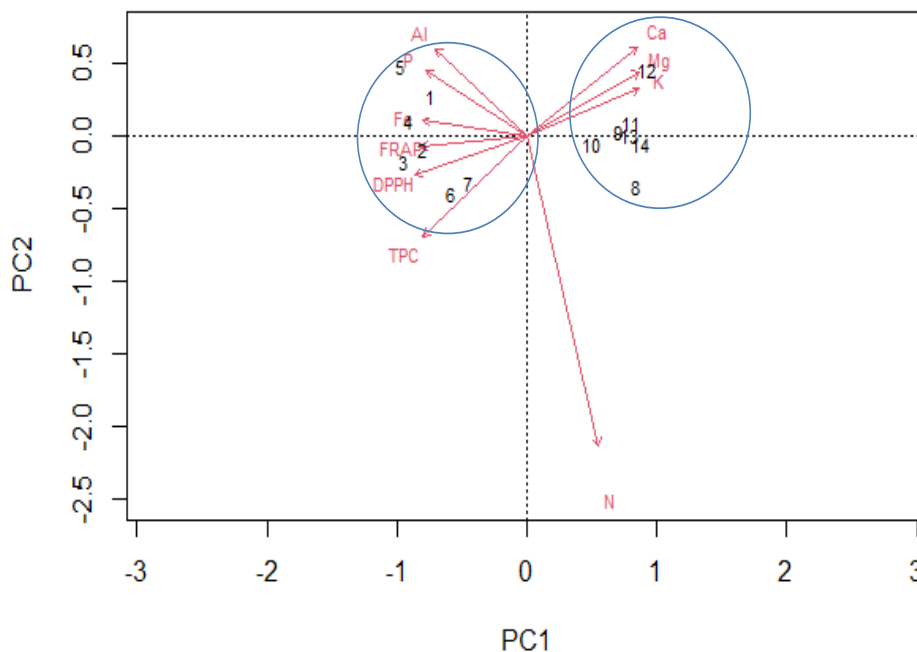


Figure 1. Biplots showing the distribution of 14 populations of tea along principal component axes 1 and 2 from PCAs summarizing variation in foliar nutrient elements and antioxidant activity assay (Note: 1–7= Lowland clones, 8–14= Highland clones).

Al (24.1 mg g⁻¹) concentration was obtained around clone TBR2020.

In terms of pH, both lowland (Table 4) and highland (Table 5) tea plantations had acidic pH, with averages of 4.23 and 3.58, respectively. The pH of lowland plantations differed significantly among clones with soil near clones AT53 and TV9 as the lowest and clones 1248 and 1294 as the highest.

Association of Soil Edaphic with Foliar Nutrient and AOA of Tea

We found that soil physicochemical properties had a significant association with foliar nutrients and AOA of tea (Table 6). Certain soil nutrients displayed direct correlation as well as inter-correlation with foliar nutrients: for instance, soil N with foliar N ($r=0.55$), soil Ca with foliar Ca ($r=0.77$), and soil Mg with foliar Mg ($r=0.59$). In addition, soil N is also significantly inter-correlated with foliar K ($r=0.83$), Ca ($r=0.64$), and Mg ($r=0.76$). Soil P is significantly inter-correlated with foliar K ($r=0.80$), Ca ($r=0.61$), and Mg ($r=0.69$). Both Ca and Mg demonstrated substantial inter-correlation with foliar K ($r=0.90$; $r=0.57$), while they showed an inter-correlation. Soil pH demonstrated a positive and significant correlation with foliar P ($r=0.81$), Fe ($r=0.53$), and Al ($r=0.55$). Furthermore, pH also had a positive and significant correlation with TPC ($r=0.59$) and AOA of tea, represented by DPPH ($r=0.84$) and FRAP ($r=0.78$).

DISCUSSION

Foliar Nutrient and AOA of Lowland and Highland Tea Plantation

Our result demonstrated that clones grown in a highland plantation have a higher foliar nutrient content than those grown at a lower elevation. However, not all nutrients would increase proportionally with altitude. Our findings indicate that the foliar

concentrations of N, K, Ca, and Mg increase with altitude. This is similar to the result of a previous study. For instance, a recent study by Xiang *et al.* (2021) also found that foliar N and P content as well as leaf C: N ratio increase significantly with altitude. Several researchers assumed that the increase in leaf nutrient content of tea with altitude could be considered a consequence of biomass production decreasing with altitude, mainly due to the cold limitation in trees in mountainous regions (Jeyakumar *et al.*, 2020).

Highland plants tend to grow at a slower pace than those grown at lower altitudes. Decreasing temperatures, lower nutrient availability, and slower rates of photosynthesis are among the factors that influence the poor growth of highland plants (Jeyakumar *et al.* 2020). Generally, every 1 km increase of elevation results in a 6.5°C decrease in temperature. Low temperatures reduce soil microbial and enzymatic activity, thus limiting nutrient availability, hence, high-altitude soils are less fertile (Xu *et al.* 2015). Furthermore, lower air density and atmospheric pressure occurring at higher altitudes produced lower CO₂ levels and slower transpiration rates, which eventually led to lower rates of photosynthesis (Wang *et al.*, 2017). Therefore, previous researchers assumed that the increase in leaf nutrient content of plants with altitude could be considered as the plants' inability to use the absorbed resources for growth.

Nitrogen is the most abundant nutrient available in tea foliar from both plantations, followed by K. However, in some cases, tea may absorb more Ca than K (Hawkesford *et al.*, 2011). This is similar to our finding, especially in lowland plantations, where K ranked as the fourth macronutrient after Ca. Sufficient K greatly boosts the yield and quality of tea, as it speeds metabolism, triggers catechin synthesis, and promotes biotic and abiotic resistance by activating and governing several enzymes (Huang *et al.*, 2022; Ruan *et al.*, 2013). Meanwhile, Ca is beneficial for improving plant resistance, enhancing photosynthesis capacity, and



Table 4. Soil physicochemical properties of lowland tea plantation.^a

Clone	N	P	K	Ca	Mg	Fe	Al	Soil pH
	%				Mg g ⁻¹			
1248	0.11±0.009	0.20±0.030ab	1.04±0.076ab	0.37±0.018	0.35±0.029a	9.45±0.31abc	14.2±1.78b	4.35±0.25ab
2024	0.12±0.012	0.12±0.038b	0.83±0.118bc	0.23±0.023	0.20±0.006b	6.16±0.61c	20.2±1.68ab	4.27±0.03ab
AT53	0.08±0.028	0.19±0.034ab	0.51±0.026cd	0.33±0.003	0.18±0.009b	6.52±0.80bc	16.2±1.75ab	3.83±0.10b
TV9	0.10±0.028	0.30±0.047ab	1.17±0.030a	0.28±0.200	0.22±0.009b	8.69±0.96abc	20.8±1.93ab	3.86±0.15b
663	0.11±0.003	0.37±0.037a	1.08±0.094ab	1.04±0.401	0.39±0.027a	10.20±0.83a	22.1±1.78a	4.80±0.21a
2026	0.16±0.021	0.09±0.012b	0.39±0.021d	0.29±0.009	0.18±0.010b	9.78±0.98ab	23.8±1.41a	4.18±0.10ab
1294	0.11±0.030	0.37±0.090a	0.77±0.030bc	0.54±0.074	0.19±0.012b	7.27±0.51abc	18.1±1.22ab	4.35±0.25ab
Mean	0.11±0.007	0.24±0.029	0.83±0.065	0.44±0.08	0.24±0.019	8.30±0.41	19.33±0.87	4.23±0.09
P-value	0.166	0.003**	0.000***	0.058	0.000***	0.007**	0.012*	0.022*

^a The significance of the values is indicated as follows: * P< 0.05; ** P< 0.01; *** P< 0.001.

Table 5. Soil physicochemical properties of highland tea plantation.^a

Clone	N	P	K	Ca	Mg	Fe	Al	Soil pH
	%				Mg g ⁻¹			
1248	0.23±0.04ab	6.63±0.8a	0.83±0.03ab	1.44±0.13	1.03±0.07ab	15.5±2.1b	20.8±3.4ab	3.35±0.2
2024	0.42±0.05ab	2.16±1.8ab	0.29±0.19b	1.56±0.15	0.59±0.22ab	8.70±4.0b	8.64±4.2bc	3.77±0.2
AT53	0.27±0.02ab	1.50±0.1b	0.16±0.03b	1.58±0.02	0.41±0.05b	3.56±0.2b	7.68±0.4c	3.41±0.5
TV9	0.41±0.08ab	4.89±2.3a	0.51±0.12b	2.16±0.56	0.62±0.13ab	28.4±0.5a	21.3±1.1ab	3.57±0.2
663	0.14±0.06b	1.67±0.2b	2.83±1.15a	1.23±0.16	3.10±1.27a	29.9±4.5a	24.1±3.8a	3.73±0.01
2026	0.49±0.04a	7.22±1.8a	0.69±0.12ab	2.62±0.52	1.28±0.46ab	12.3±1.6b	11.4±1.3abc	3.79±0.3
1294	0.32±0.12ab	2.57±0.8ab	0.23±0.09b	1.50±0.40	0.40±0.15b	7.90±2.5b	4.81±1.4c	3.46±0.3
Mean	0.32±0.03	3.80±0.6	0.79±0.24	1.73±0.15	1.06±0.26	15.19±2.3	14.12±1.8	3.58±0.1
P-value	0.032*	0.039*	0.011*	0.128	0.029*	0.000***	0.000***	0.857

^a The significance of the values is indicated as follows: * P< 0.05; ** P< 0.01; *** P< 0.001.

Table 6. Pearson correlation coefficient (r) between soil nutrients and foliar nutrient concentration and AOA from both plantations.

Soil Variables	Pearson Correlation									
	Foliar N	Foliar P	Foliar K	Foliar Ca	Foliar Mg	Foliar Fe	Foliar Al	TPC	DPPH	FRAP
Total N	0.55*	-0.67**	0.83**	0.64*	0.76**	-0.73**	-0.62*	-0.67**	-0.79**	-0.60*
Total P	0.38	-0.56*	0.80**	0.61*	0.69**	-0.65*	-0.56*	-0.50	-0.74**	-0.38
Total K	-0.46	0.07	-0.02	0.27	-0.004	0.08	0.01	-0.12	0.09	0.13
Total Ca	0.41	-0.61*	0.90**	0.77**	0.86**	-0.80**	-0.69**	-0.71**	-0.82**	-0.64*
Total Mg	-0.05	-0.45	0.57*	0.77**	0.59*	-0.47	-0.40	-0.60*	-0.49	-0.39
Total Fe	-0.15	-0.42	0.45	0.61*	0.41	-0.40	-0.39	-0.42	-0.41	-0.30
Total Al	-0.060*	0.34	-0.40	-0.26	-0.47	0.26	0.25	0.44	0.38	0.49
pH	-0.62*	0.81**	-0.76**	-0.72**	-0.76**	0.53*	0.55*	0.59*	0.84**	0.78**
Soil PC Axes										
PC1	-0.51	0.78**	-0.96**	-0.86**	-0.91**	0.80**	0.71**	0.77**	0.92**	0.71**
PC2	0.47	0.01	-0.04	-0.30	-0.02	0.04	0.05	0.10	-0.02	-0.09

^a The significance of the values is indicated as follows: * P< 0.05; ** P< 0.01; *** P< 0.001.

promoting plant growth (Huang *et al.*, 2022). Magnesium is also involved in activating and governing several other physiological processes, such as photosynthesis, respiration and nucleic acid metabolism (Pongrac *et al.*, 2020).

Our study found that lowland plantations had a higher foliar Fe and Al content than

clones derived from highland plantations. This is suggested due to the higher foliar P in lowland over highland plantation, which is supported by the PCA result (Figure 1), with both the arrow of P and Fe going in the same direction. Iron (Fe) is an important micronutrient for plants since it is involved in metabolic activities such as DNA

synthesis, respiration, and photosynthesis. In contrast, Al is toxic to most plants since a micro-molar dose of it may inhibit root growth (Mahmud *et al.* 2024). However, tea plants are recognised as Al hyper-accumulators, which was initially coined for plants containing Al exceeding 1.0 mg g^{-1} dry mass (Chenery, 1955). However, distinguishing Al accumulators requires different thresholds depending on geographic origin: tropical plants need higher levels [$2.3 - 3.9 \text{ mg Al g}^{-1}$ of leaf dry mass (Metali *et al.*, 2012)]

In tea plants, Al concentration varies between young and mature leaves, whereas young leaves contain lower Al than mature leaves. Zhang *et al.* (2018) reported that Al concentrations in young leaves ranged from $0.25 - 0.66 \text{ mg g}^{-1}$, while in mature tea leaves, they ranged from $4.3 - 10.4 \text{ mg g}^{-1}$. A recent study revealed that Al concentrations in young leaves were between 0.67 and 2.21 mg g^{-1} , and in mature leaves between 2.63 and 7.83 mg g^{-1} (Zaman *et al.*, 2024). In our study, we found that young tea leaves were even able to accumulate up to 16.61 mg g^{-1} or 8-fold higher than the result of Zaman *et al.* (2024). Al primarily enters the plant root from acidic soils ($\text{pH} < 5.0$), where Al is solubilized into its toxic form (Al^{3+}). Organic acids like malate, citrate and oxalate are critical in Al detoxification. The formation of Al-organic acid complexes (such as Al-malate) is essential for their transport within the plant. These complexes are less toxic and can be transported through the symplastic pathway and loaded into the xylem for translocation to the shoots (Wang *et al.*, 2017). Thus, we assume that high Al accumulation of tea was due to the efficiency of detoxification and/or compartmentation mechanisms. Otherwise, such high Al concentrations would be extremely toxic. In the leaves, Hajiboland *et al.* (2013) revealed that up to 60% of Al in tea plants is stored in cell walls, primarily by binding to pectin and hemicellulose components. A significant portion of Al is sequestered in the vacuoles of tea leaves, reducing its toxicity to other cellular

organelles (Gao *et al.*, 2014). In addition, Al can be deposited in vacuoles that exist as complexes with phenolic substances, such as catechin (Barceló and Poschenrieder, 2002). P is among its components. Therefore, high Al uptake is generally coupled with high uptake of P, which was supported by our PCA result (Figure 1).

In terms of AOA, clones grown at a lower elevation tend to have a superior antioxidant, as demonstrated by greater TPC and FRAP values and lower DPPH IC₅₀ values. This result was similar to a previous study conducted in Africa (Owuor *et al.*, 2011), Taiwan (Chen *et al.* 2014), Indonesia (Martono *et al.*, 2016), and China (Wang *et al.*, 2022). Altitude increases are generally linked with decreases in tea polyphenol content (Ahmed *et al.*, 2019), whereas catechin is its major constituent. Catechin was reported to be inversely correlated to cultivation altitude as the EGCG (major catechin derivative) declined when the cultivation altitude was elevated (Chen *et al.*, 2014). Wang *et al.* (2022) confirmed the inhibition of catechin biosynthesis in high altitudes by stimulating the changes induced by temperature and light at different altitudes. Light intensity plays a major role in influencing the catechin content of tea. Xiang *et al.* (2021) proved that the catechin content and photosynthetic capacity of tea plants increased under optimum light intensities ($250 - 350 \mu\text{mol m}^{-2} \text{ s}^{-1}$). However, it will decrease under shading treatment ($150 \mu\text{mol m}^{-2} \text{ s}^{-1}$) or extreme high light intensity in highland plantation ($550 \mu\text{mol m}^{-2} \text{ s}^{-1}$). Lowland-grown tea may produce up to 28% higher polyphenols compared to highland (Zhang *et al.*, 2018).

Association of Nutritional Characteristics and AOA with Soil Edaphic Factors

The PC1 of the soil properties (nutrient concentrations and pH) displayed a positive and significant correlation with foliar P, Fe, Al, and AOA (TPC, DPPH, FRAP). In PCA, PC1 is the first principal component that captures the maximum variance in the data. This suggested that PC1 represent soil fertility and nutrient availability gradient



that influences tea nutritional composition and antioxidant activity. A strong correlation between PC1 and foliar P or Fe suggested that PC1 reflects a soil axis related to P and Fe availability, essential for plant metabolism and enzymatic functions (Bhat *et al.* 2024). The presence of Al in the correlation might indicate soil acidity, which is common in tropical soil, further affects P availability and metal uptake (Ur Rahman *et al.*, 2024). Higher foliar TPC, DPPH, and FRAP linked to PC1 suggested that increasing soil P, Fe, and Al may enhance the synthesis of phenolic compounds, boosting antioxidant activity. This could be due to plant stress responses to metal presence (Fe and Al), increasing secondary metabolites such as phenolic. This suggests nutrient availability and potential soil stressors (such as Al) are key to plant biochemical composition.

Based on our analysis, total soil N was the most important element for tea growth and development, as it had a positive association not just with foliar N but also with foliar K, Ca, and Mg, as well as a negative correlation with foliar Fe and Al. In addition, total soil P, Ca, and Mg showed a substantial association with foliar K, Ca, and Mg, indicating that variations in tea's nutritional characteristics were influenced by the availability of nutrients. The results were similar to those of previous studies conducted in China (Zhao *et al.*, 2017) and Taiwan (Tseng and Lai, 2022). Nitrogen is an essential nutrient for tea production and accounts for 4-5% of tea leaf dry weight (Hamid *et al.*, 2014). Along with Mg and Mn, it up-regulated the expression of key genes for chlorophyll synthesis and promoted its synthesis (Chen *et al.*, 2021), thus resulting in a proportional increase in economic yield (Sitienei *et al.*, 2013).

Soil pH was the only soil parameter significantly correlated with foliar nutrients (P, Fe, and Al) and AOA of tea (TPC, DPPH, and FRAP assay). Soil pH from both plantations was highly acidic, with an average value of 4.23 and 3.58 for lowland and highland, respectively. Tea has also

been planted on acidic soil in Vietnam (pH 3.7-3.9) (Huu Chien *et al.*, 2018), Taiwan (pH 3.5 – 5.21) (Tseng and Lai, 2022) and China (pH 3.96-5.48) (Yan *et al.*, 2020). Soil pH significantly affects the availability of foliar P concentration. P tends to form insoluble complexes with Fe and Al in acidic soils, reducing availability. In contrast, as pH increases towards neutral, P becomes more available for plant uptake (Baquy *et al.*, 2024).

The increased Fe and Al uptake in tea plantations with increased soil pH was rare. Typically, Fe and Al solubility and availability decrease as soil pH increases (Ruan *et al.*, 2006; Alekseeva *et al.*, 2011). However, several mechanisms could explain this phenomenon. Tea plants have adapted to acidic soils and may release organic acids and chelating compounds from their roots, such as malate, citrate and oxalate. These compounds can solubilize Fe and Al even at higher pH by forming metal-organic complexes that remain plant-available. For instance, the formation of Al-malate is essential for transport within plants since these complexes are less toxic. Subsequently, they can be transported via the symplastic pathway and loaded into the xylem for translocation to the leaves (Wang *et al.*, 2017). Another mechanism includes microbial activity changes. An increase in soil pH could shift the microbial community structure. The shift had the potential to enhance siderophore-producing organisms that make Fe more available to plants (Choi *et al.*, 2024).

Higher soil pH has been associated with increased enzymatic activities in tea plants, which could enhance antioxidant defences. For instance, increasing soil pH from 3.3 to 5.3 enhances the nutrient availability in the rhizosphere. Subsequently, improving pH facilitates the uptake of essential elements including C, K, Ca, Mg, Mn, P and S, which play a vital role in phenolic biosynthesis (Jia *et al.*, 2024). The antioxidant activity, measured by DPPH and FRAP assay, tends to increase with higher soil pH. The improved nutrient uptake and enhanced

photosynthetic capacity under high pH conditions contribute to accumulating phenolic compounds, potent reducing agents. This results in higher FRAP values, indicating better antioxidant potential (Jahan *et al.*, 2022; Jia *et al.*, 2023).

CONCLUSIONS

We conclude that altitude and soil physicochemical properties are among the factors that influenced the variation of nutritional characteristics and the AOA of tea. Most foliar tea nutrients varied significantly among clonal teas, except Ca in the lowlands and Fe in the highlands. The highland tea population tended to have higher foliar nutrient concentrations, while the lowland population had better AOA performance. AT53 and 1248 were considered promising clones for having higher foliar nutrients and better AOA performance, respectively. Regarding soil edaphic factors, we found that soil nutrients and pH displayed a significant correlation with foliar nutrients, while soil pH was also significantly associated with the AOA of tea.

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غلظت عناصر غذایی برگ و فعالیت آنتی‌اکسیدانی چای (*Camellia*)
(*gsinensis* L. (O) Kuntze) کاشته شده در شبه جزیره مالزی و ارتباط آن با
عوامل خاکی

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و خیریل محمود

چکیده

این پژوهش با هدف (1) مقایسه ویژگی‌های تغذیه‌ای و فعالیت آنتی‌اکسیدانی (AOA) چای کلونال کشت شده در مزارع پایین دست و مرتفع و (2) بررسی تأثیر عوامل خاکی بر تغذیه برگ چای و AOA انجام شد. برگ‌های چای و خاک چهارده کلون چای (tea clones) بین اکتبر ۲۰۲۱ تا مارس ۲۰۲۲ از مزارع پست و مرتفع در شبه جزیره مالزی نمونه برداری شدند. برگ‌ها از نظر محتوای غذایی و فعالیت آنتی‌اکسیدانی مورد تجزیه و تحلیل قرار گرفتند، در حالی که نمونه‌های خاک تحت تجزیه و تحلیل فیزیکی و تغذیه‌ای قرار گرفتند. نتایج، تغییرات قابل توجهی را در بیشتر مواد مغذی برگ، به جز کلسیم در مناطق پایین دست و آهن در مناطق مرتفع، نشان داد. در حالی که چای کشت شده در مناطق مرتفع غلظت مواد مغذی بیشتری را نشان داد، چای کشت شده در مناطق بیشتری AOA زیادتری را نشان داد. کلون‌های AT53 و 1248 به عنوان کلون‌های امیدوارکننده در بین کلون‌ها شناسایی شدند که به ترتیب بالاترین مواد مغذی و سطوح AOA مشخص می‌شوند. در دسترس بودن مواد مغذی خاک به طور قابل توجهی بر جذب مواد مغذی برگ تأثیر گذاشت، در حالی که pH خاک با AOA مرتبط بود. بنابراین، نقش حیاتی عوامل خاکی در شکل‌دهی کیفیت چای، بینش ارزشمندی را برای چایکاران فراهم می‌کند تا خاک را بهینه کرده و عملکرد و کیفیت چای را در آینده حفظ کنند. ما دریافتیم که مواد مغذی خاک ارتباط معنی‌داری با جذب مواد مغذی دارند، در حالی که pH خاک با ویژگی‌های زراعی چای مرتبط است.

Influence of Pre-Imaginal Stages of *Bemisia tabaci* on Development, Life Table Parameters, and Predation Rate of *Euseius scutalis* (Acari: Phytoseiidae)

Mohammad Gravandian¹, Parviz Shishehbor^{1*}, Mehdi Esfandiari¹, and Elham Riahi²

ABSTRACT

The predatory mite, *Euseius scutalis* (Athias-Henriot), was studied in terms of its development, survival, and life table parameters on two preimaginal stages (egg and first instar nymph) of its prey, *Bemisia tabaci* (Gennadius) (Hem.: Aleyrodidae). The first instar nymphs of *B. tabaci* were found to be the preferred food for *E. scutalis*, resulting in decreased developmental time from egg to adult, as well as a shorter pre-oviposition period and a higher rate of oviposition. The intrinsic rate of increase (r) was found to be 0.1503 d^{-1} on the first instar nymphs and 0.0843 d^{-1} on eggs of the prey. On average, females' *E. scutalis* consumed 16.30 eggs and 29.40 nymphs from their emergence to death. When first instar nymphs of *B. tabaci* were provided, *E. scutalis* showed a higher net predation rate (C_0) and finite predation rate (ω) compared to feeding on eggs. On average, it consumed 3.52 eggs or 2.76 first instar nymphs of *B. tabaci* to produce a single egg of *E. scutalis*. In terms of sex ratio of the progeny, predatory females that fed on the first instar nymphs produced more females.

Keywords: Cotton whitefly, Growth rate, Phytoseiidae, Predation rate, Sex ratio.

INTRODUCTION

The agricultural pest known as the cotton whitefly, *Bemisia tabaci* (Gennadius) (Hem.: Aleyrodidae) is one of the major global threats to different crops. *B. tabaci* damages greenhouse vegetables and ornamental plants by sucking sap from the plants, which can reduce production (De Barro *et al.*, 2011). This is primarily due to their direct damage to host plants through leaf piercing, sap sucking, and honeydew secretion (Jones, 2003). It has a high reproductive capacity and is capable of causing significant damage through the secretion of honeydew, which promotes the rapid growth of molds (Gangwar and

Gangwar, 2018). The use of insecticides to eliminate this pest raises production costs and contributes to the development of pest resistance.

The crucial role of predatory mites of the family Phytoseiidae in biological control is widely recognized (McMurtry *et al.*, 2013). These efficient predators play a significant role in managing pest mites and other small arthropods in crops grown in both greenhouses and open fields worldwide. With over 2,700 species reported on all continents, except Antarctica, the mites from this family make up 60% of the global biological control market (Momen *et al.*, 2020). They are considered a vital solution for sustainable plant protection and for promoting integrated pest management

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against spider mites, thrips, and whiteflies (Yazdanpanah and Fathipour, 2022).

Euseius scutalis (Athias-Henriot) is a common phytoseiids in Middle East and North Africa (Bounfour and McMurtry, 1987). This predatory mite is one of the most dominant predator on economic crops in Iran (Kamali et al., 2001), and it is one of the pollen-feeding generalist species of the Phytoseiid mite (McMurtry et al., 2013). It is a significant predatory mite in biological control due to its ability to effectively manage pest populations. Although this predatory mite has the highest reproductive capacity when feeding on pollen, it is also capable of consuming a variety of small insects and mites, such as eriophyid, tenuipalpid and spider mites, eggs, and immature stages of whiteflies, thrips and scale insects (Abou-Elella et al., 2013; Stathakis et al., 2021). *E. scutalis* exhibits optimal life cycle parameters and fecundity when feeding on *Tetranychus turkestanii* (Ugarov and Nikolskii), followed by date palm pollen, making it a versatile and effective biocontrol agent (Shishehbor et al., 2022). Previous studies have shown a close association between this species and *B. tabaci* (Meyerdirk and Coudriet, 1986). The use of *E. scutalis* in integrated pest management programs can reduce reliance on chemical pesticides, promoting a safer and more sustainable agricultural ecosystem.

While some researchers have provided information on the life table of *E. scutalis* when fed on *B. tabaci* compared to other prey species or pollen grains (Nomikou et al., 2001, Nawar 2017), there is a lack of data regarding the life table of this predatory mite in relation to the different growth stages of *B. tabaci*. Therefore, this study aimed to assess the influence of two different developmental stages (eggs and first instar nymphs) of the *B. tabaci* on the biological characteristics and predation rate of *E. scutalis*, which plays a vital role in biological control.

MATERIALS AND METHODS

Mite Rearing

The population of *E. scutalis* was obtained from a laboratory-grown culture at the Faculty of Agriculture, Shahid Chamran University of Ahvaz, Iran. The initial population, identified by Dr. Farid Faraji from Mitox (Amsterdam, The Netherlands), was collected in February 2021 from marshmallow plants (*Althea officinalis* L.) infested with *T. turkestanii* on the university campus. For laboratory rearing, we used plastic green sheets measuring 3 cm in diameter, placed on a water-saturated sponge in a Petri dish measuring 6 cm in diameter and filled with water. The edges of the sheet were surrounded by strips of tissue paper. Cotton threads were placed on the plastic sheets to provide egg-laying sites and shelter for the predators (Walzer and Schausberger, 1999). As a food source, we provided date palm pollen (*Phoenix dactylifera* L.) on the plastic sheets. The mites were fed this pollen grain for approximately one month before starting the experiments. Date palm pollen was chosen because it has been recommended as one of the most suitable alternative diet sources for the easy and cost-effective rearing of *E. scutalis* (Shishehbor et al., 2022). The rearing units were held in a growth chamber at $25\pm 1^{\circ}\text{C}$, $60\pm 10\%$ RH, and a photoperiod of 16:8 (L:D).

Whitefly Rearing

A number of mature cotton whiteflies were collected using an aspirator from infested plants in the greenhouse at Shahid Chamran University of Ahvaz, Faculty of Agriculture. The whiteflies were then reared on cucumber plants inside cages (60×60×120 cm). Identification of this species was carried out using the taxonomic keys of Martin (1987). The cucumber plants (Var. Negin) used for colony maintenance

were reared at $25\pm 1^\circ\text{C}$, $60\%\pm 10\%$ RH, and a photoperiod of 16L:8D. Every week, a new cucumber plant with at least eight fully developed leaves was added to each cage.

Experimental Setup

Leaves of a cucumber plant infested with *B. tabaci* were carefully selected and cut into smaller pieces. Each piece was then examined under a stereomicroscope to confirm the presence of only 10 fresh *B. tabaci* eggs. For the first instar nymphs, the same procedure was repeated, but the leaves were cut after the eggs had developed to the first instar nymph stage (10 first instar stable nymphs). The test units were prepared the same as the units used for mite rearing, except using a piece of cucumber leaf (3 cm in diameter) containing whitefly eggs or first instar nymphs pad instead of using plastic sheet.

Effect of *B. tabaci* Immature Stages on Predatory Mite *E. scutalis*

About 50 females of *E. scutalis* were separated from the rearing unit using a fine brush and transferred to a new experimental unit. After 24 hours, the eggs that were less than 24 hour-old were placed individually in petri dishes containing one of the two pre-adult stages of *B. tabaci*. The petri dishes were checked daily to record survival and developmental durations until adulthood. Once they became adults, the females and males were paired and transferred to a new petri dish. Each day, we checked the experimental units to confirm oviposition and note the consumption. The leaf in each arena was replaced daily with a new piece of fresh food. The number of prey consumed by each pair of predatory mite was recorded daily. Additionally, the number of eggs laid by each female was recorded daily until all the females died. The survival of both females and males was monitored daily until all individuals had died. Each experiment

was repeated 60 times. The tests were conducted in a controlled-temperature cabinet at $25\pm 1^\circ\text{C}$, $60\%\pm 10\%$ RH, and a photoperiod of 16:8 (L:D). *E. scutalis* eggs collected daily and were transferred to new petri dishes containing a piece of cucumber leaf containing whitefly eggs or first instar nymphs. This procedure was repeated on all days when oviposition occurred. After they matured, the gender of the predators was determined to estimate the daily sex ratio.

Data Analysis

The computer programs TWSEX-MSChart and CONSUME-MS Chart were utilized for analyzing the life history and the daily consumption of *E. scutalis* on the two stages of *B. tabaci*, respectively (Chi and Liu, 1985; Chi, 1988; Chi, 2023a, b). The age-stage-specific survival rate (s_{xj}), age-stage-specific fecundity (f_{xj}), age-specific survival rate (l_x), age-specific fecundity (m_x), age-stage life expectancies (e_{xj}), and age-stage reproductive value (v_{xj}) along with population growth parameters were calculated using TWSEX-MS Chart program (Chi, 2023a). Based on Chi and Yang (2003), net predation rate (C_0), transformation rate (Q_p), stable predation rate (ψ), and finite predation rate (ω), were computed using CONSUME-MS Chart program (Chi and Yang, 2003; Chi, 2023b). Means and standard errors of the all parameters were calculated by bootstrap method with 100000 resamplings. Paired bootstrap test using the TWSEX-MS Chart program (Chi, 2023a) was used for mean comparisons.

RESULTS

Immature Developmental Time and Survival

Individuals of *E. scutalis* successfully developed from larvae to adults when fed on eggs and first instar nymphs of *B. tabaci*



(Table 1). The time spent by *E. scutalis* in each developmental stage was affected by the immature stage of *B. tabaci*. Development from egg to adult was significantly faster for both females and males when feeding on the first instar nymphs of *B. tabaci* compared to eggs. While the adult longevity and total lifespan of female *E. scutalis* fed on the first instar nymphs of *B. tabaci* were not different from those reared on eggs, these parameters for males were significantly longer on the first instar nymphs of *B. tabaci* than on its eggs (Table 1). Additionally, there were no significant differences between the sexes in terms of immature development on either food item. When *B. tabaci* eggs were the food source, males exhibited shorter adult longevity and total lifespan compared to the females (Table 1).

The overlapping observed in Figure 1A can be explained by the fact that individuals at different stages had varying development rates. *Euseius scutalis* exhibited high survivorship during the immature development stage. Females that were fed eggs and the first instar nymphs of *B. tabaci* survived for 39 and 32 days, respectively. The males lived for 24 days when fed on the eggs of *B. tabaci*, while they survived for 31 days when fed on the first instar nymphs.

Fecundity and Life Table Parameters

The consumption of the first instar nymphs of *B. tabaci* resulted in a shorter Average Pre-Oviposition Period (APOP) of *E. scutalis* compared to its eggs (Table 1). Similarly, significant differences in the Total

Table 1. Comparative duration (Mean±SE) of developmental time, adult longevity, total life span, APOP, TPOP, and oviposition days (days), as well as, fecundity and sex ratio of females and males of *Euseius scutalis* fed on egg and first-instar nymph of *Bemisia tabaci*.^a

Parameter	Prey stage	
	Egg	Nymph (L1)
Female egg	2.17 ±0.10 a A	2.07 ±0.05 a A
Male egg	2.27 ±0.19 a A	2.26±0.10 a A
Female larva	1.50 ±0.09 a A	1.13 ±0.06 b A
Male larva	1.64 ±0.15 a A	1.21±0.12 b A
Female protonymph	1.90 ±0.12 a A	1.57 ±0.09 b A
Male protonymph	2.00 ±0.13 a A	1.53 ±0.12 b A
Female deutonymph	2.17 ±0.14 a A	2.10 ±0.13 a A
Male deutonymph	2.36 ±0.24 a A	1.68 ±0.17 b A
Female developmental time	7.73 ±0.21 a A	6.87 ±0.15 b A
Male developmental time	8.27 ±0.24 a A	6.68 ±0.19 b A
Female adult longevity	12.80 ±1.29 a A	14.93 ±0.85 a A
Male adult longevity	9.45 ±1.27 b B	14.74 ±1.27 a A
Female total life span	20.53 ±1.40 a A	21.80 ±0.86 a A
Male total life span	17.73 ±1.28 b B	21.42 ±1.25 a A
APOP*	3.92 ±0.35 a	2.67 ±0.15 b
TPOP*	11.73 ±0.45 a	7.87 ±2.00 b
Oviposition days	5.42 ±0.83 b	8.93 ±0.54 a
Fecundity (Eggs/Female)	6.50 ± 1.09 b	15.67 ± 1.47 a
Sex ratio (Nf/N)*	0.67 ± 0.07 a	0.56 ± 0.07 a

^a Means (±SE) within rows followed by lowercase different letters are significantly different (100,000 resamplings, paired bootstrap test, $P < 0.05$). Mean values followed by different capital letters shows the significant difference between female and male for each parameter ($P < 0.05$), based on paired bootstrap test with 100,000 samples.

* APOP: Adult Pre-Oviposition Period, TPOP: Total Pre-Oviposition Period. Nf/N: Number of females to Number of individuals.

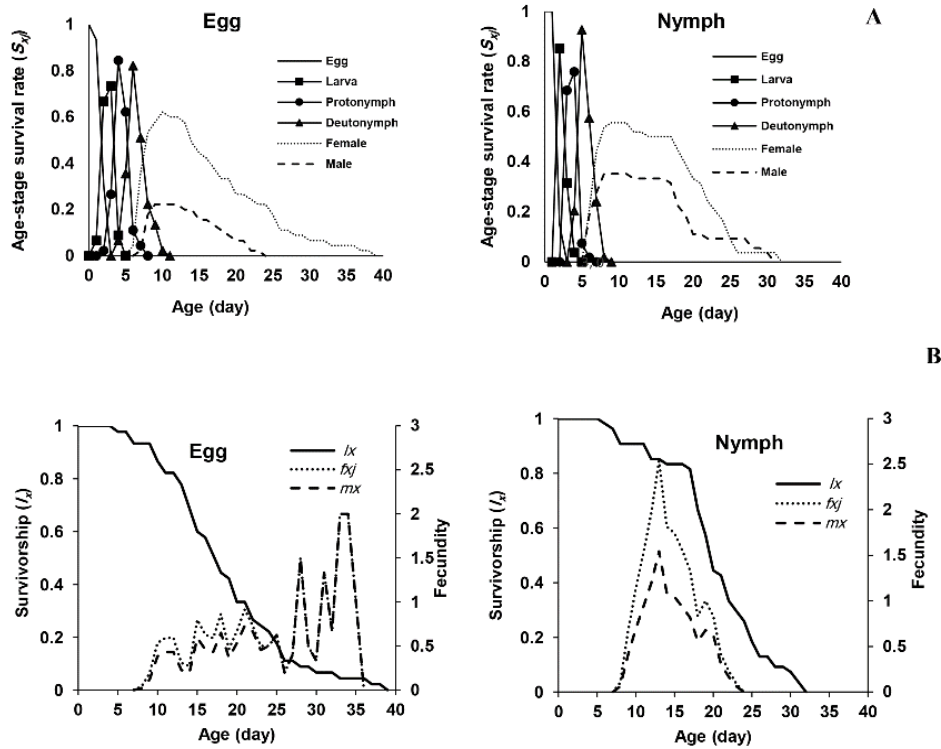


Figure 1. The age-stage survival rate (s_{xj}) (A) and the age-specific survivorship (l_x), age-stage specific fecundity of female (f_{xj}) (eggs) and age-specific fecundity (m_x) (B) of *Euseius scutalis* fed on egg and first-instar nymph of *Bemisia tabaci*.

Pre-Oviposition Period (TPOP) were observed between the different immature stages of *B. tabaci*. When the predator was fed the first instar nymphs of *B. tabaci*, the adult females laid eggs more rapidly (2.67 ± 0.15 days) compared to when eggs of this prey were offered (3.92 ± 0.35 days). *Euseius scutalis* that fed *B. tabaci* eggs had a shorter oviposition duration compared to those that used *B. tabaci* first instar nymphs.

Figure 1B summarizes the age-specific survivorship (l_x), the age-specific fecundity rate (m_x), and female age-specific fecundity (f_x) for *E. scutalis* reared on different life stages of *B. tabaci*. The maximum values of f_x for *E. scutalis* on the first instar nymphs

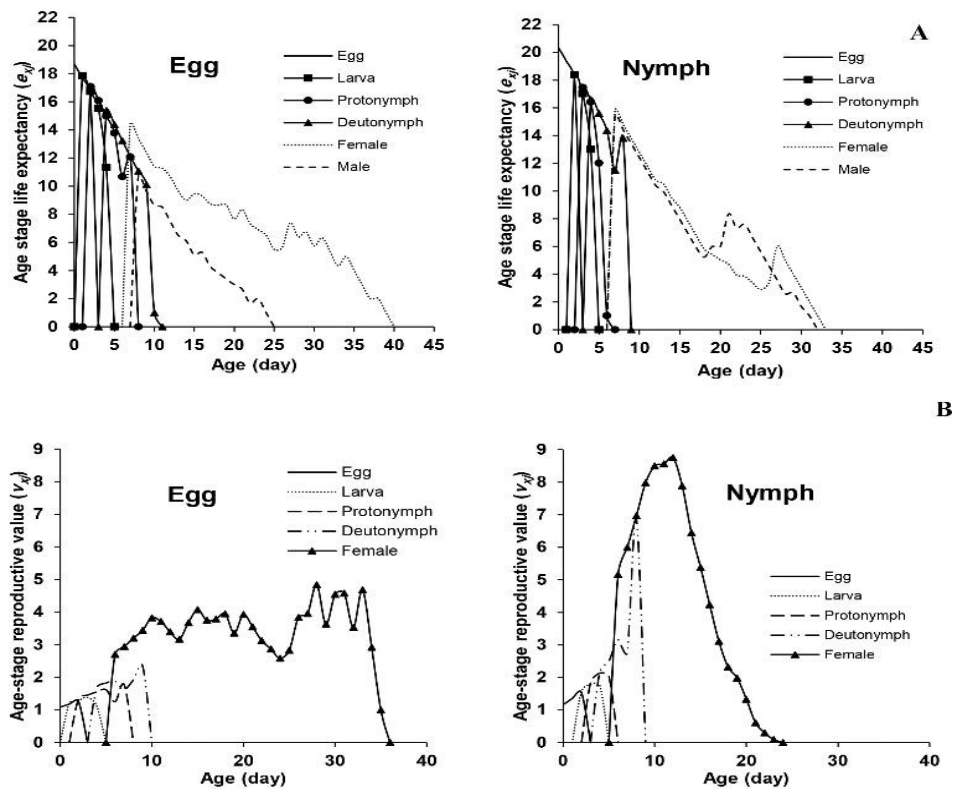
and eggs of *B. tabaci* were 2.54 and 2 eggs/individual/day, respectively, which occurred on the 13th and 33rd days, respectively.

All the population parameters of *E. scutalis* were significantly influenced by the prey stage, except for the Gross Reproductive Rate (*GRR*) (Table 2). When *B. tabaci* first instar nymphs were offered as food, R_0 , r , and λ of the predator mite were higher compared to when whitefly eggs were offered. Similarly, the mean generation time of *E. scutalis* on the former stage of prey was shorter than on the latter stage (Table 2).

**Table 2.** Population parameters of *Euseius scutalis* fed on egg and first-instar nymph of *Bemisia tabaci*.^a

Parameter	Egg	Nymph (L1)
GRR (Eggs/Individuas)	17.77 \pm 3.27 a	11.12 \pm 1.54 a
R_0 (Eggs/Individuas)	4.33 \pm 0.85 b	8.70 \pm 1.34 a
r (d^{-1})	0.084 \pm 0.01 b	0.150 \pm 0.01 a
λ (d^{-1})	1.088 \pm 0.01 b	1.162 \pm 0.01 a
T (Day)	17.38 \pm 1.11 a	14.39 \pm 0.18 b

^a Means (\pm SE) within rows followed by different letters are significantly different (100,000 resamplings, paired bootstrap test, $P < 0.05$). GRR : Gross Reproductive Rate, R_0 : Net reproductive rate, r : Intrinsic rate of increase, λ : Finite rate of increase, T : Mean generation time.

**Figure 2.** The age-stage specific life expectancy (e_{xj}) (A) and the age-stage reproductive value (v_{xj}) (B) of *Euseius scutalis* fed on egg and first-instar nymph of *Bemisia tabaci*.

The age-stage life expectancies (e_{xj}) of *E. scutalis*, which represent the expected lifespan of individuals at age x and stage j after age x , are illustrated in Figure 2A for two different life stages of *B. tabaci*. As age increased, the e_{xj} values gradually decreased. Notably, e_{xj} was higher for nymphs compared to eggs (Figure 2-A), indicating that *E. scutalis* feeding on nymphs of *B. tabaci* lived longer than those

that fed on eggs. The age-stage reproductive value (v_{xj}), which indicates the contribution of an individual at age x and stage j to the future population, increased significantly when adults emerged and the peak of v_{xj} for the predator occurred at ages 28 and 12 days on the egg and nymph stages of *B. tabaci*, respectively (Figure 2-B).

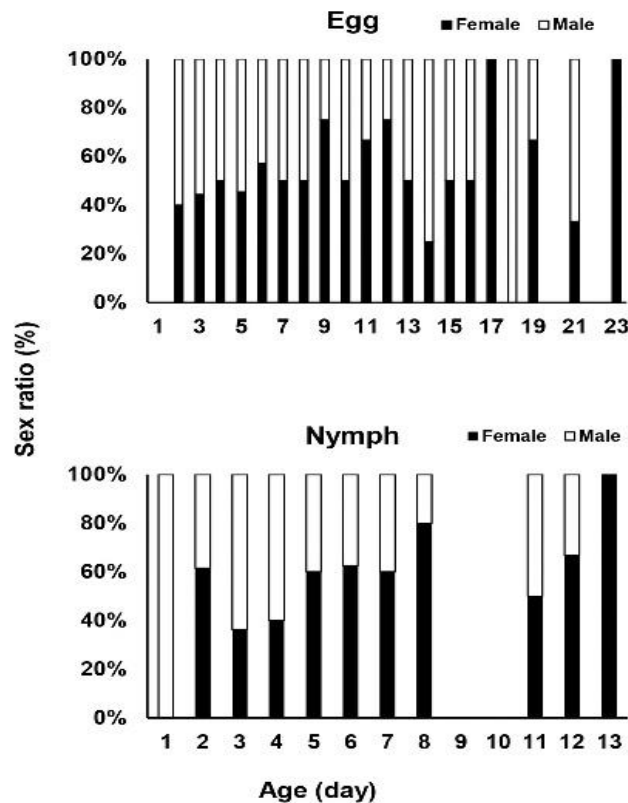


Figure 3. Offspring sex ratio of females of *Euseius scutalis* fed on egg and first-instar nymph of *Bemisia tabaci*. White and black bars indicate the percentages of male and female offspring, respectively.

Sex Ratio

During the first days of oviposition, sex ratio was males-based (Figure 3). The percentage of female to male progeny per female parent reared on *B. tabaci* eggs during the initial three 24-hour periods were 00:00, 40:60 and 44:55 (on the first day, all collected eggs died before adult emergence). For *E. scutalis* females reared on *B. tabaci* first instar nymphs, similar measurements were 0:100, 61:38, and 36:63.

Consumption Rate

The larval stage of *E. scutalis* did not prey on the eggs and the first instar nymphs of *B. tabaci*. The amount of prey consumed by *E. scutalis* immatures when *B. tabaci* eggs were provided did not differ from that

obtained when *B. tabaci* first instar nymphs were offered (Table 3). The predation rate of *E. scutalis* adults and their total life span were significantly affected by the prey stage. The consumption rate of both female and male *E. scutalis* was higher when they were fed on the first instar nymphs of *B. tabaci* rather than its eggs. When the first instar nymphs of *B. tabaci* were provided, *E. scutalis* showed a higher net predation rate (C_0) and finite predation rate (ω) compared to when its eggs were provided. On average, it takes either 3.52 eggs or 2.76 first instar nymphs of *B. tabaci* to produce a single egg of *E. scutalis*, respectively (Table 3).

The age-specific predation rate (k_x) refers to the average number of *B. tabaci* consumed by *E. scutalis* at age x (Figure 4). The highest value of this parameter was 2.08 and 1.62 on the first instar nymphs and eggs of *B. tabaci*, respectively, which was



observed at age 10. The age-specific net predation rate (q_x) can be determined by taking into account the survivorship (Figure 4). Its highest value was estimated to be 1.88 and 1.40 on the aforementioned prey stages.

DISCUSSION

In our study, we found that the different immature stages of *B. tabaci* as prey had a significant impact on the duration of *E. scutalis*' pre-adult stages and its total lifespan. We observed that motile prey stage (nymph L1) was more conducive to the growth of *E. scutalis*. Regardless of previous studies showing that the type of prey influences the fecundity and reproductive parameters of *E. scutalis* (Momen and Hussein, 2011), our research has revealed that the developmental stage of the prey can also impact these parameters. Female *E. scutalis* that fed on the first instar nymphs of *B. tabaci* had a shorter pre-oviposition period, longer oviposition period, and produced more eggs than those reared on eggs of *B. tabaci*. This suggests that prey on the first instar nymphs are more favorable to this predator than prey on eggs.

Prey stage preferences vary considerably among generalist and specialist phytoseiid predatory mites, with specialists typically preferring the egg stage over other stages of their prey (Blackwood *et al.*, 2001). According to the literature, some other predators, including *Macrolophus caliginosus* (Wagner) (Hem.: Miridae) and *Serangium parcesetosum* Sicard (Col.: Coccinellidae) also preferred the nymph stage of *B. tabaci* for optimal reproductive success (Bonato *et al.*, 2006; Firas and Sengonca, 2007). However, our findings differ from those reported by Meyerdirk and Coudriet (1986), who found that eggs, followed by the first instars of *B. tabaci*, were the most suitable whitefly host stage for *E. scutalis*, while the second instar

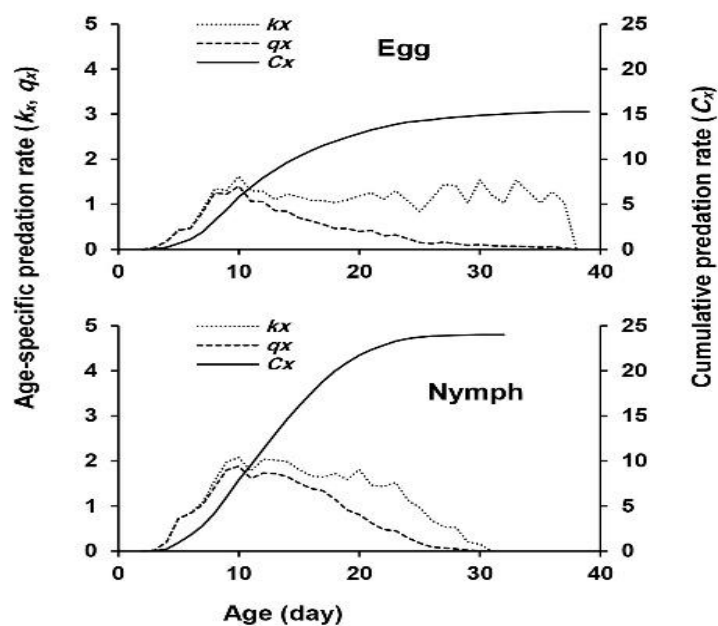
nymph was the least suitable stage. The predation of *E. scutalis* on the eggs and first instar larvae of *B. tabaci* was not significantly different (Nomikou *et al.*, 2004), which contrasts with our results. This discrepancy can be explained by the fact that Nomikou *et al.* (2004) reported the predation rate over only one day, while we examined the predation rate throughout the lifespan. Additionally, variations in predator and prey strains, as well as the previous food source of the predatory mite, can also account for this difference. Furthermore, it appears that insect nymphs have a higher nutritional content compared to insect eggs. Shah *et al.* (2022) indicated that insect nymphs can contain a protein content ranging from 40-73% of their body weight, depending on the species. Additionally, higher concentrations of fatty acids are found in insect nymphs compared to their eggs. However, information on the nutritional value of insect eggs is not as extensively covered in the literature. It has been noted that the hardness of the egg's chorion and the length of time required for handling them may pose difficulties for the mites when feeding on them (Meyerdirk and Coudriet, 1986; Carrillo and Pena, 2012; Ganjisaffar and Perring, 2015). Furthermore, it is evident that there is a difference in size and biomass between the eggs and nymphs of *B. tabaci*. The nymphs were found to be larger than the eggs. However, the potential benefits of this size difference are somewhat diminished by the fact that the nymphs are active stages, requiring the predator to exert energy and time in capturing them. Nevertheless, due to the smaller size of the leaf discs compared to normal leaves and the high density of prey, the predator females did not have to exert much energy in searching for active prey (Bruce-Oliver and Hoy, 1990).

Similar to previous studies on *E. scutalis* (Bounfour and McMurtry, 1987;

Table 3. Mean (\pm SE) predation rates of *Euseius scutalis* fed on egg and first-instar nymph of *Bemisia tabaci*.

Parameter	Egg	Nymph (L1)
Protonymph female	0.27 \pm 0.07 a A	0.23 \pm 0.05 a A
Protonymph male	0.36 \pm 0.08 a A	0.26 \pm 0.08 a A
Deutonymph female	1.33 \pm 0.10 a A	1.47 \pm 0.07 a A
Deutonymph male	1.18 \pm 0.12 a A	1.11 \pm 0.15 a A
Pre-adult female	1.60 \pm 0.19 a A	1.70 \pm 0.18 a A
Pre-adult male	1.55 \pm 0.25 a A	1.37 \pm 0.22 a B
Adult female	16.30 \pm 5.48 b A	29.40 \pm 5.48 a A
Adult male	11.33 \pm 3.32 b B	17.67 \pm 4.36 a B
Total longevity female	17.90 \pm 1.55 b A	32.10 \pm 1.65 a A
Total longevity male	12.87 \pm 1.55 b B	19.04 \pm 1.29 a B
C_0	15.26 \pm 1.30 b	23.99 \pm 1.62 a
Q_p	3.52 \pm 0.54 a	2.76 \pm 0.34 a
ψ	0.58 \pm 0.03 a	0.63 \pm 0.03 a
ω	0.63 \pm 0.03 b	0.73 \pm 0.04 a

^a Mean values followed by different lowercase letters within the same row are significantly different ($P < 0.05$), based on paired bootstrap test with 100,000 samples. Mean values followed by different capital letters shows the significant difference between female and male for each parameter ($P < 0.05$), based on paired bootstrap test with 100,000 samples. C_0 : Net predation rate; ω : Finite predation rate; Q_p : Transformation rate; ψ : Stable predation rate.

**Figure 4.** Age-specific predation rate (k_x), age-specific net predation rate (q_x) and cumulative predation rate (C_x) of predator mite *Euseius scutalis* fed on egg and first-instar nymph of *Bemisia tabaci*.



Bazazzadeh *et al.*, 2025), the pre-adult development for male was incomparable to that of females in our study. This developmental similarity can be attributed to some biological and genetic factors. Research indicates that the sex-lethal gene (*Ppsxl*) plays a role in sex determination and reproductive processes, but its influence on sexual differentiation in *Phytoseiulus persimilis* Athias-Henriot is not as pronounced as in insect (Li *et al.*, 2023). This suggests that both sexes may share similar developmental pathways during their immature stages. While the similarities in immature development are evident, it is important to consider that environmental factors and specific ecological roles may also influence the developmental trajectories of these mites, potentially leading to variations not solely explained by genetic mechanisms.

Based on the results of the current study and regardless of the tested immature stages, most of the eggs give rise to male offspring at the beginning of the oviposition period and to female offspring throughout the rest of the oviposition period. A similar trend has also been reported for a Morocco (Marrakech) strain of *E. scutalis* (Bounfour and McMurtry, 1987) and for other phytoseiid such as *Typhlodromus caudiglans* Schuster (Putman, 1962), *Phytoseiulus persimilis*, *Amblyseius bibens* Blommers (Schulten *et al.*, 1978), *Galendromus helveolus* (Chant) (Caceres and Childers, 1991), and *Kampimodromus aberrans* (Boroufas *et al.*, 2007). Sabelis (1985) stated that increased male offspring production at the beginning of the oviposition period could result in the early insemination of females that would afterwards start to search for suitable food.

The predatory mite *E. scutalis* showed a higher *r*-value when preying on the first instar nymphs of *B. tabaci* compared to its predation on *B. tabaci* eggs. The *r*-value estimated in this study was lower than the *r*-value of *E. scutalis* on *B. tabaci* (0.191 d⁻¹) (Fouly *et al.*, 2013). By contrast to our study, when *Amblyseius orientalis* Ehara

was fed on *B. tabaci*, the *r* was negative, indicating that this prey is not a sufficient food source for sustaining *A. orientalis* populations (Zhang *et al.*, 2015). In contrast, when *Typhlodromus negevi* Swirski and Amitai was fed on *B. tabaci* eggs, the intrinsic rate of increase (0.271 d⁻¹) was higher than our study (Momen *et al.*, 2009), indicating that this prey is more suitable for *T. negevi* than *E. scutalis*.

CONCLUSIONS

The pre-immature stage of the prey appears to be a crucial factor in developing an integrated pest management program for *B. tabaci* using *E. scutalis*. Our results demonstrate that *E. scutalis* has the potential to control *B. tabaci* due to its ability to feed on both eggs and the first instar nymphs of the prey. Predators that can consume multiple life stages of their prey are more effective at controlling pest populations. Furthermore, *E. scutalis* showed a preference for feeding on nymphs rather than eggs. Field evaluations should be conducted to further assess its biological control potential, particularly with strategies aimed at enhancing its population establishment and long-term control efficiency, such as providing supplementary food.

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Preference and Life Table of *Amblyseius orientalis* on *Bemisia tabaci* and *Tetranychus cinabarinus*. *PLoS One*, **10(10)**: e0138820.

تأثیر دو مرحله رشدی *Bemisia tabaci* بر رشد، پارامترهای جدول زندگی، و نرخ شکارگری کنه شکارگر، (*Euseius scutalis* (Acari: Phytoseiidae)

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چکیده

رشد، بقا و پارامترهای جدول زندگی کنه شکارگر، *Euseius scutalis* - (Athias-Henriot)، با تغذیه از دو مرحله پیش از بلوغ (تخم و پوره سن اول) (*Bemisia tabaci* (Gennadius) مورد مطالعه قرار گرفت. پوره‌های سن اول *B. tabaci* به عنوان غذای ارجح برای *E. scutalis* شناخته شد زیرا منجر به کاهش دوره رشد از تخم تا بلوغ و همچنین دوره پیش از تخم‌گذاری و افزایش نرخ تخم‌گذاری شد. نرخ ذاتی افزایش جمعیت (r) با تغذیه از پوره‌های سن اول 0/1503 بر روز و با تغذیه از تخم 0/0843 بر روز بود. به طور متوسط ماده‌های *E. scutalis، 16/30 تخم و 29/40 پوره را در کل دوره زندگی (از زمان ظهور تا مرگ) تغذیه کردند. هنگامی که پوره‌های سن اول *B. tabaci* ارائه شد، *E. scutalis* نرخ خالص شکارگری (C_0) و نرخ متناهی شکارگری (ω) بالاتری را در مقایسه با تغذیه از تخم‌ها نشان داد. به طور متوسط کنه شکارگر 3/52 عدد تخم یا 2/76 عدد پوره اول *B. tabaci* را برای تولید یک تخم *E. scutalis* مصرف کرد. از نظر نسبت جنسی نتاج، ماده‌هایی که از پوره‌های سن اول تغذیه کردند، ماده‌های بیشتری تولید کردند.*

Synthesis of Poly- γ -Glutamate in Solid-State Fermentation and Its Application in Biocontrol

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ABSTRACT

Poly- Gamma- Glutamic Acid (γ -PGA) is a natural polymer with diverse applications across multiple industries. However, its use in agriculture is limited due to high production costs. This study aimed to (1) Optimize the cost-effective production of γ -PGA through Solid-State Fermentation (SSF) using *Bacillus velezensis* UTB96, (2) Evaluate the concentration and molecular weight of γ -PGA suitable for agricultural applications, particularly in strawberry cultivation, and (3) Explore the impact of γ -PGA on extending the shelf-life of strawberry fruits during cold storage. Initially, the production of γ -PGA using SSF with *B. velezensis* UTB96 was investigated, along with evaluation of the influence of physicochemical factors on the molecular weight of γ -PGA. Based on the results, three different molecular weights of γ -PGA were identified: 1156.43 kDa, 734.38 kDa, and 296.55 kDa. These were selected for greenhouse trials to assess their effectiveness in controlling gray mold on strawberry plants. The results showed that, by utilizing agricultural wastes including sesame flour, wheat straw, and banana peel in SSF methodology, γ -PGA could be produced at a rate of 70 g/kg of dry weight of the culture medium. Analyzing the impact of γ -PGA on reducing gray mold revealed that this compound could enhance the plant's defense. A significant increase in the activity of ascorbate peroxidase and Phenylalanine Ammonia-Lyase (PAL) enzymes was observed, along with the production of polyphenolic compounds such as ellagic acid. Consequently, these mechanisms improved the plant's flexibility and tolerance to the fungus, helping to maintain the quality of the fruits during cold storage.

Keywords: *Botrytis cinerea*, Controlling gray mold, Cold storage, γ -PGA.

INTRODUCTION

There is increasing interest in finding effective and sustainable alternatives to chemical pesticides. One promising option is biological control. Various microbial Biological Control Agents (BCAs) have been developed in recent decades to tackle fungal and bacterial diseases. Many studies have focused on microorganisms such as *Pseudomonas* spp., *Bacillus* spp., and *Streptomyces* spp. (Bonaterra *et al.*, 2022).

Bacillus velezensis is a well-known strain recognized for its beneficial effects on plant growth and its role in biocontrol as a gram-positive rhizobacterium (Fan *et al.*, 2018). Recent research has investigated the microencapsulation of this bacterium with natural polymers and nanoparticles to control diseases like the *Rhizoctonia solani* fungus in beans (Moradi Pour *et al.*, 2021) and pistachio gum (Moradi Pour *et al.*, 2022).

Numerous studies have highlighted the

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potential of bacteria, particularly *Bacillus* species, in direct antibiosis, competition, and the secretion of various secondary metabolites in the rhizosphere (Fan *et al.*, 2018). A significant secondary metabolite produced by *B. velezensis* is Poly-Gamma-Glutamic Acid (γ -PGA). This biopolymer is biodegradable, non-toxic, environmentally safe, and hypoallergenic, making it highly sought after in various industries.

Research on this biological compound has shown that γ -PGA can significantly enhance plant performance and morphological characteristics. It also improves Soil Microbial Biomass Carbon (SMBC) and Nitrogen (SMBN), boosts soil enzyme activity, and increases plant resistance to both biotic and abiotic stresses (Song *et al.*, 2019).

Given the widespread application of γ -PGA across various industries, its industrial production through Submerged Fermentation (SmF) using *Bacillus* bacteria is common. However, this method is expensive, which restricts its use in agriculture. In contrast, Solid-State Fermentation (SSF) presents a cost-effective alternative by utilizing inexpensive and readily accessible raw materials. Furthermore, SSF provides various economic and engineering benefits, such as lower energy consumption and simplified equipment and infrastructure requirements (Chen *et al.*, 2005).

Therefore, this study investigated innovative approaches for the cost-effective production of γ -PGA through solid-state fermentation, utilizing affordable waste materials for agricultural purposes. Additionally, it examined the effects of environmental factors on the molecular weight of γ -PGA. Another critical aspect addressed in this article is the mechanisms by which γ -PGA enhances the resistance of strawberry plants to necrotrophic fungi, particularly *Botrytis cinerea*, the most prevalent and destructive fungal pathogen affecting strawberries. Additionally, the study investigates the effect of pre-harvest

application of γ -PGA on the shelf life of strawberry fruits during cold storage.

MATERIALS AND METHODS

γ -PGA Production in Solid-State Fermentation (SSF) and Its Identification

Preparation of *Bacillus velezensis* UTB96 Bacteria

In this study, the *Bacillus velezensis* strain UTB96 was obtained from the microbial collection at Tehran University. The bacteria were cultured in a Nutrient Broth medium (NB) at 37 °C and 200 rpm for 16 hours to serve as the inoculum. This medium contained approximately 3×10^7 cells per mL.

Substrate for Solid-State Fermentation

To achieve optimal γ -PGA production, a balanced combination of protein, sugar, and carbon sources is essential. For the economical production of γ -PGA, the recommended medium consists of a blend of sesame flour (as the protein source), wheat straw (as the carbon source), banana peel (providing sugar and minerals), and cow manure (as a mineral source). Various experiments were conducted with different ratios of these components to determine the most effective combination for production.

Extraction and Purification of γ -PGA

The extraction and purification of γ -PGA were performed according to the method described by Goto and Kunioka (1992).

Identification and Characterization of γ -PGA

The produced γ -PGA was identified and characterized using SDS-PAGE (Yu *et al.*,

2016), FT-IR (Khalil *et al.*, 2018), and spectrophotometric methods (Zeng *et al.*, 2012).

Determining the Molecular Weight of γ -PGA

The effects of the following physicochemical factors on the molecular weight of γ -PGA were studied by varying one factor at a time:

- a) Incubation temperature (27 to 42°C)
- b) Initial moisture level of the fermentation medium (50-75%)
- c) Initial pH level of the fermentation medium (5 to 8)
- d) Fermentation time (12 to 96 hours)

The average molecular weights of the γ -PGA product obtained under these specified environmental conditions were determined using Gel Permeation Chromatography (GPC). The mobile phase consisted of 50 mM phosphate buffer at pH 6.8, with a flow rate of 1.0 mL min⁻¹. Molecular weights were calculated relative to polystyrene standards.

Studying the Impact of γ -PGA on Strawberry Resistance to *B. cinerea* Fungus

The effect of γ -PGA on the resistance of strawberry plants to *B. cinerea* was evaluated under greenhouse conditions. For this research, all fermented culture media containing γ -PGA in three different molecular weights (High: 1156.43 kDa, Medium: 734.38 kDa, and Low: 296.55 kDa) were selected for experimentation. These media were dried in an oven at 70°C for 3 days and then crushed using a mill. The resulting powders were mixed with a combination of perlite and cocopeat in a 50:50 ratio to serve as the substrate for strawberry cultivation.

The concentrations of γ -PGA investigated were 10, 20, 50, and 100 mg kg⁻¹ of the cultivation bed. Each treatment involved

five 1.5-liter pots, with two strawberry cv. 'Camarosa' seedlings planted in each pot, which were irrigated daily with 100 cc of drinking water. The light-dark photoperiod was set to 8 hours of light and 16 hours of darkness. At the end of the flowering stage, the plants were inoculated with a *B. cinerea* fungus suspension containing 10⁶ spores per mL. The control treatment (Sh0) was inoculated with water.

Treatments

The treatments consisted of two control groups and γ -PGA groups:

a) Control treatment group:

- Sh0: Control treatment without fermentation substrate, with or without γ -PGA, and without contamination by *B. cinerea* fungus.
- Sh-: Control treatment without fermentation substrate, with or without γ -PGA, and with contamination by *B. cinerea* fungus.
- Sh 10, 20, 50, and 100 mg: In this investigation, the entire solid-state fermentation substrate was added to the pots. To assess the impact of compounds produced by bacteria other than γ -PGA, the fermentation substrate without the γ -PGA compound was added to the culture medium at concentrations of 10, 20, 50, and 100 mg/kg of soil. This group of control treatments was inoculated with the *B. cinerea* fungus.

b) γ -PGA treatment group: The treatments consisted of fermentation substrate containing γ -PGA with molecular weights of 296.55, 734.38, and 1156.43 kDa. Each of these molecular weights was investigated at four concentrations: 10, 20, 50, and 100 mg kg⁻¹ of soil. The γ -PGA group treatments were inoculated with the fungus *B. cinerea*.



Ascorbate Peroxidase (APX) Enzyme Activity Assay

Sampling of strawberry leaves for the investigation of biochemical characteristics was conducted immediately before inoculation and on days 3, 7, 10, 20, and 30 post-inoculations, coinciding with the first fruit harvest. The samples were promptly transferred to a -80 freezer for storage until examination. The activity of the APX enzyme was determined using the method described by Braga *et al.* (2009).

Phenylalanine Ammonia Lyase (PAL) Enzyme Activity Assay

The activity of phenylalanine ammonia-lyase was assessed by converting L-phenylalanine into trans-cinnamic acid following the protocol outlined by Tovar *et al.* (2002). PAL enzymatic activity was reported as units per gram of fresh leaf weight.

Assay of Ellagic Acid (EA)

The concentration of ellagic acid was measured using the spectrophotometric method established by Wilson and Hagerman (1990).

Measurement of Physicochemical Characteristics of Fruits in Storage Conditions

To determine the effectiveness of γ -PGA on the shelf life and quality of fruits in storage, physicochemical tests were conducted. These tests included assessing weight loss, firmness, Total Soluble Solids (TSSs), and Titratable Acidity (TA) using established methods (Farida *et al.*, 2023). Forty fruits were harvested from each treatment and stored in a cold room at a temperature of 4°C and a humidity of 90%

for 10 days. The samples were analyzed on days 0, 3, 6, and 10.

Apparent Decay of Fruits

To assess decay, 20 fruits from each treatment were randomly selected, and the average decay for each treatment was calculated using the numerical scale defined by Babalar *et al.* (2007). Fungal decay was evaluated through visual examination and microscopic observation of fungal mycelium growth. The numerical scale ranged from 5 (indicating no decay) to 1 (indicating more than 16% decay), with intermediate values representing different levels of decay: 4 (less than 5%), 3 (6-10%), and 2 (11-15%) (Babalar *et al.*, 2007).

Statistical Analysis

This study utilized a completely randomized design. The data were analyzed using SAS 9.1.3 statistical software (2001). Duncan's multiple range test was employed at the 5% significance level to compare the means. The experiments were conducted in triplicate.

RESULTS AND DISCUSSION

γ -PGA Production by SSF and Its Identification

Substrate of SSF

Studies have shown that nitrogen and carbon sources, particularly sugars, play a crucial role in γ -PGA production (Sung *et al.*, 2005). Therefore, the optimal ratio between sesame flour that served as a nitrogen source, and banana peel, which acted as a sugar source, was determined. In all the examined samples, the amounts of wheat straw and manure in the substrate were kept constant at 150 and 50 g, respectively (Tables 1 and 2). Subsequently,

Table 1. Main composition in selected substances for solid-state fermentation.

The main composition	Minerals	Carbohydrate	Carbon source (sugar)	Protein
Substances	Cow manure	Wheat straw	Banana peel	Sesame flour
Amount	34 %	42.56 g 100 g ⁻¹	39.8 g 100 g ⁻¹	45.8 g 100 g ⁻¹

Table 2. The optimal ratio of sesame flour to banana peel (g).

Sample	Sesame flour	Banana peel	Kg of the	The average production of γ -PGA per dry weight of the substrate
1	200	600		55.75 ^d
2	300	500		61.5 ^c
3	400	400		65 ^b
4	500	300		68.33 ^a
5	600	200		69.16 ^a
6	700	100		60.2 ^c

Table 3. The optimal ratio of wheat straw to manure and between sesame flour and banana peel (g).^a

Sample	Sesame flour	Banana peel	Wheat straw	Manure	The average production of γ -PGA per Kg of the dry weight of the substrate
1	600	200	180	20	62.5 ^c
2	600	200	170	30	62.5 ^c
3	600	200	160	40	70 ^a
4	600	200	150	50	69 ^b
5	600	200	140	60	52.5 ^e
6	600	200	100	100	33.25 ^g
7	600	200	160	40	67.5 ^b
8	400	200	380	20	54 ^c
9	400	200	360	40	57.5 ^d
10	400	200	340	60	56.75 ^d
11	400	200	320	80	42.5 ^f

^a Different letters indicate significant differences between production values of γ -PGA ($P < 0.05$).

based on this ratio, the proportion of cow manure to wheat straw was calculated. To determine the optimal ratio, various combinations were examined (Table 3). The most effective composition of the fermentation substrate comprised (g kg⁻¹ dry weight of substrate): 600g sesame flour, 200g banana peels, 160g wheat straw, and 40g cow manure, resulting in a γ -PGA production rate of 70 g kg⁻¹ Dry Weight (DW) of the substrate.

Identification and Characterization of γ -PGA

Various analytical tools were employed to identify and characterize the γ -PGA produced by *B. velezensis* UTB96 through SSF. As shown in Figure 1-a, the Fourier-Transform Infrared (FT-IR) spectrum of γ -PGA displayed amide bands at 1,648.6 cm⁻¹ and carbonyl group C=O at 1,402.43 cm⁻¹. Additionally, it exhibited C-N stretching vibrations at 1075.76 cm⁻¹ and an O-H stretching band at 3447.26 cm⁻¹.

The chemical structure of the γ -PGA obtained in this study was consistent with the structure elucidated by Ho *et al.* (2006)



and Khalil *et al.* (2018). Notably, based on previous studies (Rajan *et al.*, 2014) and the absorption bands of amide groups, the secondary structure of γ -PGA was identified as an α -helix motif.

Figure 1-b displays the UV absorption spectrum of γ -PGA in deionized water, covering the range of 190–340 nm. According to the orbital law, peptide excitation absorption peak, resulting from the presence of carbonyl and amide groups, typically occurs around 200 nm (Braga *et al.*, 2009). This aligns with our study's findings that the γ -PGA biopolymer exhibited maximum absorption at 200 nm (216 nm).

Studies have demonstrated the effectiveness of methylene blue staining as a qualitative method for measuring γ -PGA production (Yu *et al.*, 2016). In this investigation, the production of γ -PGA by *B. velezensis* UTB96 was successfully confirmed using methylene blue staining, as shown in Figure 1- c.

The mechanism by which the cationic dye methylene blue is absorbed by γ -PGA is linked to the active sites (COO^-) present on the surface of the polymer. The absorption of methylene blue occurs spontaneously (Ogata *et al.*, 2017). The increases of the

concentration or molecular weight of γ -PGA is indicating on a longer peptide chain and increasing the number of active sites (COO^-). This increase in active sites enhances dye absorption, as demonstrated by the results.

Poly- γ - Glutamic Acid Molecular Weight

Microbial production of γ -PGA can result in molecular weights ranging from 100 to over 2,000 kDa. Several factors influence the efficiency and molecular weight of γ -PGA (Sung *et al.*, 2005). To investigate the effect of temperature on the molecular weight of γ -PGA, five experiments were conducted at varying temperatures. As shown in Figure 2-a, the maximum γ -PGA molecular weight (1117.53 kDa) was observed at 37°C, with similar values around 1112.9 kDa at 32°C. A decrease in the molecular weight was noted at 42°C, although this change was not significant compared to the 37°C. This reduction may be attributed to rapid cell growth at 42°C during the initial stages, which depletes nutrients in the medium and leads to the utilization of γ -PGA as a nitrogen and carbon source for bacterial cells in the later

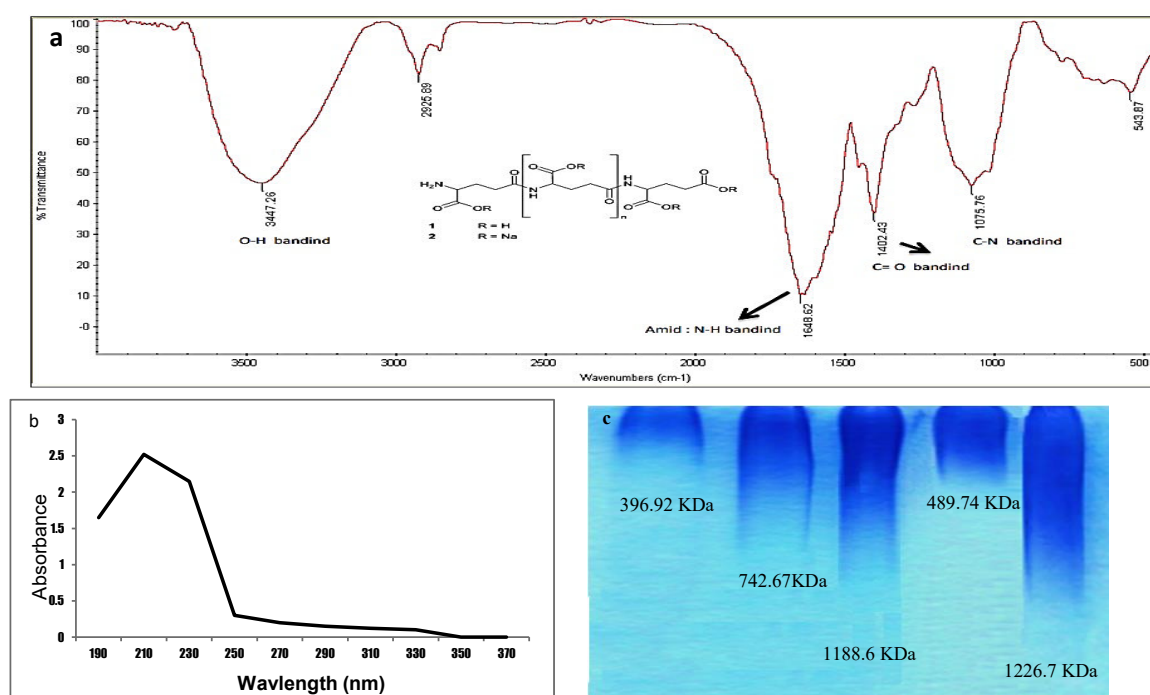


Figure 1. γ -PGA Identification, a: FT-IR absorption peaks (cm^{-1}), b: UV spectral analysis, c: SDS-PAGE for methylene blue stained samples containing purified γ -PGA.

stages of fermentation. Additionally, temperature can influence the activity of enzymes involved in synthesizing the γ -PGA amino acid chain, potentially affecting the molecular weight (Ajayeoba *et al.*, 2019).

The effect of moisture on the molecular weight of γ -PGA is illustrated in Figure 2-b. There was a direct relationship between humidity and the molecular weight of γ -PGA; as humidity increased to 65%, the molecular weight of γ -PGA also rose. However, a subsequent decrease in molecular weight was observed beyond this point. Moisture plays a vital role in oxygen and mass transfer during Solid-State Fermentation (SSF). Reports indicate that the activity of the pgsBCA enzyme complex is ATP-dependent, with ATP levels being influenced by oxygen availability. Therefore, when media humidity is at its optimal level, both enzyme complex activity and the molecular weight of γ -PGA are expected to increase (Sung *et al.*, 2005).

molecular weight between pH values from 6.5 to 8. Under acidic conditions, the carboxylic acid groups do not ionize, causing the γ -PGA structure to adopt an α -helical conformation, which results in decreased stability and decomposition of the compound (Seo *et al.*, 2008), leading to a reduction in molecular weight. In contrast, at higher pH levels, the ionic hydration of γ -PGA induces a conformational shift from α -helix to random coil (Seo *et al.*, 2008), thereby increasing the compound's molecular weight.

At 96 hours of fermentation, the molecular weight of γ -PGA increased significantly between 12 and 36 hours. However, after 48 hours, a downward trend in molecular weight was observed. By the end of the 96-hour fermentation period, the molecular weight of γ -PGA showed a 46% decline compared to the 36-hour fermentation period (Figure 2-d). When γ -PGA consists of longer monomeric chains,

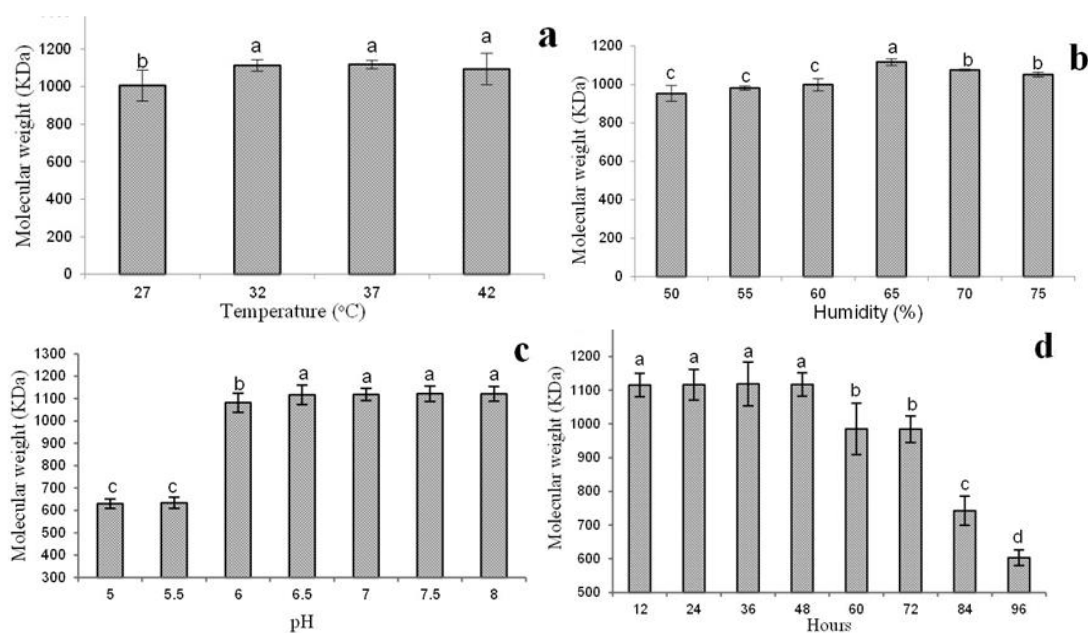


Figure 2. Effect of different factors on γ -PGA molecular weight: (a) Temperature, (b) Initial moisture, (c) Initial pH, and (d) Incubation time. Different letters in each figure indicate significant differences between the states of the investigated factor ($P < 0.05$).

In Figure 2-c, it is shown that as the pH increased from 5 to 8, the molecular weight of γ -PGA rose from 630 kDa to 1121.4 kDa. There was no significant difference in

its molecular weight is higher. Therefore, extending the fermentation time up to 36 hours provides the polymerase enzyme systems with more time to polymerize

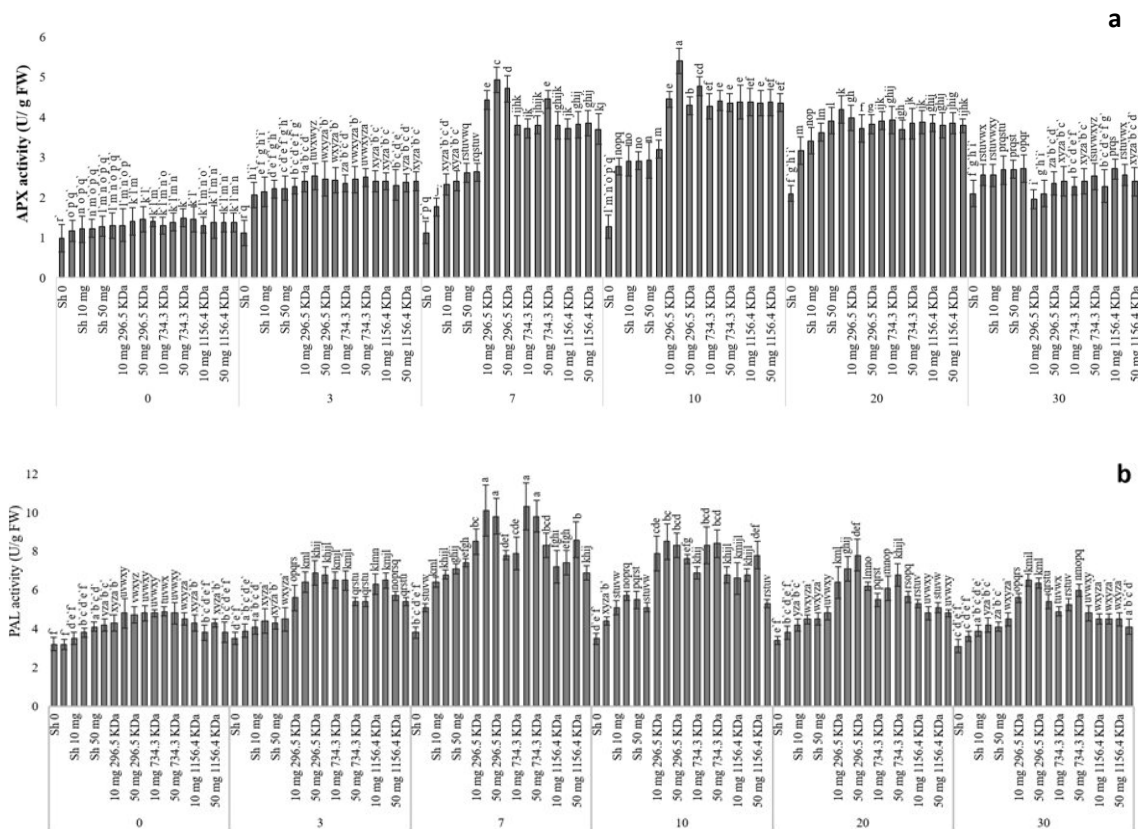


monomers, increasing the molecular weight of γ -PGA. Prolonging fermentation beyond 36 hours can create adverse conditions such as nutrient scarcity, oxygen depletion, and low humidity levels, which subsequently reduce the activity of the γ -PGA polymerase enzyme. In response, bacteria may hydrolyze γ -PGA as a source of carbon and nitrogen to ensure their survival (Cao et al., 2018), leading to a decrease in the molecular weight of γ -PGA.

γ -PGA Effect on Strawberry Plant Resistance to Gray Mold Disease

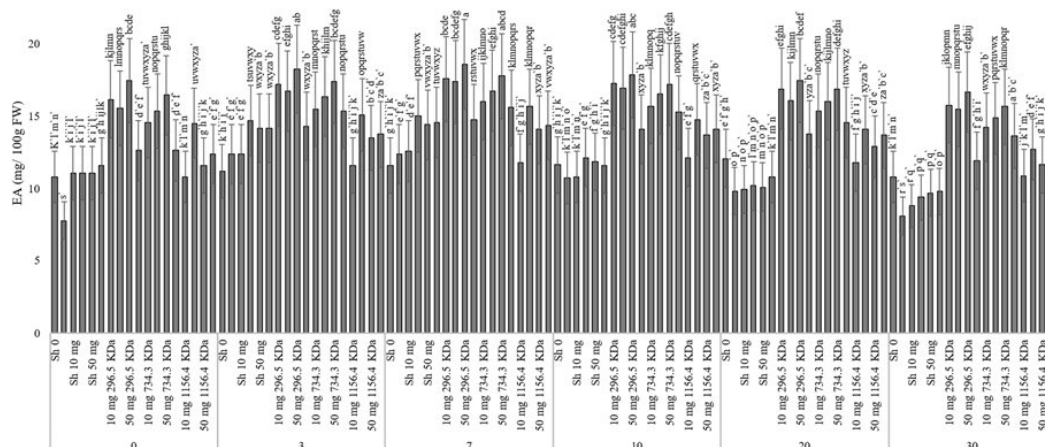
Ascorbate Peroxidase (APX) Enzyme Assay

The activity of the APX enzyme in the leaves of plants treated with γ -PGA showed an increasing trend until the tenth day. Notably, the increase in enzyme activity was significantly higher in the γ -PGA treatments compared to the infected control (Sh-). On the tenth day, the highest enzyme activity was recorded in the treatment of 20 mg



* The order of the columns in the days examined is: Sh0, Sh-, Sh10, Sh20, Sh50, Sh100, 10 mg 296.5 kDa, 20 mg 296.5 kDa, 50 mg 296.5 kDa, 100 mg 296.5 kDa, 10 mg 734.3 kDa, 20 mg 734.3 kDa, 50 mg 734.3 kDa, 100 mg 734.3 kDa, 10 mg 1156.4 kDa, 20 mg 1156.4 kDa, 50 mg 1156.4 kDa and 100 mg 1156.4 kDa.

Figure 3. The effect of γ -PGA on the activity of enzymes involved in strawberry plant defense: (a) Ascorbate Peroxidase (APX), and (b) Phenylalanine Ammonia-Lyase (PAL).



*The order of the columns in the days examined is: Sh0, Sh-, Sh10, Sh20, Sh50, Sh100, 10 mg 296.5 kDa, 20 mg 296.5 kDa, 50 mg 296.5 kDa, 100 mg 296.5 kDa, 10 mg 734.3 kDa, 20 mg 734.3 kDa, 50 mg 734.3 kDa, 100 mg 734.3 kDa, 10 mg 1156.4 kDa, 20 mg 1156.4 kDa, 50 mg 1156.4 kDa and 100 mg 1156.4 kDa. **Figure 4.** γ -PGA effect on the activity of Ellagic Acid (EA) in strawberry leaves inoculated with *B. cinerea*.

296.55 kDa, which was 50 times higher than that of the negative control (Figure 3-a). After the tenth day, the activity of the ascorbate peroxidase enzyme began to decline. In contrast, the control treatments continued to show an increase in enzyme activity until the twentieth day (Figure 3-a). This continued increase in the control could be attributed to the emergence of new infections or the spread of the fungal pathogen *B. cinerea* within the plant tissues.

APX is a key enzyme that converts ascorbate to dehydroascorbate, effectively removing peroxides, particularly H_2O_2 , from plant cells (Navari-Izzo *et al.*, 1997). Based on the results obtained, it can be concluded that γ -PGA positively influences the plant's antioxidant system, facilitating the metabolism of Reactive Oxygen Species (ROS) and preventing the penetration and spread of the necrotrophic fungus *B. cinerea* in strawberry plants.

Phenylalanine Ammonia Lyase (PAL) Enzyme Assay

The application of γ -PGA significantly increased the activity of the PAL enzyme

from the third to the seventh day following the inoculation of *B. cinerea* in the γ -PGA treatments compared to the control groups. The highest increases in PAL activity were observed in the treatments with 20 and 50 mg of 296.55 kDa, as well as 50 and 20 mg of 734.38 kDa. In contrast, the uninfected (Sh0) and negative (Sh-) control groups exhibited the lowest levels of phenylalanine ammonia-lyase activity (Figure 3-b). PAL plays a crucial role in the biosynthesis of polyphenolic compounds, including flavonoids, phenylpropanoids, and lignin in plants. Research suggests that γ -PGA can enhance the activity of the PAL enzyme by activating the ROS signaling pathway, leading to increased enzyme activity (Lei *et al.*, 2015). Furthermore, studies have indicated that stimulating the phenylpropanoid pathway to produce polyphenolic and flavonoid compounds can help neutralize and prevent excessive ROS production caused by *B. cinerea* infection (Kumar *et al.*, 2020; Perkowski and Warpeha., 2019).

Impact of γ -PGA on the Ellagic Acid (EA) Content in Leaves

The level of ellagic acid in strawberry leaves continued to rise until the seventh



day, with the greatest increase observed in the treatment with 50 mg of 296.55 kDa (18.6 mg 100 g⁻¹ FW). Subsequently, a significant decline in ellagic acid was noted in the control treatments, while in the γ -PGA treatments this decline exhibited a more gradual trend (Figure 4).

Within plant cells, ellagic acid exists in both free and covalently-bound forms, such as EA glycosides and ellagitannins. Although the level of free ellagic acid in cells is initially low, it tends to increase during biotic and abiotic stresses. Ellagic acid has demonstrated efficiency in absorbing free radicals (Williams *et al.*, 2014). Therefore, the rise in ellagic acid levels during the compatible interaction between *B. cinerea* and strawberries may indicate its direct involvement in protecting plant cells through the modulation of the redox balance. Additionally, this compound contributes to defence against fungal pathogens by damaging ergosterol (Lei *et al.*, 2015). As a result, ellagic acid leads to pathogen death by disrupting ergosterol and compromising the integrity of the fungal

membrane.

Measurement of Physicochemical Characteristics of Fruits in Storage Conditions

As storage time increased, fruits exhibited a decrease in weight, firmness, and titratable acidity. Notably, the control treatments showed the most significant reduction compared to the γ -PGA treatments (Table 4). The lowest reduction was observed in the treatments with 50 mg and 20 mg of 296.55 kDa, as well as 50 mg of 734.38 kDa.

In terms of TSS analysis, a gradual increase was noted across all treatments throughout the storage period. The smallest increase was recorded in the treatment with 50 mg of 296.55 kDa, which was 27.4% lower than the Sh- treatment.

Physicochemical changes during fruit storage are primarily caused by respiration, water loss, the consumption and breakdown of stored compounds and metabolites during metabolic activities, and the activity of degrading enzymes (Fawole *et al.*, 2020).

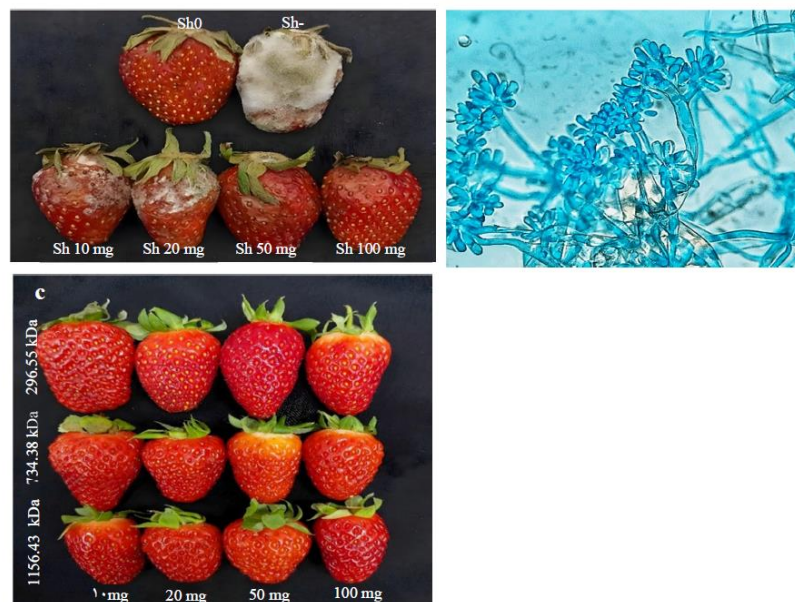


Figure 5. Examination of the apparent decay in strawberry fruits after 10 days of storage at 4°C and 90% humidity. (a) Control treatments, (b) Microscopic examination of fungal mycelium grown on the fruits of control treatments, and (c) γ -PGA treatments.

Table 4. The impact of different treatments on the physicochemical properties of strawberry fruits after 10 days of cold storage.^a

Indicator Treatment	Weight loss (%)	TA (%)	TSS (%)	Firmness (kg m ⁻²)	Apparent decay
Sh 0	5.3 ^b	0.62 ^{cdefg}	8.3 ^a	0.64 ^g	3.11 ^b
Sh-	5.9 ^a	0.47 ^g	8.4 ^a	0.6 ^g	1.04 ^c
Sh 10 mg	4.5 ^c	0.5 ^{fg}	8 ^{ab}	0.62 ^g	1.95 ^d
Sh 20 mg	4.28 ^{cd}	0.53 ^{efg}	7.9 ^{ab}	0.73 ^{fg}	1.95 ^d
Sh 50 mg	4.2 ^{cd}	0.58 ^{defg}	7.6 ^{bc}	0.81 ^{ef}	2.83 ^c
Sh 100 mg	4.12 ^d	0.6 ^{defg}	7.3 ^{cd}	0.84 ^{def}	2.52 ^c
10 mg 296.5 kDa	2.9 ^{hi}	0.72 ^{bcd}	6.7 ^{ef}	0.95 ^{cde}	4.79 ^a
20 mg 296.5 kDa	1.6 ^{kl}	1.15 ^a	6.5 ^{fg}	1.14 ^{ab}	4.79 ^a
50 mg 296.5 kDa	1.5 ^l	0.8 ^b	6.1 ^g	0.9 ^{de}	4.79 ^a
100 mg 296.5 kDa	2.3 ^j	0.72 ^{bcd}	6.7 ^{ef}	0.98 ^{cd}	4.79 ^a
10 mg 734.3 kDa	2.9 ^{hi}	0.69 ^{cde}	7 ^{def}	0.88 ^{de}	4.79 ^a
20 mg 734.3 kDa	3.06 ^{gh}	0.74 ^{bcd}	6.5 ^{fg}	0.83 ^{ef}	4.79 ^a
50 mg 734.3 kDa	1.9 ^k	0.7 ^{dc}	6.5 ^{fg}	1.2 ^a	4.79 ^a
100 mg 734.3 kDa	3.23 ^{fg}	0.64 ^{cdef}	6.8 ^{def}	0.91 ^{cde}	4.79 ^a
10 mg 1156.4 kDa	2.7 ⁱ	0.77 ^{bc}	6.6 ^{ef}	1.05 ^{bc}	4.79 ^a
20 mg 1156.4kDa	2.8 ^{hi}	0.68 ^{cde}	7.1 ^{cde}	0.9 ^{de}	4.79 ^a
50 mg 1156.4 kDa	3.6 ^e	0.66 ^{cdef}	6.9 ^{def}	0.92 ^{cde}	4.79 ^a
100 mg 1156.4 kDa	3.5 ^{ef}	0.64 ^{cdef}	7.2 ^{cde}	0.98 ^{cd}	4.79 ^a

^a Different letters in columns show significant differences (P<0.001).

Research has shown that γ -PGA operates through various mechanisms, including reducing the activity of cell wall degrading enzymes (Wang *et al.*, 2020), inhibiting abscisic acid signal transmission (Shan *et al.*, 2023), and enhancing the antioxidant capacity in fruits. These actions collectively contribute to maintaining cell integrity, preventing water loss, reducing decay, and delaying aging in fruits. As a result, γ -PGA plays a crucial role in preserving the quality of fruits during storage.

Apparent Decay of Fruits

At the end of ten days, no symptoms of fungal infection were observed in the γ -PGA treatments, while the fruits in the control treatments exhibited moderate to severe rotting (Table 4). As storage time increases, the texture of strawberries softens due to changes in the cell wall structure (Brummell *et al.*, 1999). The antioxidant activity of γ -PGA and its ability to maintain the integrity of the cell wall, helps prevent rapid deterioration of the fruit tissue and maintains

its hardness. This, in turn, inhibits the penetration and spread of fungi into the fruit tissue.

CONCLUSIONS

The findings of this research demonstrate that it is possible to produce γ -PGA compounds in significant quantities with diverse molecular weights through solid-state fermentation and manipulation of fermentation conditions. The results indicate that γ -PGA, particularly at low to medium molecular weights and concentrations, could enhance plant defense against necrotrophic pathogens, such as *B. cinerea*, by activating antioxidant mechanisms and boosting the generation of defense compounds. This enhancement could also prolong the storage life of strawberry fruit. The outcomes of this study present a promising avenue for researchers and experts in agriculture to further investigate the potential applications of γ -PGA in various fields, including biological control.



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تولید پلی- γ -گلوتامات به روش فرمانتاسیون بستر جامد و کاربرد آن در کنترل زیستی

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چکیده

پلی- گاما- گلوتامیک اسید (γ -PGA) یک پلیمر طبیعی با کاربردهای متنوع در صنایع مختلف است. با این حال، استفاده از آن در کشاورزی به دلیل هزینه‌های بالای تولید محدود است. اهداف این مطالعه: تولید بهینه و مقرون به صرفه γ -PGA از طریق تخمیر حالت جامد (SSF) با استفاده از باکتری *Bacillus velezensis* UTB96، تعیین غلظت و وزن مولکولی مناسب γ -PGA برای کاربردهای کشاورزی، به‌ویژه در کشت توت فرنگی، و در نهایت بررسی تأثیر γ -PGA تولید شده بر افزایش عمر مفید میوه‌های توت فرنگی در طول انبارمانی است. ابتدا تولید γ -PGA با استفاده از SSF و باکتری *Bacillus velezensis* UTB96 مورد بررسی قرار گرفت و تأثیر عوامل فیزیوشیمیایی بر وزن مولکولی γ -PGA ارزیابی شد. بر اساس نتایج، سه وزن مولکولی مختلف γ -PGA شناسایی شد: 1156.43، 734.38 و 296.55 کیلوالتون. این وزن‌ها برای آزمایش‌های گلخانه‌ای به منظور ارزیابی اثربخشی آن‌ها در کنترل کپک خاکستری روی گیاه توت فرنگی انتخاب شدند. نتایج نشان داد که با استفاده از ضایعات کشاورزی، از جمله کنجاله کنجد، کاه گندم و پوست موز در روش SSF، می‌توان γ -PGA را با نرخ 70 گرم در کیلوگرم وزن خشک محیط کشت تولید کرد. تحلیل تأثیر γ -PGA بر کاهش بیماری کپک خاکستری نشان داد که این ترکیب می‌تواند مقاومت گیاه را بهبود بخشد. افزایش قابل توجهی در فعالیت آنزیم‌های آسکوربات پراکسیداز و فنیل‌آلانین آمونیا لیاز (PAL) همراه با تولید ترکیبات پلی‌فنولی مانند اسید الازیک مشاهده شد. در نتیجه، این مکانیسم‌ها انعطاف‌پذیری و تحمل گیاه را در برابر قارچ بهبود بخشیدند و منجر به حفظ کیفیت میوه‌ها در طول نگهداری در سردخانه شدند.

Role of Melatonin and Pomegranate Seed Oil in Maintaining the Quality of Mexican Limes during Storage

Mahbobeh Mohammadi¹, Somayeh Rastegar^{1*}, and Abbas Rohani²

ABSTRACT

Mexican limes have a limited shelf life due to color changes and reduced freshness. This study evaluated the effects of Melatonin (ML) and Pomegranate Seed Oil (PSO) on maintaining postharvest quality at 20±2°C and 50-60% relative humidity. The results showed that melatonin at 100 and 200 µM+PSO exhibited the highest overall fruit acceptability. The control group displayed the most weight loss (20.8%), while PSO demonstrated the least (12.5%). With the exception of the PSO treatment, all other treatments exhibited significantly elevated levels of phenols, flavonoids, and antioxidants. The PSO and control treatments demonstrated the lowest catalase (69 U mg⁻¹ FW) and peroxidase (53.5 U mg⁻¹ FW) activities, while the highest polyphenol oxidase activity (99.6 U mg⁻¹ FW) was also observed in these groups. The control and PSO treatments also exhibited the highest Total Soluble Solids (TSSs) content (8.2%) and the lowest acidity (8.5%). Overall, most traits exhibited significant differences between the treatment groups and the control, excluding the PSO treatment.

Keywords: Antioxidant, Citrus, Postharvest quality, Shelf life.

INTRODUCTION

Citrus aurantifolia, is a citrus cultivar extensively farmed in tropical zones (Romero-Romero *et al.*, 2020). Citrus are fruits valued for their color, taste, and nutrients – including sugars, acids, vitamins, fiber, polyphenols, and flavonoids. Storage alters lime quality, causing peel discoloration, weight loss, lowering fruit quality and market value (Chen *et al.*, 2019). This process negatively impacts fruit quality, leading to significant losses in nutritional value, taste, and overall sensory appeal. This ultimately results in a substantial amount of agricultural produce being discarded (Pott *et al.*, 2020). Despite the inevitability of fruit overripening, the development of sustainable and innovative postharvest technologies is crucial to delay

these changes across the entire supply chain for various fruits.

Melatonin is a tryptophan-derived substituted indolamine, being found in almost all living organisms. Melatonin, a bioactive compound, has recently become a focus of interest in agriculture for its role in regulating plant physiological processes (Arabia *et al.*, 2022). Melatonin plays a crucial role in preserving plant proteins, chlorophyll levels, and photosynthetic efficiency. This ultimately leads to improved postharvest quality in various horticultural products (Madebo *et al.*, 2022). Postharvest treatments are typically performed by immersing the fruit in melatonin prior to storage. In numerous fruits, postharvest melatonin dipping has been shown to delay ripening, enhance antioxidant systems, and maintain the quality of cherries (Wang *et al.*,

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2019a) and mangoes during both cold storage (Rastegar *et al.*, 2020) and ambient conditions (Liu *et al.*, 2020). In citrus fruits, dipping in 1.0 g L⁻¹ melatonin solution preserved freshness, ascorbic acid, and total soluble solids (Wang *et al.*, 2019b).

Pomegranate Seed Oil (PSO) is a recycled product obtained from the extraction of pomegranate seeds and its production is currently the preferred method for valorizing the non-edible part of pomegranate (Kaseke *et al.*, 2020; Gumus *et al.*, 2020). Furthermore, Pomegranate Seed Oil (PSO), which is rich in Conjugated Linoleic Acid (CLA) including punicic acid, exhibits potent antioxidant activity. Its application as a natural food additive offers the potential for extending fruit shelf life and valorizing agricultural waste (Cortez-Trejo *et al.*, 2021). The presence of minor secondary metabolites with beneficial effects on human health has encouraged the utilization of PSO as a pharmaceutical ingredient in food supplement formulations (Paul and Radhakrishnan, 2020). Teodosio *et al.* (2018b) examined the impact of *Chlorella sp.* and Pomegranate Seed Oil (PSO) coatings on extending the storage life of *Spondias tuberosa* fruits under refrigeration, finding that these treatments effectively delayed ripening, maintained fruit firmness, and preserved a vibrant green color compared to untreated controls.

Extending the shelf life of lime fruit is crucial for minimizing postharvest losses.

This research explores a novel approach by investigating the potential of melatonin to improve the postharvest quality and longevity of lime fruit. The purpose of this study was to investigate how melatonin and pomegranate oil, both individually and in combination, affect the quality and shelf life of Mexican lime fruits. These treatments may provide promising potential in developing sustainable methods for improving fruit quality and prolonging shelf life. This research will advance our understanding of fruit preservation and provide valuable solutions for the agricultural sector.

MATERIALS AND METHODS

Mexican lime samples were obtained from a commercial orchard in Rodan City, Hormozgan Province, Iran, at approximately 57° E and 27° N, at 185 meters above sea level. The fruits, collected at the mature green stage, were selected for uniformity and health, then, washed for one minute before testing.

Treatment of Fruits

This study employed six treatment groups, as detailed in Figure 1. The fruits were flooded in their designated treatment solutions at ambient temperature for 10 min.

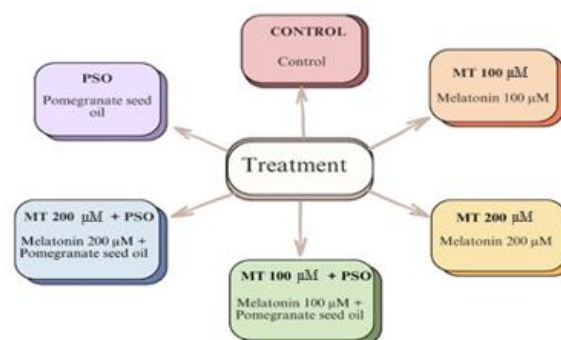


Figure 1. Experimental treatments and the corresponding abbreviations.

Following a 2-hour drying period, the fruits were individually placed in disposable plastic containers and stored. Each treatment comprised 3 replicates, each containing 9 fruits in separate, sealed containers. The containers were maintained at a consistent temperature of $20\pm 2^\circ\text{C}$ and relative humidity of 50-60% for 24 days.

Overall Visual Acceptability (OVA)

The study utilized a standardized subjective approach to evaluate the Overall Visual Acceptability (OVA) of the fruits, employing a detailed four-point scoring system derived from established criteria (Mohammadi *et al.*, 2024b) to comprehensively assess visual attributes and overall fruit conditions as detailed in Table 1.

Weight Loss

The weight of the fruits was measured using a digital scale with an accuracy of 0.01 g and the percentage of weight loss was calculated using Equation (1) (Dong and Wang, 2018):

$$WL = \frac{W_0 - W_f}{W_0} \times 100 \quad (1)$$

Where, WL represents is the Weight Loss percentage, W_0 is the initial Weight and W_f is the final Weight.

Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

Fruit juice samples were blended with

80% methanol to extract phenolic and flavonoid compounds. After centrifugation at $4,000\times g$, the supernatant was collected for further analysis.

TPC of the lime extracts was measured using a modified version of a method by Azizi *et al.* (2010). Its absorbance at 750 nm was measured using a spectrophotometer (Cecil 2501).

A method (Chang *et al.*, 2002) was used to quantify Total Flavonoid Content (TFC). The absorbance at 415 nm was measured using a UV-visible spectrophotometer.

Antioxidant Activity

The antioxidant potential of lime extracts was evaluated using the DPPH assay, following a modified method from Azlim Almey *et al.* (2010). The percentage of DPPH radical scavenging activity was computed utilizing Equation (2):

$$AA = \frac{A_c - A_s}{A_c} \quad (2)$$

Where, AA is the Antioxidant Activity percentage, A_c is the control and A_s is the sample.

Activities of Antioxidant Enzymes

Lime peel was homogenized in 20 mL of sodium phosphate buffer containing EDTA, Triton X-100, and PVPP, then, filtered and centrifuged. The supernatant was collected as the crude extract for antioxidant enzyme assays.

Catalase (CAT) enzyme activity was measured based on the decrease in hydrogen peroxide absorption at a wavelength of 240

Table 1. Visual acceptability scoring criteria.

Score	Quality description
4 (Excellent)	Fruits exhibit exceptional freshness, firmness, and overall quality. The peel is glossy and free of dehydration, shriveling, or discoloration.
3 (Good)	Fruits are marketable and meet acceptable quality standards despite minimal shriveling and softening.
2 (Not saleable but edible)	Fruits display moderate symptoms of shriveling, dryness, color loss (from green to orange), and ripening. Fruits remain edible but lack optimal aroma, taste, and color.
1 (Poor quality)	Fruits exhibit severe shriveling, darkened peel color, and signs of decay.



nm.

Peroxidase (POD) activity was measured by the conversion of guaiacol to tetraguaiacol. The reaction mixture included sodium phosphate buffer, guaiacol, H₂O₂, and crude enzyme extract, with absorbance at 470 nm recorded every 30 seconds for 2 min (Wang and Han 2009).

Polyphenol Oxidase (PPO) was extracted from 0.5 g of sample homogenized in phosphate buffer and centrifuged. The reaction mixture, including catechol and phosphate buffer, was incubated, and 60 µL of PPO was added. Absorbance at 420 nm was measured.

Total Soluble Solids (TSS) and Titratable Acidity (TA)

The TSS concentration in fruit juice was quantified by employing a digital refractometer (DBR 95) and was expressed as a percentage. TA was assessed through titration of samples using a 0.1 M sodium hydroxide solution until a pH of 8.2 was reached. The outcomes are expressed in terms of citric acid equivalents (Kumar *et al.*, 2021).

Fruit Color

The peel color of the fruits was objectively assessed using a Konica Chroma meter CR-400, measuring color characteristics such as Hue angle (H°), Chromaticity (C), and Luminosity (L*), based on the CIE Lab system. While a* and b* values vary from negative to positive and negative to positive, respectively, L* values indicate brightness (Fawole *et al.*, 2020).

Statistical Analysis

The study utilized a Completely Randomized Design (CRD) within a factorial experimental framework. Statistical analysis was performed using Least

Significant Difference (LSD) tests at $P \leq 0.05$ with SAS software. It should be noted that the nonparametric Kruskal-Wallis test was used for the overall visual acceptance trait. Principal component analysis (PCA) was conducted using the XLSTAT program. For Pearson correlation analysis, the tool available at <https://www.bioinformatics.com.cn/en> was employed.

RESULTS

Overall Visual Acceptability (OVA)

Figures 2-a and -b illustrate that both the control and PSO-treated fruits experienced color changes and quality decline after 24 days. In contrast, fruits treated with a combination of Melatonin (ML) and PSO retained better quality and freshness, with minimal color changes. Among the treatments, the combination of different melatonin concentrations with PSO achieved the highest marketability.

Weight Loss

The proportion of weight loss in treated lime fruit was much lower than that of the control group, according to a comparison of weight loss in stored lime fruit (Figure 3). The control group exhibited the maximum weight loss at 20.8%, while the minimum weight loss was observed in the PSO treatment at 12.5%.

TPC, TFC, and Antioxidant Capacity

A general decline in TPC was observed across most treatments during storage, indicative of phenolic degradation. The control and PSO-treated samples exhibited the most pronounced decrease in phenolic levels. In contrast, samples treated with 100 µM ML+PSO demonstrated the highest retention of phenolic content, followed by -

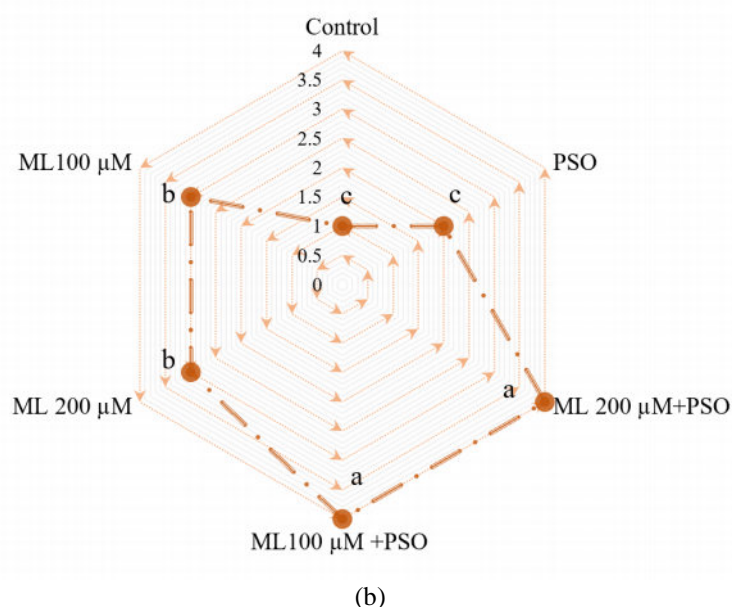
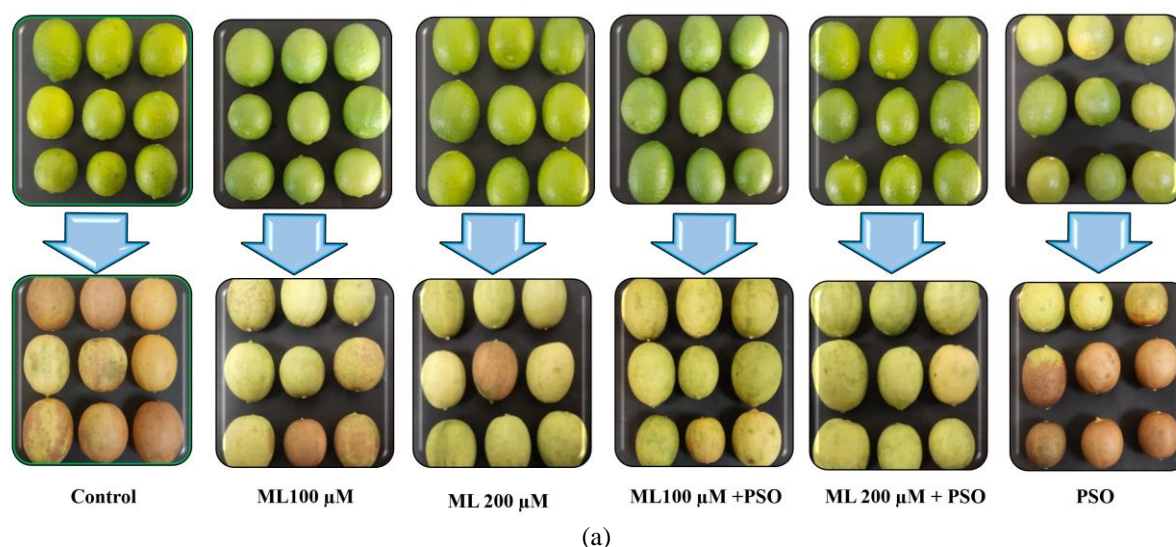


Figure 2. The effect of treatments (Distilled water, 100 μM ML: 100 μM Melatonin, 200 μM ML: 200 μM Melatonin, 100 μM ML+PSO: 100 μM Melatonin with Pomegranate Seed Oil (PSO), 200 μM ML+PSO: 200 μM Melatonin with PSO) on overall visual acceptability of Mexican lime fruit stored for 24 days at $20\pm 2^\circ\text{C}$ and 50-60% RH.

those treated with 100 μM ML (Figure 4-a). Figure 4-b shows the total flavonoid content of the fruit after storage. Fruits treated with 200 and 100 μM ML did not show a notable decrease in flavonoids. On the other hand, the total flavonoid content of the PSO-treated and the control samples significantly decreased throughout storage. Notably, the control sample had the lowest flavonoid content, at 0.57 mg g^{-1} .

Analysis of the results showed that the antioxidant capacity of all samples decreased during storage. This decrease was significantly more pronounced in the control and PSO-treated groups compared to the other treatment groups. In contrast, samples treated with melatonin alone or in combination with PSO showed superior antioxidant capacity retention, with no significant difference observed between these treatments (Figure 4-c).

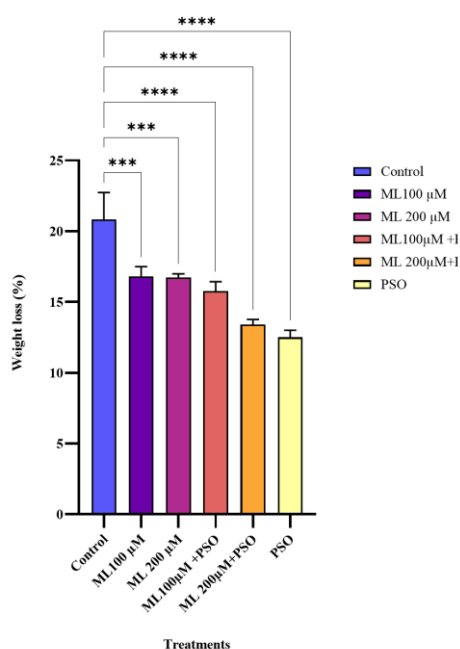


Figure 3. The effect of treatments (Distilled water, 100 µM ML: 100 µM Melatonin, 200 µM ML: 200 µM Melatonin, 100 µM ML+PSO: 100 µM Melatonin with pomegranate seed oil, 200 µM ML+PSO: 200 µM Melatonin with Pomegranate Seed Oil (PSO) on weight loss of Mexican lime fruit stored for 24 days at $20\pm 2^{\circ}\text{C}$ and 50-60% RH. Data represent mean values of $n=3$, and the error bars represent Standard Errors (SE) of the means. Statistical analysis was performed using an LSD test at $P\leq 0.05$ level.

The Activity of Antioxidant Enzymes

CAT

CAT activity remained constant in the control and PSO-treated samples throughout the storage period (Figure 5-a). Conversely, fruits treated with other combinations of ML and PSO exhibited a significant increase in CAT activity.

PPO

A significant increase in enzymatic activity was observed in both the control (99

$\text{U mg}^{-1}\text{ FW}$) and PSO-treated samples ($98 \text{ U mg}^{-1}\text{ FW}$) after 24 days of storage. In contrast, no significant differences in enzymatic activity were found among the other treatment groups when compared with the initial values (Figure 5-b).

POD

After a 24-day storage period, treated fruits showed a rise in POD activity. The highest levels of enzyme activity were shown by the 100 and 200 µM ML+PSO treatments. On the other hand, the control and PSO-treated groups showed a minor drop-in POD enzyme activity in comparison to the original values.

TSS and TA

Figure 6-a illustrates a gradual increase in TSS content across all samples during storage. The highest TSS concentrations were observed in fruits treated with PSO (8.16%) and in the control group (8.03%). Conversely, fruits treated with 100 µM ML exhibited the lowest TSS concentration.

A general decline in TA was observed across all samples during storage, as depicted in Figure 6-b. After 24 days of storage, the lowest TA values were observed in both the PSO-treated and the control groups. In contrast, fruits treated with 100 and 200 µM ML+PSO exhibited significantly higher acidity compared to the other treatments.

Color Parameters

During storage, color analysis of all samples consistently showed increasing L^* values, with the PSO treatment having the lowest L^* at the end, while the a^* index shifted from negative to positive. The highest a^* was found in PSO treatment. The b^* index gradually rose, with higher values in the ML 100 and 200 µM+PSO treatments

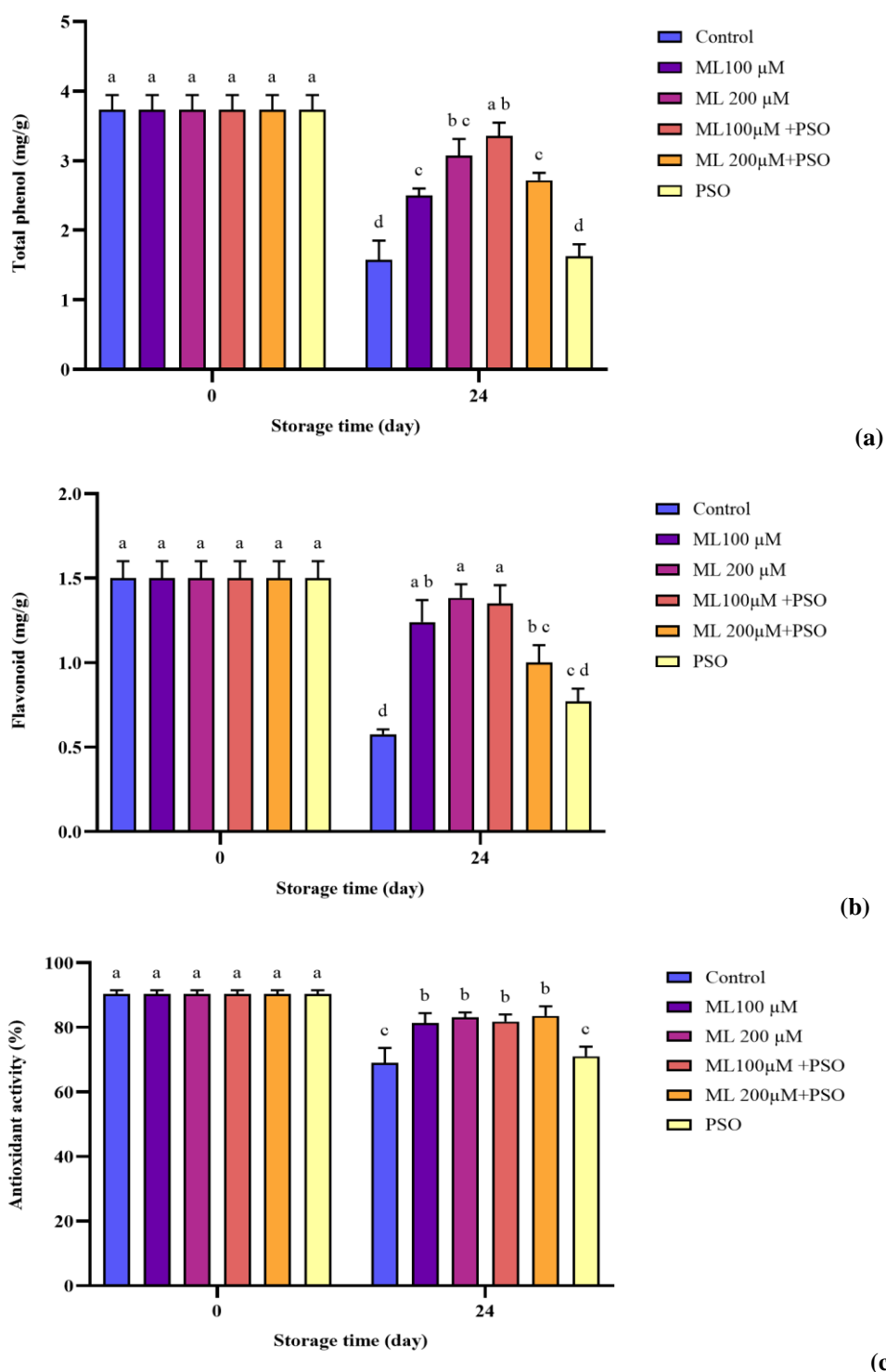
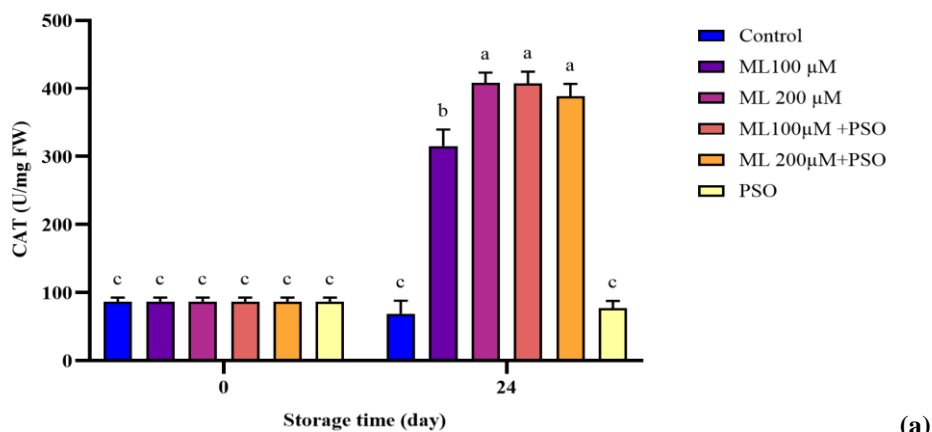
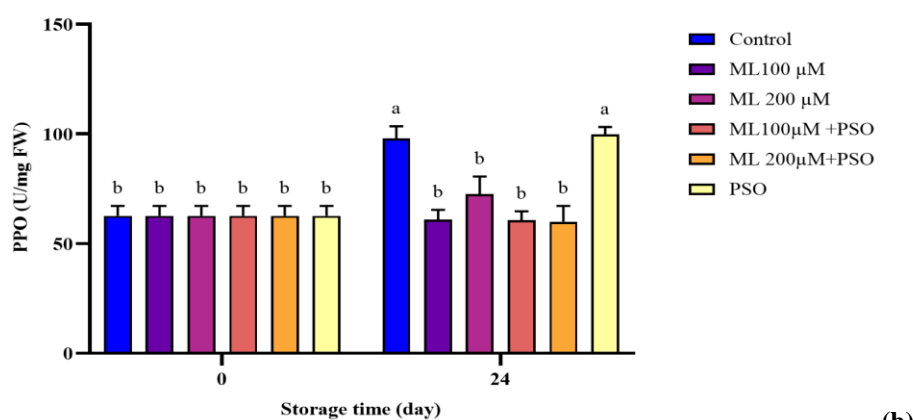


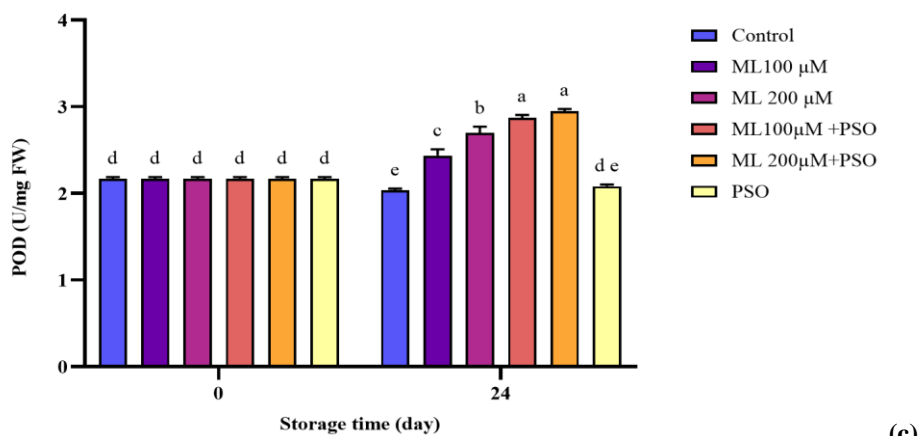
Figure 4. The effect of treatments (Distilled water, 100 μM ML: 100 μM Melatonin, 200 μM ML: 200 μM Melatonin, 100 μM ML+PSO: 100 μM Melatonin with pomegranate seed oil, 200 μM ML+PSO: 200 μM Melatonin with PSO) on: (a) Total phenols, (b) Flavonoids, and (c) Antioxidant activity of Mexican lime fruit stored for 24 days at 20±2°C and 50-60% RH. Data represent mean values of n= 3 and the error bars represent Standard Errors (SE) of the means. Statistical analysis was performed using the LSD test at P≤ 0.05 level.



(a)



(b)



(c)

Figure 5. The effect of treatments (Distilled water, 100 μ M ML: 100 μ M Melatonin, 200 μ M ML: 200 μ M Melatonin, 100 μ M ML+PSO: 100 μ M Melatonin with pomegranate seed oil, 200 μ M ML+PSO: 200 μ M Melatonin with PSO) on: (a) Catalase, (b) Peroxidase, and (c) Polyphenol oxidase of Mexican lime fruit stored for 24 days at $20\pm 2^\circ\text{C}$ and 50-60% RH. Data represent mean values of $n=3$ and the error bars represent Standard Errors (SE) of the means. Statistical analysis was performed using the LSD test at $P\leq 0.05$ level.

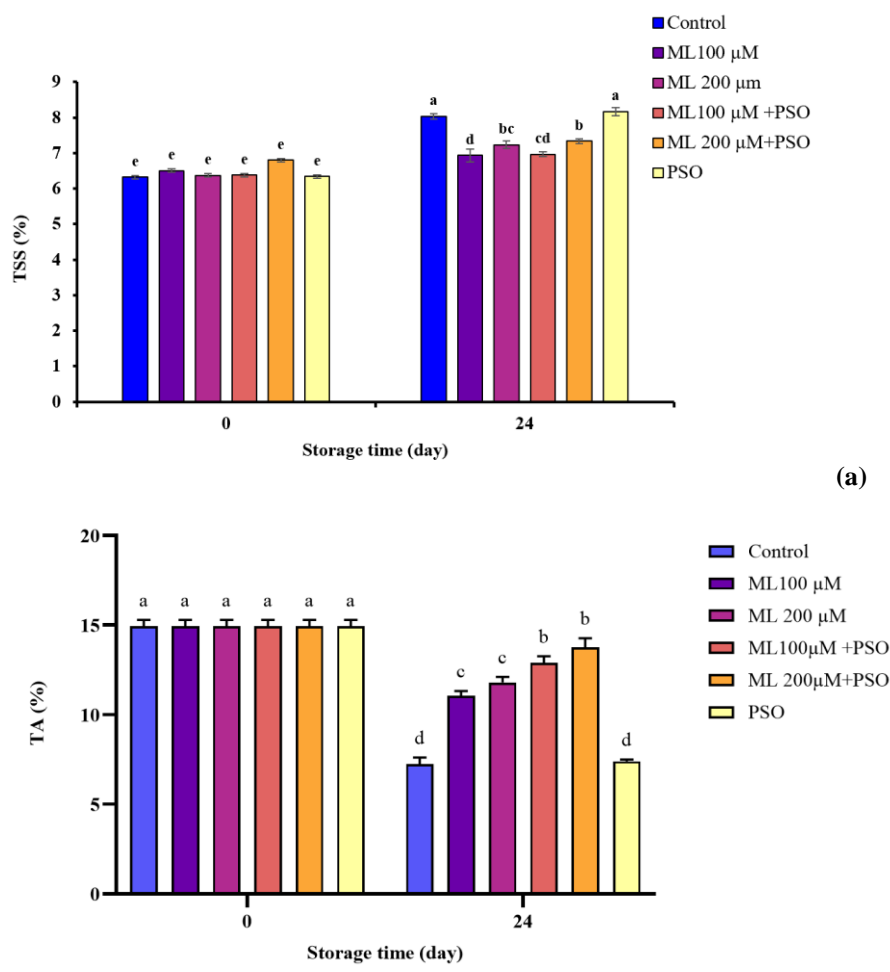


Figure 6. The effect of treatments (Distilled water, 100 μM ML: 100 μM Melatonin, 200 μM ML: 200 μM Melatonin, 100 μM ML+PSO: 100 μM Melatonin with Pomegranate Seed Oil (PSO), 200 μM ML+PSO: 200 μM Melatonin with PSO) on: (a) Total Soluble Solids (TSSs) and (b) Titratable Acidity (TA) of Mexican lime fruit stored for 24 days at 20±2°C and 50-60% RH. Data represent mean values of n= 3 and the error bars represent Standard Errors (SE) of the means. Statistical analysis was performed using the LSD test at P≤ 0.05 level.

and the lowest in PSO alone. The Hue angle decreased uniformly across all treatments, and chroma values slightly increased, with lower values observed in the PSO treatment. The Color Change Index (CCI) decreased throughout the experiment, with the control group exhibiting the lowest value at the end.

PCA and Pearson Correlation

The study employed Principal Component Analysis (PCA) to examine the relationships

between various parameters and treatments. F1 explained 58.8% of the variance, showing positive associations with total phenol, flavonoid, Titratable Acidity (TA), Overall Visual Color (OVC), and antioxidant activity, and negative correlations with weight loss, Total Soluble Solids (TSSs), and L* value. F2 accounted for 32.3% of the variance, with positive loadings for catalase (CAT), Peroxidase (POD), and pH, and negative loadings for Polyphenol Oxidase (PPO) and a* value. The PCA biplot revealed a strong association of PPO with PSO24 and control



treatments, while the ML (100 μ M)+PSO treatment was closely related to POD. Pearson correlation analysis showed a significant positive correlation between overall visual acceptability and phenol, flavonoid, antioxidant capacity, and TA, with a negative correlation to TSS and PPO.

Technique for Order Preference by Similarity to Ideal Solution (TOPSIS) computation

TOPSIS, a prominent Multi-Criteria Decision-Making (MCDM) approach, offers a more comprehensive assessment by simultaneously evaluating and ranking treatments based on their performance across all relevant factors. TOPSIS

effectively integrates multiple criteria into a single ranking system by considering both the advantages and disadvantages of each treatment. This method determines the distance of each treatment from ideal solutions, allowing for an evaluation of how closely it approaches optimal performance. This comprehensive analysis considers all relevant parameters, providing a more holistic understanding of the treatment options (Heidari *et al.*, 2022). As shown in Table 2. The study employed the TOPSIS technique to evaluate and rank various treatments based on multiple criteria. After 24 days of storage, the highest-ranked treatment for Mexican lime fruit was ML

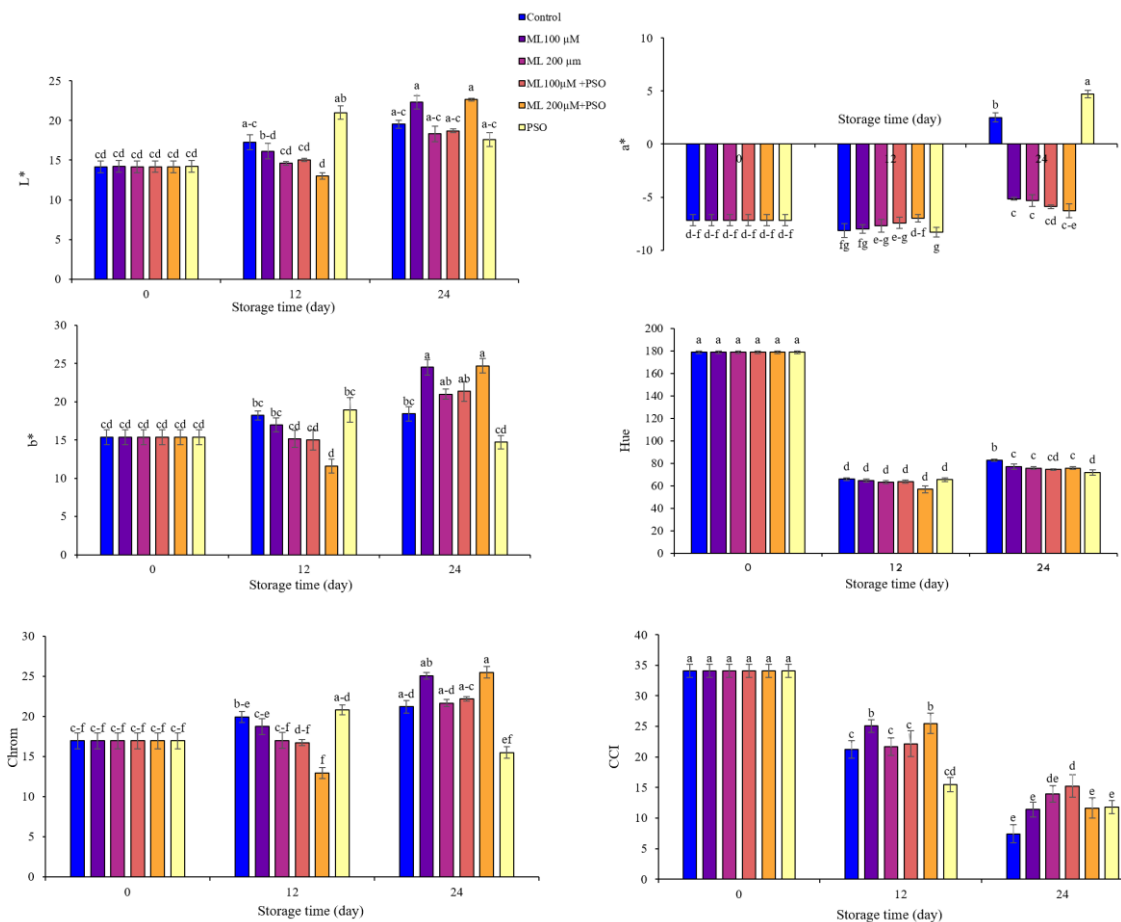
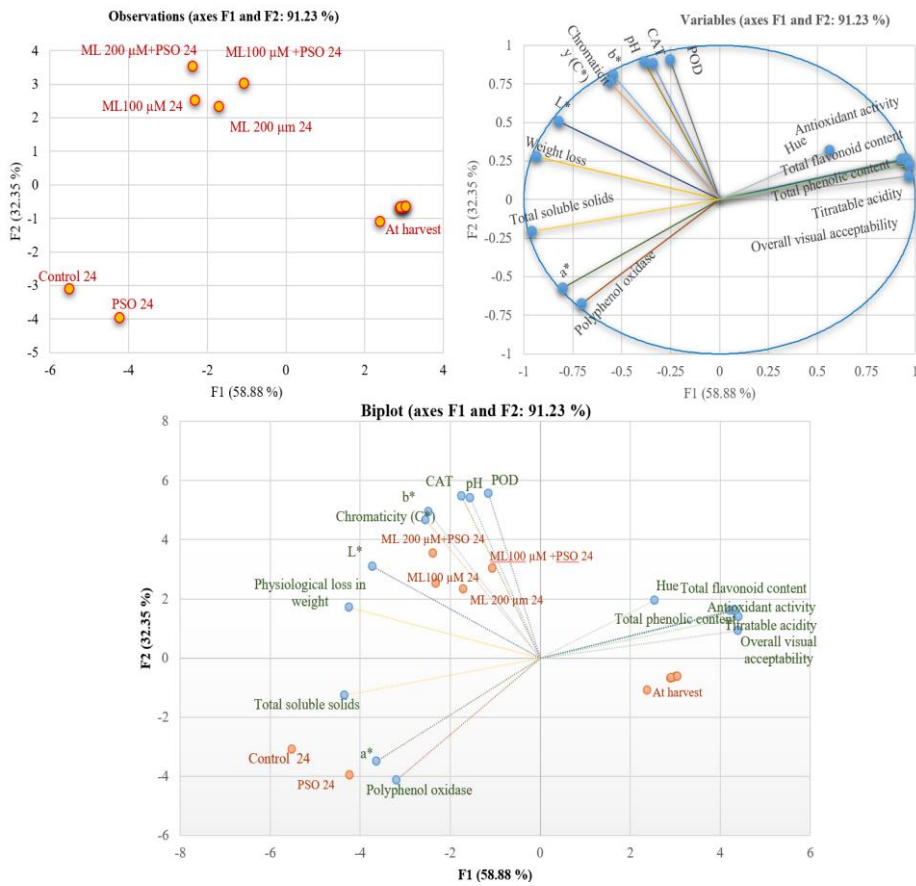
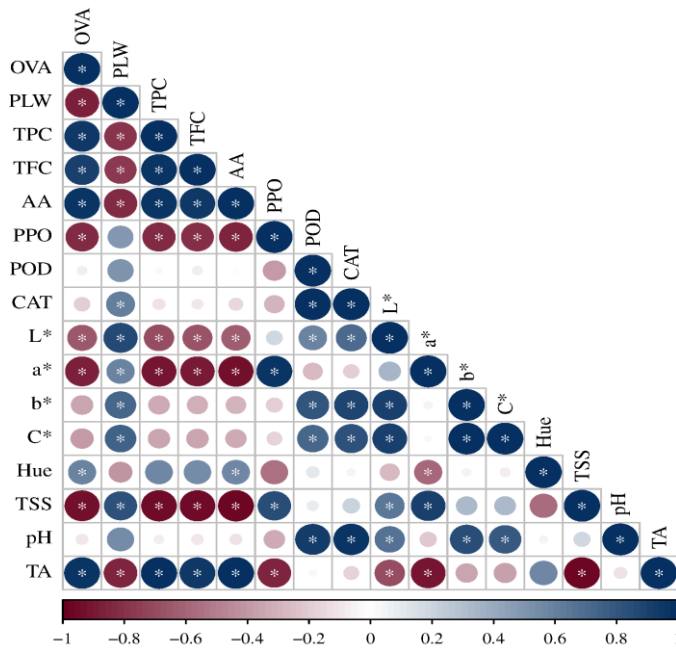


Figure 7. The effect of treatments (Distilled water, 100 μ M ML: 100 μ M Melatonin, 200 μ M ML: 200 μ M Melatonin, 100 μ M ML+PSO: 100 μ M Melatonin with Pomegranate Seed Oil (PSO), 200 μ M ML+PSO: 200 μ M Melatonin with PSO) on: (a) L*, (b) a*, (c) b*, (d) Hue, (e) Chroma, and (f) CCI of Mexican lime fruit stored for 24 days at 20 \pm 2 $^{\circ}$ C and 50-60% RH. Data represent mean values of n= 3 and the error bars represent Standard Errors (SE) of the means. Statistical analysis was performed using the LSD test at P \leq 0.05 level.



(a)



(b)

Figure 8. Principal Component Analysis (PCA) of the treatments and variable trait relationships in Mexican lime fruit, including PCA loading plots of the examined variable traits (a). Pearson's correlation coefficients among physio-biochemical traits in Mexican lime under cold stress of 0 (a) and -6°C (b).



(100 μ M)+PSO, with a score of 0.85, followed by ML (200 μ M)+PSO, with a score of 0.83.

DISCUSSION

Visual appeal is key for consumer preference in food, especially limes. Aroma, flavor, and color strongly influence this. Fruit quality assessments often involve evaluating physicochemical parameters and their changes during storage (Carrión-Antolí *et al.*, 2022). Excessive water loss (over 5%) in fruits can significantly diminish their visual appeal, leading to wilting and skin drying. This accelerates aging, reduces marketability, and results in economic losses (Lufu *et al.*, 2020, Liu *et al.*, 2020). Melatonin may preserve fruit quality by acting as an antioxidant, maintaining color, texture, and flavor. Studies show it improves kiwi fruit's appearance by increasing chlorophyll and carotenoid levels (Zhang *et al.*, 2023). By creating an effective barrier against evaporation and oxygen penetration, pomegranate seed oil reduces oxidation and maintains the freshness of fruits, and can be used as a useful ingredient in the formulation of edible coatings to increase the shelf life of fruits (Teodosio *et al.*, 2018a).

Weight loss in fresh produce, primarily due to water loss through respiration and transpiration, indicates quality deterioration. This loss of weight in fresh fruits corresponds to the reduction of water through metabolic processes and product degradation during storage (Ferreira *et al.*,

2020). The mechanism behind this weight loss reduction might be related to the maintenance and improvement of membrane function, as suggested by Wang *et al.* (2019a). Melatonin reinforces cell membranes and reduces water loss to help preserve fruit quality (Rastgoo *et al.*, 2024). Furthermore, our findings support the notion that ML treatment can significantly inhibit weight loss in fruits, as observed in kiwi berries (Zhang *et al.*, 2023). In line with our results, Shahsavari and Rastegar (2025) also found that pomegranate seed oil coating significantly limited and minimized post-harvest weight loss in guava.

Citrus fruits are packed with flavonoids and antioxidant phenolics. These compounds fight oxidative stress by scavenging Reactive Oxygen Species (ROS), boosting the antioxidant defenses and protecting against damage (Promyou *et al.*, 2023). Studies show that melatonin effectively reduces ROS accumulation in fruits by activating both enzymatic and non-enzymatic antioxidants, thus delaying fruit aging (Liu *et al.*, 2025). Similarly, strawberries treated with melatonin exhibit increased levels of total phenolics and flavonoids, leading to delayed ripening (Liu *et al.*, 2018). Melatonin treatment in peaches enhances antioxidant activity, minimizes oxidative stress, protects cell membranes, and delays aging after harvest (Gao *et al.*, 2016). Finally, studies on jujube have shown that melatonin application increases phenolic compounds, delaying senescence and enhancing overall fruit quality (Wang *et al.*, 2021). Melatonin increases the activity of key enzymes such as phenylalanine

Table 2. The values of indices and their rankings for each treatment.

Score	Rank	Treatment
0.19	6	Control
0.77	4	ML 100
0.80	3	ML 200
0.85	1	ML 100+ PSO
0.83	2	ML 200 +PSO
0.21	5	PSO

ammonia lyase by stimulating the phenylpropanoid pathway. This increased enzyme activity leads to the production of more phenolic compounds (Sharafi *et al.*, 2021). Pomegranate seed oil creates a barrier layer on the surface of the fruit, limiting oxygen penetration, thereby helping to preserve phenolic compounds and reduce oxidative processes. This feature plays an effective role in preserving the quality of fruits after harvest (Chavan *et al.*, 2023)

Our findings are consistent with those of Tavallali (2018), who observed a decrease in TPC in lime fruits during storage. Similar results have been reported for other fruits, such as mangoes and litchi, where ML dipping preserved higher TPC levels for longer shelf durations. However, Xu *et al.* (2022) found a trend toward lower TPC increases in 'Friar' plum flesh following melatonin treatment. Ma *et al.* (2021) suggested that the accumulation of phenolic compounds in ML-treated fruits contributes to maintaining postharvest quality and mitigating physiological senescence. Edible coatings containing pomegranate seed oil, by retaining 70% of phenolic compounds, played an effective role in preserving the quality and bioactive compounds of strawberries during storage, which was consistent with the results of our study (Melikoğlu *et al.*, 2022). Research on *Spondias tuberosa* fruits revealed that coatings integrating *Chlorella* sp. with pomegranate seed oil generated a modified atmosphere that significantly slowed the ripening process; specifically, the formulation containing 2.0% *Chlorella* sp. combined with PSO offered the most effective preservation of ascorbic acid and phenolic compounds (Teodosio *et al.*, 2020).

Limes, rich in flavonoids with antioxidant properties, may benefit from ML, which modulates total phenolic content and mitigates physiological senescence (Khatam *et al.*, 2024). Similar to its effect in oranges, where it led to the accumulation of phenolics and maintained postharvest quality, our findings suggest a potential role for melatonin in mitigating physiological

senescence in limes. Melatonin's antioxidant function extends beyond direct free radical scavenging, also enhancing the concentration of other antioxidants and antioxidant enzyme activity, ultimately increasing the postharvest life of fruits and vegetables (Ahmed *et al.*, 2020). Melatonin's amphiphilic properties enable it to penetrate cell membranes and neutralize reactive species within cells. Exogenous melatonin regulates ROS levels, preserving cell integrity and reducing oxidative damage (Sharafi *et al.*, 2021). Pomegranate seed oil acts as an antioxidant, and prevents nutrient oxidation, moisture loss, and color changes, thus helping to maintain the quality and shelf life of the fruit (Drinić *et al.*, 2020). Studies have also confirmed that PSO can substantially improve the oxidative stability of plant-based oils by enhancing antioxidant potential and slowing down the degradation of beneficial compounds during storage periods (Siraj *et al.*, 2019).

Melatonin, being amphiphilic, easily penetrates cells, neutralizes reactive species, regulates ROS levels, protects cell integrity, and minimizes oxidative damage (Onik *et al.*, 2021). Melatonin treatment in peaches boosts antioxidant enzyme activity, reduces ROS levels, and delays deterioration, highlighting its potential for enhancing postharvest fruit quality (Gao *et al.*, 2016). Melatonin is a powerful antioxidant that can neutralize up to 10 ROS molecules per molecule, significantly surpassing other antioxidants like vitamin C, vitamin E, and glutathione (Galano and Reiter, 2018). PPO, which accelerates tissue browning by converting phenolics to quinones, increases with storage but is reduced in melatonin-treated fruits. Studies, including those by Rastegari *et al.* (2020) and Gao *et al.* (2018), found that melatonin decreases PPO activity in mangoes and peaches. Our results support Marak *et al.* (2023) findings, showing that melatonin administration successfully suppresses PPO and POD activity in litchi fruits, postponing browning and maintaining antioxidant levels throughout storage. This demonstrates how melatonin may improve



the quality and shelf life of fruit (Marak *et al.*, 2023).

TSS and TA are key maturity indices for citrus juice quality. During storage, TSS increases due to factors such as water loss, starch degradation, and the conversion of polysaccharides and pectin to sugars (Hussain *et al.*, 2022). TSS rises with fruit maturation and storage-induced dehydration, while TA decreases due to organic acid consumption. Melatonin has been shown to delay TSS increase and preserve TA in fruits like mangoes and strawberries (Promyou *et al.*, 2023, Ma *et al.*, 2021). Our results confirm melatonin's effectiveness in regulating TSS and TA in limes, with changes linked to metabolic processes such as organic acid consumption during respiration (Bhardwaj *et al.*, 2022b). Our findings align with previous observations, suggesting melatonin's potential to regulate lime quality. The observed decrease in both TSS and TA in both treatment groups is likely due to metabolic processes within the fruit, including the consumption of organic acids during respiration (Bhardwaj *et al.*, 2022a). These results collectively indicate that ML application could be a viable approach for preserving the postharvest quality of fruits by modulating the TSS and TA.

Peel color affects consumer preferences by signaling internal quality. During storage, decreased L^* indicates browning and reduced hue angle shows chlorophyll loss, allowing carotenoids to turn fruit color from green to yellow as it ripens (Liu *et al.*, 2021). In limes, as maturity progresses, chlorophyll degradation allows for the accumulation and expression of carotenoids in the flavedo (outermost layer of the peel), resulting in the fruit's color changing from green to yellow (Sun *et al.*, 2019). Melatonin use significantly reduces chlorophyll degradation and increases carotenoid production at metabolic levels in various vegetables, including broccoli. (Yan *et al.*, 2024). In ripening limes, a^* increases due to chlorophyll loss and carotenoid gain, shifting color towards red. Melatonin

treatment lowers a^* and b^* , delaying ripening. However, b^* increases during storage due to chlorophyll decline and carotenoid rise. Melatonin preserves chloroplast integrity, delaying color changes and enhancing lime sensory quality. Melatonin treatment maintains chloroplast integrity by inhibiting genes and enzymes involved in chlorophyll degradation, thereby enhancing the sensory quality of fruit. Our results are consistent with those of Promyou *et al.* (2023) who showed that melatonin treatment effectively maintains brightness and delays color changes in strawberries. Similarly, the observed delay in fruit color development in our melatonin-treated lemons is consistent with observations in various other fruits, including papaya (Borthakur *et al.*, 2024) and mango (Liu *et al.*, 2020). Furthermore, our findings are consistent with those of Zhang *et al.* (2018), who reported that melatonin application slows down the changes in L^* and a^* parameters and delays the onset of senescence in litchi fruit. Furthermore, melatonin treatment has been shown to preserve the color and appearance of lychee fruit, reduce skin browning, and improve overall fruit quality (Xie *et al.*, 2022). In a study by Mohammadi *et al.* (2024a), the use of pomegranate seed oil (PSO) in combination with xanthan gum showed a significant effect on preserving the color of the fruit peel. These results indicate that pomegranate seed oil is effective in preserving the natural color and increasing the apparent shelf life of the fruit by reducing chlorophyll decomposition and delaying the synthesis of carotenoids. In addition, studies demonstrated that the application of a coating composed of 3% tamarind starch and 0.24 mL/mL pomegranate seed oil resulted in greater efficiency in maintaining fruit luminosity (L^*) and delaying color development, as indicated by the C^* values (Onias *et al.*, 2019).

CONCLUSIONS

Our study investigated the efficacy of enriching melatonin using Pomegranate Seed Oil (PSO) for the postharvest preservation of *Citrus aurantifolia*. The applied treatments demonstrated a promising ability to mitigate PPO activity, thereby reducing browning, a major quality concern in limes. Additionally, the treatments significantly improved the function of antioxidant enzymes, notably POD and CAT, indicating a strengthened antioxidant defense system within the fruit. These combined effects highlight the effectiveness of the treatments, excluding PSO alone, in enhancing the overall quality and prolonging the storage duration of Mexican lime. Thus, our findings underscore the potential of melatonin as a valuable postharvest strategy for preserving the quality and marketability of limes.

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نقش ملاتونین و روغن دانه انار در حفظ کیفیت لیموترش مکزیکی در طول نگهداری

محبوبه محمدی، سمیه رستگار، و عباس روحانی

چکیده

لیموترش‌های مکزیکی به دلیل تغییر رنگ و کاهش تازگی، ماندگاری محدودی دارند. این مطالعه اثرات ملاتونین (ML) و روغن دانه انار (PSO) را بر حفظ کیفیت پس از برداشت در دمای 20 ± 2 درجه سانتیگراد و رطوبت نسبی 50-60% ارزیابی کرد. نتایج نشان داد که ملاتونین در غلظت‌های 100 و 200 میکرومولار + PSO بالاترین میزان پذیرش کلی میوه را نشان داد. گروه کنترل بیشترین کاهش وزن (20.8%) را نشان داد، در حالی که PSO کمترین کاهش وزن (12.5%) را نشان داد. به استثنای تیمار PSO، سایر تیمارها به طور قابل توجهی سطح فنول‌ها، فلاونوئیدها و آنتی‌اکسیدان‌ها را افزایش دادند. تیمارهای PSO و کنترل کمترین فعالیت کاتالاز (69 واحد در گرم وزن تر) و پراکسیداز (53.5) واحد در گرم وزن تر ([JK1.1] FW) را نشان دادند، در حالی که بالاترین فعالیت پلی فنول اکسیداز (99.6 واحد در گرم وزن تر) نیز در این گروه‌ها مشاهده شد. تیمارهای شاهد و PSO همچنین بالاترین میزان کل مواد جامد محلول (8.2%) (TSSs) و کمترین میزان اسیدیته (8.5%) را نشان دادند. در مجموع، اکثر صفات، به استثنای تیمار PSO، تفاوت معنی‌داری بین گروه‌های تیمار و شاهد نشان دادند.

Co-Composting of Municipal Solid Waste with Activated Biochar: A Promising Approach to Improve the Quality of Compost

Kamal Khalkhal¹, Adel Reyhanitabar^{1*}, Shahin Oustan¹, and Nasser Aliasgharzad¹

ABSTRACT

Biochar reduces composting problems and improves compost quality. However, Activated Biochar (AB) and its size are often overlooked. This research aimed to evaluate the impact of co-composting of Municipal Solid Waste (MSW) with different-sized biochar and AB on the quality of the resulting co-compost. The MSW were mixed thoroughly with different-sized (B_{2-4mm}, B_{1-2mm} and B_{0.5-1mm}) and activated biochars (H₂SO₄-AB_{0.5-1mm} and NaOH-AB_{0.5-1mm}) and co-composted for 90 days until compost maturity. The results revealed that the activation of biochar with NaOH and H₂SO₄ caused the appearance of a mesh structure on the biochar surface, leading to improved stability and maturity, enhanced biodegradation and humification indices. Specifically, NaOH-AB (5%, w/w) showed the highest temperature (71.5°C), germination index (130.9%), and total nitrogen content (1.37%) and the longest thermophilic period (7 days). The highest Organic Matter content (OM) (37.9%) and the lowest electrical conductivity (7.4 dS m⁻¹) were recorded in B_{1-2mm} (10%, w/w). Furthermore, the lowest nitrate concentration (254.4 mg kg⁻¹) and the highest C/N ratio (18.1) were in H₂SO₄-AB (10%). Principal Component Analysis (PCA) highlighted the critical role of the C/N ratio and OM content during the composting. The study recommends the addition of biochar to MSW to achieve an appropriate C/N ratio and prevent nitrogen loss. Overall, incorporating NaOH- and H₂SO₄-activated biochars was found to be a valuable strategy for the composting of municipal solid wastes. Our findings provide valuable insights into the potential of biochar in optimizing the composting process.

Keywords: Biochar activation, C/N ratio, Composting process, Humification.

INTRODUCTION

The management of Municipal Solid Waste (MSW) is a global concern. According to the United Nations Environment Program, the global MSW generation will reach 3.8 billion tons by 2050 (UNEP, 2024). In the Middle East, nearly 65% of MSW consists of organic waste, which is higher than the global average and more than 70% of this MSW is primarily disposed of in unsanitary landfills (UNEP, 2024). Consequently, effective management of MSW has emerged as a pivotal 21st-century challenge, which

requires innovative technologies to recover resources and facilitate the transition to a sustainable bio-economy, particularly in developing countries. From both economic and environmental perspectives, composting offers a promising solution for managing the organic fraction of MSW, while also reducing waste disposal costs (Bhattacharjee *et al.*, 2023). Composting involves various stages where diverse microbial communities (bacteria, fungi, and actinomycetes) operate at different temperatures, generating heat as they decompose organic matter. The resulting compost is a nutrient-rich organic fertilizer that improves the physicochemical,

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and biological properties of soil, enhances soil fertility and reduces the reliance on chemical fertilizers (Babu *et al.*, 2021). Additionally, composting can be associated with challenges such as greenhouse gas emissions, nitrogen loss, and the contamination of soil and water resources (Barthod *et al.*, 2018; Nguyen *et al.*, 2022). To address these challenges, "co-composting", which involves the use of additives at the beginning of the composting, has emerged as a promising approach (Barthod *et al.*, 2018).

Recently, biochar has been recognized as a valuable additive and a key component to reduce the adverse effects of MSW composting and improve the quality of the final compost (Feng *et al.*, 2024). Biochar possesses a range of beneficial characteristics, including a porous structure, high specific surface area, high ion exchange capacity, active oxygen functional groups, and resistance to decomposition. These properties enhance aeration and accelerate the decomposition of toxic substances through co-metabolism, reducing heavy metal bioavailability, nitrogen loss, greenhouse gas emissions, and composting time (Nguyen *et al.*, 2022). In addition, the physicochemical properties of biochar, including particle size and activation, can affect its efficiency in improving compost quality. The particle size affects aeration, moisture, porosity, turning efficiency, and the uniformity of the compost pile (He *et al.*, 2019). However, previous studies have provided limited insights into the effects of biochar particle size on the co-composting process.

The main purpose of the activation process is to enhance the oxygen-containing functional groups, surface area, pore volume and diameter, and increase the porosity of the Activated Biochar (AB) (Panwar and Pawar, 2020). Chemical activation (includes acid and alkaline activation) is the most widely adopted process for activation, with several advantages over physical methods. Among various chemical activators, NaOH and H₂SO₄ are considered more suitable due

to their cost-effectiveness and lower environmental impact (Panwar and Pawar, 2020). Ye *et al.* (2019) reported that chemical-activated biochar increased available habitats for microorganisms, thereby improving the intensity of microbial respiration. Given the limited studies on AB composting, further investigation is warranted.

The objective of this study was to evaluate the impact of co-composting of MSW with different-sized biochar and AB on the quality of the resulting co-compost. The compost quality characteristics were evaluated, including maturity and stability, enzyme activity, and humification indices.

MATERIALS AND METHODS

Preparation and Activation of Biochars

MSW was collected from the Municipal Waste Management Organization, Tabriz, Iran. The waste biomass for the production of biochar, was prepared by mixing the pruning branches of plum and pomegranate trees. Biochar was produced by slow pyrolysis at 400 °C with a heating rate of 10°C per minute and a one-hour holding time at the target temperature. The biochar was separated using 0.5-1 mm (B_{0.5-1 mm}), 1-2 mm (B_{1-2 mm}), and 2-4 mm (B_{2-4 mm}) sieves. For biochar activation, the biochar (B_{0.5-1mm}) was mixed with solutions of 2M NaOH and/or H₂SO₄ at a solid-to-solution ratio of 1:2 (w/v) with gentle stirring for 2 hours under the hood. Then, the suspension was filtered, and the residual solid was washed several times with distilled water until the pH was fixed at approximately 7.0. Finally, the washed biochar was dried in an oven at 65°C for 12 hours (Fan *et al.*, 2010).

Chemical Analysis of Biochars and MSW

For the chemical analysis, the pH and EC were determined in a 1:10 (w/v) compost to

water ratio (Singh *et al.*, 2017). The contents of carbon, hydrogen, nitrogen, and sulfur was measured by CHNS analysis (Vario ELIII Elementary Analyzer, Germany). The biochar Cation Exchange Capacity (CEC) was determined by the method of Wang *et al.* (2013). The ash content was obtained by the Singh *et al.* (2017) method. The total concentrations of P, K, Na, Fe, Mn, Zn, Cu, Pb, and Ni were determined by ash digestion with a 3:1 mixture of two acids (HNO₃:HCl, 1:3, v/v) (Jones Jr. and Case, 1990). The available P concentration was determined by the Olsen method (Kuo, 1996), and the NH₄⁺ concentration was determined by the indophenol blue method (Li *et al.*, 2015). Water soluble K and Na concentrations were determined at 1:10 ratio (w/v). The concentration of heavy metals was measured by an atomic absorption spectrometer

(Shimadzu, AA-6300). Biochar functional groups were determined by the FTIR technique as a general characterization technique (Bruker Tensor 27 FTIR spectrometer). Furthermore, the biochar surface morphology was evaluated by SEM (Tuscan FEG-SEM, MIRA3). The characteristics of the produced biochars and the MSW used in this study are presented in Table 1.

Composting System and Experimental Design

The experiment was carried out with two factors of biochar type at eleven levels, and time at eight levels of biochar, with two repetitions for three months. For this purpose, biochars of 2-4 mm, 1-2 mm, and

Table 1. Basic characteristics of the MSW and biochars.^a

Properties	MSW	B* _(0.5-1mm)	B* _(1-2mm)	B* _(2-4mm)	NaOH-AB**	H ₂ SO ₄ -AB**
Ash (g 100 g ⁻¹)	72.05±3.04	25.57±0.58	19.75±0.35	19.02±0.31	18.80±0.14	17.95±0.35
C (g 100 g ⁻¹)	16.03±1.00	54.47±5.67	54.47±5.67	54.47±5.67	63.61±4.28	50.67±2.86
H (g 100 g ⁻¹)	0.75±0.01	1.60±0.04	1.60±0.04	1.60±0.04	1.69±0.07	1.54±0.06
N (g 100 g ⁻¹)	1.28±0.02	1.01±0.00	0.88±0.00	0.85±0.00	1.22±0.00	0.95±0.00
S (g 100 g ⁻¹)	0.74±0.00	0.36±0.01	0.36±0.01	0.36±0.01	0.27±0.00	1.81±0.00
O (g 100 g ⁻¹)	8.88±1.59	16.99±6.29	22.94±5.35	23.7±5.4	14.41±4.06	27.08±2.44
O/C	0.42±0.05	0.23±0.15	0.32±0.14	0.44±0.15	0.17±0.08	0.40±0.08
H/C	0.56±0.01	0.35±0.00	0.35±0.00	0.35±0.00	0.32±0.00	0.36±0.00
C/N	12.52±0.98	53.93±6.58	61.9±6.68	64.08±6.6	52.14±4.73	53.34±3.26
CEC (Cmolc kg ⁻¹)	-	25.04±1.47	14.74±2.88	11.76±1.7	28.34±2.7	25.57±0.74
EC (dS m ⁻¹)	8.2±0.31	0.55±0.00	0.34±0.00	0.28±0.01	0.47±0.01	1.76±0.01
Total K (g kg ⁻¹)	10.66±0.20	5.03±0.01	5.03±0.01	5.13±0.01	2.67±0.00	2.47±0.01
Total P (g kg ⁻¹)	1.41±0.08	1.16±0.01	1.13±0.01	1.12±0.03	0.94±0.01	0.72±0.01
Total Na (g kg ⁻¹)	2.64±0.06	0.86±0.00	0.78±0.01	0.79±0.01	2.04±0.01	0.35±0.00
Total Fe (g kg ⁻¹)	1.28±0.33	1.18±0.01	1.07±0.01	0.83±0.03	1.1±0.00	1.01±0.01
Total Mn (mg kg ⁻¹)	143.34±14.11	110.78±2.86	68.73±2.86	61.20±2.97	98.87±2.85	84.04±1.46
Total Zn (mg kg ⁻¹)	49.47±1.56	30.1±0.21	28.3±1.41	25.4±0.57	163.71±4.26	138.21±2.86
Total Cu (mg kg ⁻¹)	58.01±5.67	20.67±0.44	13.85±0.57	14.18±0.45	24.24±0.74	16.77±1.81
Total Pb (mg kg ⁻¹)	180.52±28.34	161.13±7.11	163.90±4.53	163.9±14.27	172.21±2.98	166.67±11.46
Total Ni (mg kg ⁻¹)	81.91±12.76	73.12±4.38	71.66±7.79	70.19±14.3	81.18±1.5	74.59±5.69
pH	Min: 7.53 Max: 7.61	Min: 8.58 Max: 8.74	Min: 8.92 Max: 9.20	Min: 9.15 Max: 9.21	Min: 9.06 Max: 9.34	Min: 7.63 Max: 7.77

^a Values indicate the mean±standard deviation based on determination with two replications.

*B= Biochar **AB= Activated biochar



0.5-1 mm Activated Biochar (AB) with H₂SO₄ and NaOH (H₂SO₄-AB_{0.5-1mm} and NaOH-AB_{0.5-1mm}) were thoroughly mixed with MSW, each at two levels of 5 and 10% (w/w). One treatment with no addition of biochar was considered the control. The substrates were accommodated in 100-L plastic barrels (44 cm diameter and 76 cm height) with an approximate weight of 70 kg. In each barrel, one kg of cow manure was added to increase the microbial activity, and 30 holes were drilled for better ventilation. The temperature of the composting mixture was monitored daily. Once a week, the compost materials were turned and thoroughly mixed. The moisture of the materials was kept in the range of 50-60% by weight until the end of the experiment. Sampling was performed on days 1, 7, 14, 28, 42, 56, 70, and 90, and each time at least five subsamples (approximately 500 g) were taken from different depths of the barrel and mixed thoroughly, and a composite sample was taken (Vandecasteele *et al.*, 2016). The composite sample was divided into two subsamples (air-dry and moist). The air-dried samples were used to measure the basic physicochemical properties and elemental concentrations, and the moist samples were used to measure the biological indicators and concentrations of NO₃⁻ and NH₄⁺. Both samples were stored in the refrigerator at 4°C prior to analysis.

Compost Analysis

The pH and EC were measured in 1 to 5 (w/v) compost to water ratio (Awasthi *et al.*, 2017a). The Organic Matter (OM) content was determined by ashing at 550 °C for 6 hours (Haug, 1993). Total N (TN) was determined by the Kjeldahl method (Bremner, 1996). The NH₄⁺ concentration was determined using the methods of indophenol blue (Li *et al.*, 2015). The NO₃⁻ concentration was measured using the sulfosalicylic acid (Cataldo *et al.*, 1975). The Germination Index (GI), as an indicator

of phytotoxicity, was evaluated according to the method proposed by Zucconi *et al.* (1981). The activity of enzymes, including urease (Tabatabai, 1994), and dehydrogenase (Schinner *et al.*, 2012) and microbial respiration (Anderson, 1983) were also determined in three composting periods of mesophilic, thermophilic, and maturing (1, 7, and 90 days, respectively). The humic acid was extracted and purified from compost according to the method described by Sánchez- Monedero *et al.* (2002). To determine the E₄/E₆ (absorbance ratio at 465 nm to 665 nm) and E₃/E₅ (absorbance ratio at 300 nm to 500 nm) ratios, the absorbance was measured using a spectrophotometer (SU-6100, Philler Scientific) at wavelengths of 465, 665, 300, and 500 nm (Chen *et al.*, 1977).

Statistical Analysis

After the normality test, a repeated-measures ANOVA was performed to evaluate the main and interaction effects of treatments and time on some dynamic response characteristics. The mean comparisons were performed by the Duncan method (P ≤ 0.05). Principal component analysis (PCA) and cluster analysis were also used to group similar individuals. All statistical analyses and drawing graphs were performed using SPSS 27.0 and Origin software.

RESULTS AND DISCUSSION

Effect of Chemical Activation on Biochar Properties

The chemical activation of biochar caused the appearance of a mesh structure with irregularly sized cavities and deep pores by modifying the biochar surface structure (Figures 1-a, -b, and -c). An *et al.* (2020) also reported that the chemical activation of biochar significantly changed the biochar

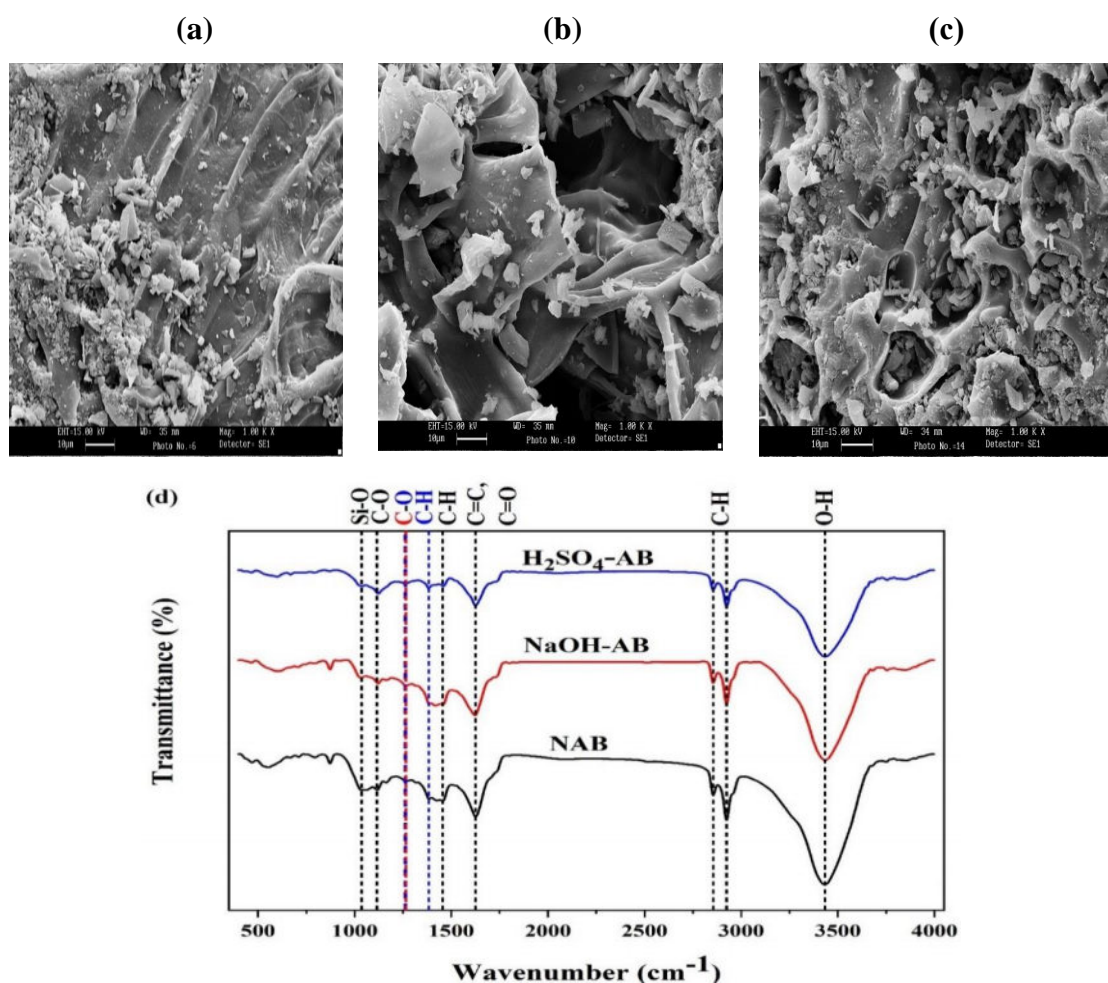


Figure 1. Scanning Electron Microscope (SEM) images of NAB (a), NaOH-AB (b), H₂SO₄-AB (c), and FTIR spectra of NAB and NaOH-AB and H₂SO₄-AB (d).

surface structure and increased its specific surface area.

Figure 1-d shows the FTIR spectra of the activated and Non-Activated Biochars (NAB). No distinct differences in the position of the relevant peaks were observed between the FTIR spectra of the non-activated and activated biochars. However, similar to the results of Dehkhoda *et al.* (2014), the chemical activation of biochar diminished the intensity of the peaks, indicating removal or reduction of some compounds. The activation of biochar each produced a peak of approximately 1,260 to 1,270 cm⁻¹ related to the presence of C-O in aryl esters (aromatic structure). On the other hand, H₂SO₄-AB produced a peak of

approximately 1384 cm⁻¹ compared to NaOH-AB and NAB (related to the presence of C-H in CH₂ or CH₃ (aliphatic structure)).

Impact of Biochar on Some Compost Maturity and Stability Indices

The addition of biochar increased the temperature in the thermophilic phase ($P < 0.05$), indicating the effect of biochar on microbial activity (Figure 2-a). Except for the control and H₂SO₄-AB (5%) treatment, in which the thermophilic period was five days, the rest of the biochar treatments had a longer thermophilic period (6 and 7 days). On the first day of the thermophilic period,



the minimum temperature (54.5°C) was related to the control and the maximum (71.5°C) was associated with the longest thermophilic period (7 days) for the NaOH-AB (5%) treatment. Compared to the control, biochar treatments led to the early onset of the thermophilic phase, increasing the temperature and prolonging this phase, which agrees with the results of Manu *et al.* (2021).

The compost pH fluctuated between 7.4 and 7.9. As shown in Figure 2-b, at the initial phase of composting, the pH decreased, probably due to the release of organic acids, then, decomposition of proteins started and resulted in an increase in pH due to NH₃ production. On days 28 to 42, the pH increased again due to the continued decomposition of organic acids, which was consistent with Wang *et al.* (2023). Finally, pH of the compost pile stabilized at values between 7.49 and 7.74 and the compost produced in the NaOH-AB (10%) treatment had the highest pH (7.74) ($P < 0.05$), in response to NaOH solution used in the biochar activation process. There is confusing literature on biochar effects. Both increasing (Vandecasteele *et al.*, 2016) and decreasing (Mao *et al.*, 2018) effects of biochar addition on the final pH of the compost piles have been reported. However, some studies similar to our study, did not observe a significant difference in the pH of the final compost (Manu *et al.*, 2021; Janczak *et al.*, 2017). Much of the confusion has probably arisen from the different nature of feedstock as well as biochar and different conditions of co-composting processes.

According to Figure 2-c, the maximum and minimum values of final EC were recorded in the control (9.32 dS m⁻¹) and B_{1-2mm} (10%) treatments (7.41 dS m⁻¹), respectively. The increase in EC was more intense in the thermophilic period and can be related to the increase in the activity of microorganisms and mineralization of OM. The most significant decrease was found in the treatments with 10% biochar, which indicated the dilution or absorption effect of added biochar. Qu *et al.* (2020) also reported

the potential of biochar to reduce compost EC values through dilution and/or absorption.

The content of OM decreased over the time of composting process due to the consumption of carbon by microorganisms (Figure 2-d). After the thermophilic period, the decrease in OM content slowed. In the final compost, the highest and lowest OM contents were observed in the B_{1-2mm} (10%) treatment (37.93%) and the control (27.1% based on dry weight), respectively ($P < 0.05$). In general, the level of OM in the 10% biochar treatments was almost higher than that in the 5% biochar treatments. Due to the chemical recalcitrance of biochar aromatic structure, it does not go through severe degradation during composting. This is consistent with the results of Manu *et al.* (2021). The reduction in OM content was the highest in the H₂SO₄-AB (10%) treatment (12.26%) and the lowest in the control (7.18%).

The NH₄⁺ concentration increased at the beginning of the thermophilic period and then decreased (Figure 2-e). The initial increase could be due to ammonification reactions. In the final compost, the NaOH-AB (10%) treatment had the lowest NH₄⁺ concentration (70 mg kg⁻¹), and the control had the highest NH₄⁺ concentration (153 mg kg⁻¹), with significant differences from the rest of the treatments ($P < 0.05$), indicating the positive effect of biochar on the reduction in NH₄⁺ concentration. The activated biochars, especially NaOH-AB (5%), had the highest impact on reducing NH₄⁺ concentration, while the control had the lowest. This observation can be explained by combined roles of adsorption (the high absorption capacity of the activated biochars for NH₄⁺) and microbial immobilization in reducing nitrogen loss. On the other hand, the carboxylic and phenolic functional groups attached to the surface of biochar had negative charges to adsorb NH₄⁺, as a result of the aging process (Nguyen *et al.*, 2017) or activation with NaOH.

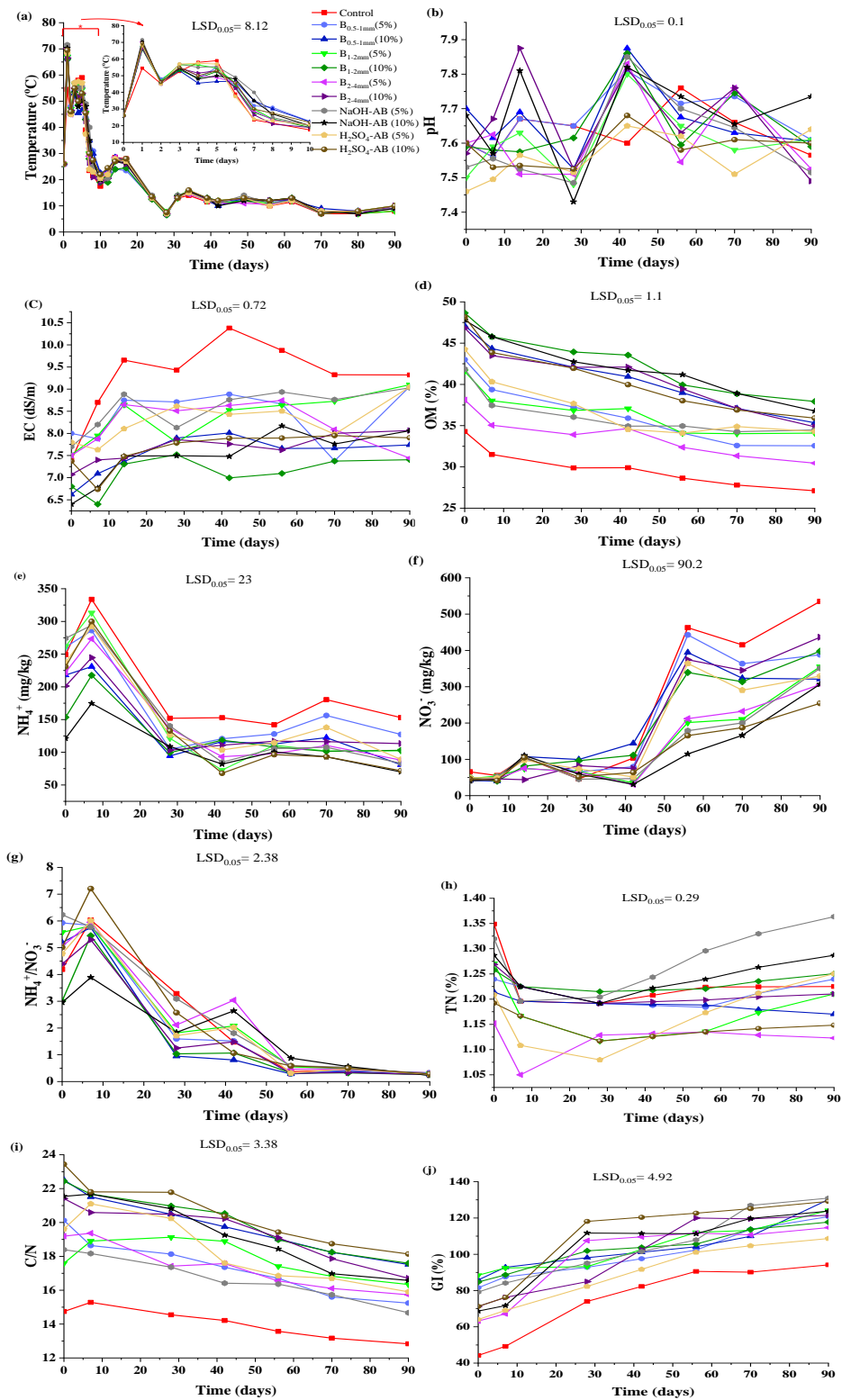


Figure 2. Changes in temperature (a), pH (b), EC (c), OM (d), NH₄⁺ (e) and NO₃⁻ concentrations (f), NH₄⁺/NO₃⁻ ratio (g), TN (h), C/N ratio (i), and GI% (j) during composting.



During composting, the concentration of NO_3^- , in contrast to NH_4^+ , increased (Figure 2-f). In the first few weeks of the composting process, the NO_3^- concentration was low, and there were no significant changes in its concentration, because the temperature, pH, or NH_4^+ concentration was high enough to prevent the activity and growth of nitrate-producing bacteria (Ren *et al.*, 2019; Wang *et al.*, 2023). After 42 days, the NO_3^- concentration rapidly started to increase and, finally, reached a relatively constant level. The control and the H_2SO_4 -AB (10%) treatment had the highest and lowest NO_3^- concentrations, respectively ($P < 0.05$). These results were consistent with the results of Manu *et al.* (2021). The higher concentration of NO_3^- in the control was due to its lower temperature, which favored the nitrification process.

The $\text{NH}_4^+/\text{NO}_3^-$ ratio is used as a nitrification index to check the compost maturity and stability. Ratios less than 0.5 are considered fully mature compost. All the composts produced in this research were fully mature regarding the $\text{NH}_4^+/\text{NO}_3^-$ ratio (Figure 2-g). The highest and lowest ratios in the final compost were detected in the control (0.33) and the NaOH-AB (10%) treatment (0.23), respectively. It was observed that the treatments with 10% biochar had lower $\text{NH}_4^+/\text{NO}_3^-$ molar ratios and reached the mature phase earlier. Furthermore, in the NaOH-AB treatments, the $\text{NH}_4^+/\text{NO}_3^-$ ratio decreased with decreasing biochar particle size ($B_{0.5-1\text{mm}} < B_{1-2\text{mm}} < B_{2-4\text{mm}}$).

In this study, the TN content first decreased sharply and then gradually increased during the composting process ($P > 0.05$) (Figure 2-h). The most significant decrease in TN content coincided with a sharp increase in NH_4^+ concentration. These results were consistent with the reports of Wang *et al.* (2023). The reason for the reduction in TN content in the thermophilic phase can be associated with de-nitrification and/or ammonia volatilization. The TN content decreased in all treatments from the first to the seventh day (thermophilic

period). However, the highest initial TN content (1.35%) and the maximum of its subsequent decrease from the first day to the seventh day (11.35%) were both observed in the control ($P < 0.05$). This sharp decrease was accompanied by a significant increase in NH_4^+ concentration (Figure 2-e). Manu *et al.* (2021), reported that N losses during the composting averaged 31.4% TN, 17.2% NH_3 , and 1.4% N_2O .

The decline in the C/N ratio in the control was less than that in the biochar-amended treatments (Figure 2-i). The lowest C/N ratio at both the beginning and end of the composting process was detected in the control (14.74 and 12.83, respectively), which indicated the loss of nitrogen through ammonia volatilization and/or NO_3^- leaching from the compost pile. At low C/N ratios, carbon is consumed before nitrogen fixation, and an unpleasant smell is generated due to ammonia volatilization. In the conditions of this research, the decline in the C/N ratio in the composts that received biochar was 28-46% higher than the decline in the C/N ratio in the control. The average C/N ratio in the composts receiving 10% biochar was higher than those receiving 5% biochar. Wang *et al.* (2023) recommended the use of biochar to decrease the C/N ratio of the final compost, likely due to enhanced nitrogen conservation and OM degradation in the compost pile. However, other researchers, such as Vandecasteele *et al.* (2016), found the addition of biochar to be inappropriate, likely due to the high doses used. Some studies also reported no significant difference in the C/N ratio between composts with and without biochar incorporation (Malińska *et al.*, 2014). One of the reasons for these conflicting results is the difference in the C/N ratio of the raw materials. Considering the carbon content in the biochar used in this research (51-64%), it is recommended to add biochar to MSW in order to increase the C/N ratio to the appropriate range of 20-30 and prevent nitrogen loss.

The most important tests to evaluate compost quality characteristics is the GI.

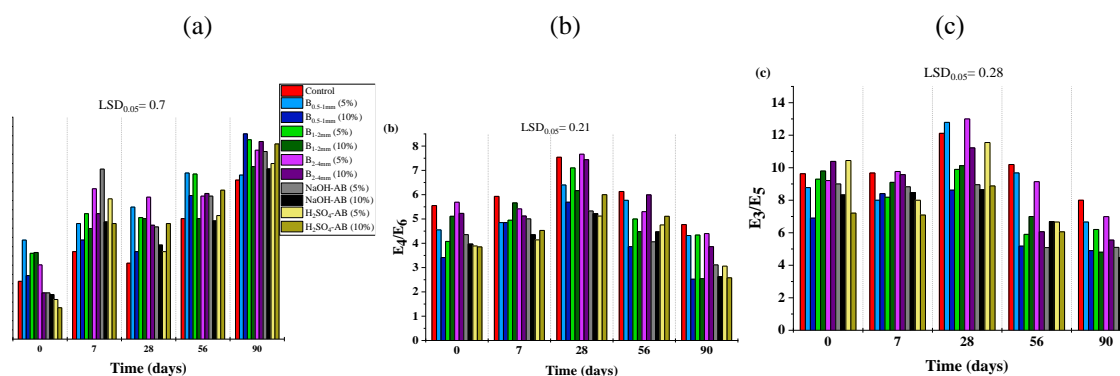


Figure 3. Time dependence of humification indices during the co-composting process: Humic acid yield (a), E_4/E_6 ratio (b), and E_3/E_5 ratio (c).

The standard value of the GI is reported to be $\geq 80\%$ for compost maturity (Zucconi *et al.*, 1981). The GI had an increasing trend during composting (Figure 2-j). However, in the final compost, the control had the lowest GI (94%), and the NaOH-AB (5%) treatment had the highest GI (131%) ($P < 0.05$). Similar to the results of this research, some researchers also reported that the GI in composts with biochar was higher than that in composts without biochar (Manu *et al.*, 2021; Wang *et al.*, 2023). The higher GI in the biochar treatments compared to the control in this study can be attributed to their higher temperature and longer thermophilic period, which helped remove pathogenic bacteria and improve compost quality.

Humic Acid Yield, E_4/E_6 and E_3/E_5 Ratios

The HA yield increased during composting, mainly in the thermophilic phase (Figure 3-a). The highest and lowest HA% were related to the $B_{0.5-1mm}$ (10%) treatment (8.9%) and the control (6.9%), respectively. By adding 0 to 10% biochar to the compost, the HA% of the final compost increased, on average, from 0 to 22.54%, which is consistent with the results of Jindo *et al.* (2016). The enhanced humic acid production induced by biochar addition during composting may be due to the release of aromatic precursors from biochar or the adsorption of soluble organic compounds on

the active surfaces of biochar (Jindo *et al.*, 2016).

The E_4/E_6 ratio is inversely related to the degree of aromatic condensation of the humic substances (Ren *et al.*, 2019). The E_3/E_5 ratio also denotes a more or less similar concept. In this research, the trends of both the E_4/E_6 and E_3/E_5 ratios over time increased first and then decreased (Figures 3-b, and -c). For composts with low degrees of humification due to the presence of proteins and carbohydrates, the ratios of E_4/E_6 and E_3/E_5 were high (Chen *et al.*, 1977). As the degree of humification increased, large molecules were formed, and the E_4/E_6 and E_3/E_5 ratios decreased. The E_4/E_6 ratios of the extracted HA varied between 2 and 10. The ratios near 2 are considered to be mature compost. In this research, the control had the highest E_4/E_6 and E_3/E_5 ratios (4.77 and 8, respectively), and the $B_{0.5-1mm}$ (10%), B_{1-2mm} (10%), NaOH-AB (10%), and H_2SO_4 -AB (10%) treatments had the lowest ratios of E_4/E_6 (2.52 to 2.63) and E_3/E_5 (4.23 to 4.9) ($P < 0.05$). Wang *et al.* (2023) also reported E_4/E_6 ratios of 2.84 to 3.47 for biochar-treated composts. The control in the compost production process decreases in the E_4/E_6 and E_3/E_5 ratios, indicating the lower production of humic substances without biochar application. These results were consistent with those of Wang *et al.* (2023) and Manu *et al.* (2021).



Microbial Respiration, Activity of Urease, and Dehydrogenase

The biochar treatments, compared to the control, caused a significant increase in microbial respiration for the thermophilic period only ($P < 0.05$). Microbial respiration increased in the thermophilic period due to the presence of easily degradable compounds, whereas it decreased in the final days of composting (Figure 4-a). In the thermophilic period, the control and the AB treatments had the lowest and highest microbial respiration, respectively. Biochars with smaller sizes ($B_{0.5-1\text{mm}}$ and $B_{1-2\text{mm}}$) had greater rates of microbial respiration than biochars with larger sizes ($B_{2-4\text{mm}}$). This is probably because of their higher specific surface area supporting a large proportion of the total microbial community in the compost pile. The biochar treatments had higher levels of microbial respiration (17.5%, on average) than the control. This finding is consistent with the results of Steiner *et al.* (2011).

Urease is an enzyme whose activity is determined by measuring the concentration of NH_4^+ produced. In the final compost, the highest and lowest urease activities were observed in the control and the $\text{H}_2\text{SO}_4\text{-AB}$ (10%) treatment, respectively. Similar

increased from the beginning of composting to the thermophilic phase and then decreased toward the maturing phase (Figure 4-b).

The activity of dehydrogenases enzyme often matches the microbial activity. In this study, the changes in dehydrogenase activity were similar to those observed for microbial respiration and urease activity, with a maximum in the thermophilic period (Figure 4-c). Zhang and Sun (2014) also reported that the incorporation of biochar can increase the activity of dehydrogenase in the thermophilic phase. The possible reason for this is the supply of nutrients from the biochar.

Principal Component Analysis (PCA)

Based on the results obtained, 61% of the cumulative variance is explained by the first and second components (Figure 5). Among the quality parameters of the produced composts, those with the most impact on the first component were C/N > OM > Urease activity > Temperature > NO_3^- > EC > E_3/E_5 ratio > E_4/E_6 ratio > NH_4^+ concentration; and those with the most influence on the second component were HA > Dehydrogenase > GI . In addition, PCA enabled the grouping of different composts. As shown in Figure 5

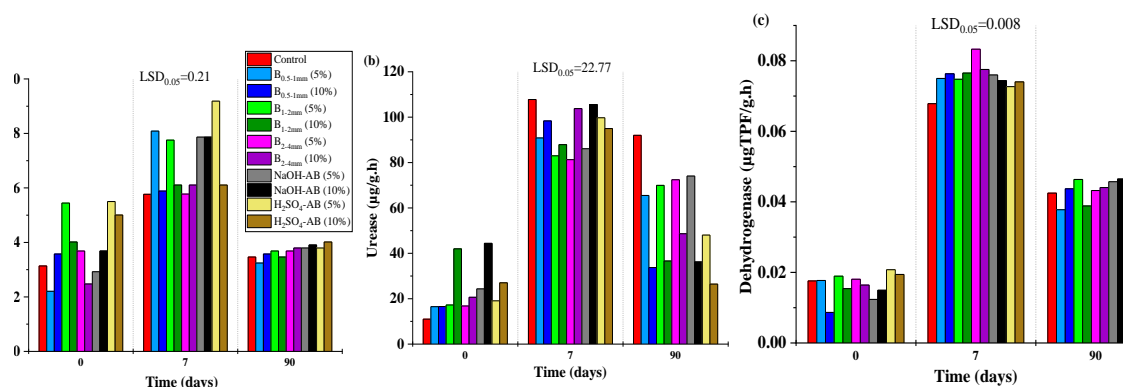


Figure 4. Changes in biological indices during composting: Respiration (a), urease (b), and dehydrogenase (c).

results were obtained for the NH_4^+ concentration. As expected, urease activity

(red points), there was a significant distance between the control and the biochar-treated

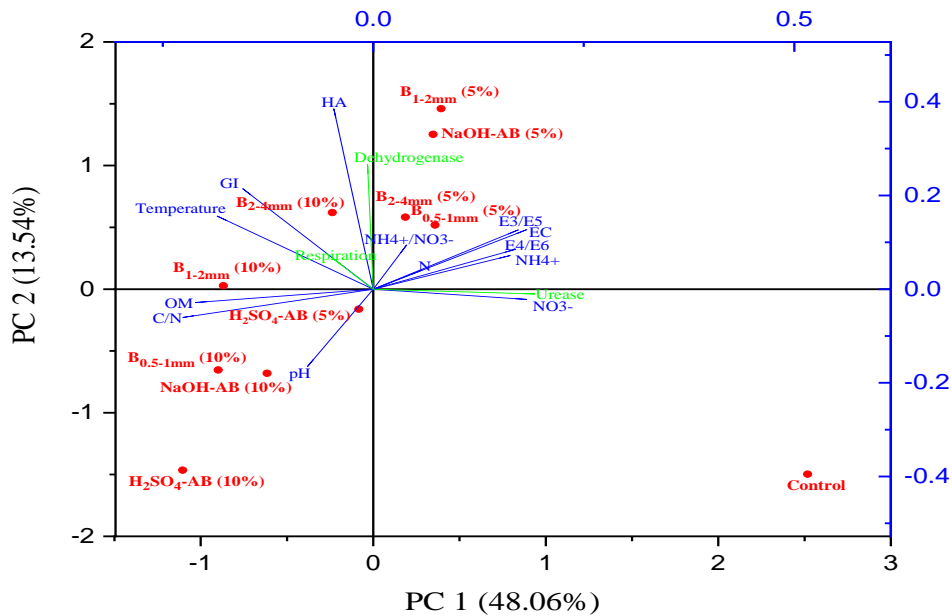


Figure 5. PCA biplot during composting.

composts, indicating the significance of biochar incorporation in the composting process. To elaborate on specific interactions between variables, it can be noted that the reduction in the C/N ratio was associated with an increase in OM, indicating improved decomposition of OM in the presence of AB. The NaOH/H₂SO₄-activated biochars enhanced microbial activity and surface interactions, which contributed to a faster reduction in the C/N ratio. Additionally, a positive correlation between OM content and temperature during the thermophilic phase highlighted biochar's role in enhancing microbial activity and enhancing compost quality. Similar to our findings, Awasthi *et al.* (2017a) reported that the PCA showed the strongest correlation with OM degradation and the C/N ratio.

CONCLUSIONS

The results indicated that, chemically, AB (NaOH-AB and H₂SO₄-AB) enhanced stability and maturity indices, promoted OM biodegradation, and improved humification

indices, particularly with small-sized biochar (0.5-2 mm) and when incorporating 10% biochar compared to 5% (w/w). This presents an effective strategy for improving the quality of MSW co-compost. PCA further highlighted the critical role of the C/N ratio and OM content in the co-composting. We recommend that future studies compare various activation methods, optimize biochar activation conditions to maximize composting efficiency, and conduct cost-benefit and carbon footprint analyses.

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کمپوست سازی همزمان زیاله‌های جامد شهری با بیوچار فعال: رویکردی امیدوارکننده برای بهبود کیفیت کمپوست

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چکیده

بیوچار مشکلات کمپوست‌سازی را کاهش داده و کیفیت کمپوست را بهبود می‌بخشد. با این حال، بیوچار فعال (AB) و اندازه ذرات آن اغلب نادیده گرفته می‌شوند. این تحقیق با هدف ارزیابی تأثیر کمپوست‌سازی مشترک پسماند جامد شهری (MSW) با بیوچار و بیوچار فعال با اندازه‌های مختلف بر کیفیت کمپوست حاصل انجام شد. MSW به طور کامل با بیوچارهای با اندازه‌های مختلف ($B_{0.5-1mm}$, B_{1-2mm} , B_{2-4mm}) و همچنین بیوچارهای فعال‌شده با H_2SO_4 (-) مختلف ($AB_{0.5-1mm}$, $NaOH$ - $AB_{0.5-1mm}$) مخلوط شد و به مدت 90 روز تا زمان بلوغ کمپوست، فرایند کمپوست‌سازی مشترک انجام گرفت. نتایج نشان داد که فعال‌سازی بیوچار با $NaOH$ باعث ایجاد ساختار مشبک روی سطح بیوچار شده و منجر به بهبود شاخص‌های پایداری و رسیدگی کمپوست، افزایش شاخص‌های تجزیه‌زیستی و هوموسی‌شدن می‌شود. به‌طور خاص، w/w (5% $NaOH$ - AB) بالاترین دما (71/5 درجه سانتی‌گراد)، شاخص جوانه‌زنی (130/9%) و محتوای نیتروژن کل (1/37%) و طولانی‌ترین دوره گرمادوستی (7 روز) را نشان داد. بالاترین میزان ماده آلی (37/9%) (OM) و کمترین میزان قابلیت هدایت الکتریکی (7/4 دسی‌زیمنس بر متر) در B_{1-2mm} (10% w/w) ثبت شد. علاوه بر این، کمترین غلظت نیترات (254/4 میلی‌گرم بر کیلوگرم) و بالاترین نسبت C/N (18/1) در H_2SO_4 - AB (10%) به دست آمد. تجزیه و تحلیل مؤلفه‌های اصلی (PCA) نقش حیاتی نسبت C/N و میزان OM را در طول کمپوست‌سازی برجسته کرد. این مطالعه افزودن بیوچار به MSW را برای دستیابی به نسبت C/N مناسب و جلوگیری از هدررفت نیتروژن توصیه می‌کند. به‌طور کلی، ترکیب بیوچارهای فعال شده با $NaOH$ و H_2SO_4 به عنوان یک استراتژی ارزشمند برای کمپوست‌سازی پسماند شهری شناخته شد و یافته‌ها بینش‌های ارزشمندی در مورد پتانسیل بیوچار در بهینه‌سازی فرایند کمپوست‌سازی ارائه می‌دهند.

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