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RESEARCH

Valorization of grape pomace through melanin extraction and its biostimulant effects on yield and development of winter barley

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ABSTRACT

Recent advancements in agricultural research have focused on the development of plant- and animal-derived substances with biostimulant properties. Among these, melanin extracted from grape pomace has garnered significant attention due to its diverse biological activities and promising potential as an agricultural biostimulant. This study aims to identify the optimal technological parameters for extracting natural melanin from plant-based industrial waste. Additionally, it seeks to determine the optimal application rate of the extracted melanin and evaluate its effects on the growth and yield parameters of winter barley (*Hordeum vulgare* L.) Melanin content was determined using gravimetric and spectroscopic methods. Field trials followed the Community Plant Variety Office (CPVO) distinctness, uniformity and stability (DUS) testing protocol for barley. The optimal conditions for melanin extraction from grape pomace were determined to be a solid-to-liquid ratio of 1:14, a 0.4 M NaOH solution concentration, an extraction temperature of 60 °C, and a duration of 2.5 h. Under these conditions, the melanin yield reached 90% of the initial content present in the raw material. Field experiments were conducted from 2021 to 2024 under irrigated conditions on brown semi-desert soils, using a completely randomized design with three replicates. Melanin acts as a biostimulant for winter barley when applied at a concentration of 0.028% for seed treatment and 0.014% for three foliar applications, starting at the stem extension stage and continuing weekly through the heading stage. The results demonstrate that melanin enhances seed germination by 7.6% in the field conditions, accelerates plant development, reduces maturation time by 7-11 d, and increases yield by 2.41 t. The incorporation of melanin extract into agricultural systems offers a practical strategy for improving crop productivity, promoting sustainability, and addressing the challenges faced in modern farming.

Key words: Biostimulant, extraction, melanin, productivity, winter barley.

INTRODUCTION

Agriculture plays a crucial role in the Republic of Armenia (RA), but the country faces significant challenges in achieving self-sufficiency in cereal production, leading to a reliance on grain imports. Over the past decade, cereal production has declined (Avagyan et al., 2024) due to factors such as small farm sizes, water scarcity, climate change, limited technology, and knowledge gaps among farmers.

Addressing these challenges requires strategies to enhance productivity and ensure food security, including the adoption of high-yield crops, modern cultivation technologies (Avagyan et al., 2024), and naturally derived biostimulants like melanin. Research shows that melanin is a potent phytostimulant, promoting plant growth and development, thus making it an effective tool for sustainable crop cultivation (Avetisyan et al., 2024). Biostimulants, sourced from natural materials, provide a sustainable alternative to conventional agricultural inputs. They promote eco-friendly farming by enhancing plant growth, nutrient uptake, and crop yields, while also reducing dependence on chemical fertilizers and pesticides (Rouphael and Calla, 2018).

Melanins are natural pigments with diverse physicochemical properties, making them valuable for applications in medicine, pharmacology, and agriculture (Zheng et al., 2019; Tran-Ly et al., 2020). In plants, melanin functions as a potent biostimulant, primarily due to its strong antioxidant capacity that enables it to scavenge reactive oxygen species (ROS) and neutralize harmful radicals (Muñoz-Torres et al., 2024).

Beyond its antioxidant role, melanin modulates plant hormonal signaling by mimicking auxins, thereby promoting root and shoot development and influencing the activity of stress-related hormones (Ikram et al., 2024). It also enhances N metabolism by stimulating root growth and enzymatic activity, which leads to improved N uptake, assimilation, and protein synthesis (Guo et al., 2023). In addition, melanin supports C metabolism by protecting chloroplasts, boosting photosynthetic efficiency, and facilitating C fixation, thereby contributing to overall plant growth. The biosynthesis of melanin in plants is closely linked to C metabolism through its involvement in phenylpropanoid and fatty acid biosynthetic pathways (Glagoleva et al., 2020).

Agronomically, melanin has been applied as a pre-sowing seed treatment and foliar spray, with significant positive effects on crop yield across a variety of species including cereals, potatoes, chickpeas, tomatoes, peppers, and eggplants (Sadoyan et al., 2023; Aghajanyan et al., 2024b; Haggag et al., 2024). Its auxin-like activity fosters root and shoot formation while improving plant resilience under stress conditions.

Despite its promising agricultural potential, the widespread adoption of melanin faces significant challenges. High production costs, limited scalability, and a lack of long-term studies across different crops and environmental conditions hinder its broader use (Muñoz-Torres et al., 2024). Moreover, melanin's effectiveness can vary depending on soil type and climate, and the absence of regulatory frameworks further limits its integration into mainstream agricultural practices.

Melanin is traditionally extracted from plant and animal sources or produced through chemical synthesis; however, large-scale production remains a major bottleneck due to the high cost of precursors and the complexity of synthesis processes, ultimately limiting its economic feasibility (Martínez et al., 2019; Pavan et al., 2020; Kurian, 2022; Tsouko et al., 2023; Yang et al., 2023). Overcoming these limitations is essential for the broader application of melanin-based materials in agriculture and other industries.

Future progress depends on the development of cost-effective, scalable production methods that yield melanin with consistent chemical structures (Tran-Ly et al., 2020). Microbial synthesis using fungi and bacteria has emerged as a promising, sustainable alternative (Singh et al., 2021). Additionally, low-cost melanin extracts derived from industrial plant waste have demonstrated multifunctional biostimulant properties, including antioxidant, antimicrobial, radioprotective, and immunomodulatory effects (Aghajanyan et al., 2024a).

The biosynthesis of water-soluble melanin using *Bacillus thuringiensis* strains has been extensively studied (Margaryan et al., 2019; Zhu et al., 2022; Sadoyan et al., 2023; Avetisyan et al., 2024; Muñoz-Torres et al., 2024). These studies show that the fermentation process, using a high-cost multi-component nutrient medium, not only promotes melanin production but also results in the synthesis of insecticidal toxins. Therefore, the potential presence of these toxins in ripened plants and fruits must be carefully considered.

Recent research emphasizes the value of grape pomace and skins as sources of bioactive compounds (Panić et al., 2019; Souza et al., 2022). The biostimulant melanin extraction method from winery waste, involving 3-4 technological stages, matches microbial melanin in terms of physicochemical properties and biological activity (Aghajanyan et al., 2022; 2024a). This makes the proposed biostimulant extraction method both relevant and beneficial.

The objective of this research was to develop a cost-effective method for extracting natural melanin with pronounced biostimulatory properties from plant-based industrial waste, using a low-concentration NaOH solution and characterized by low energy consumption. Additionally, the study aims to determine the optimal application rate of the extracted melanin and evaluate its effects on the growth and yield parameters of winter barley.

MATERIALS AND METHODS

Melanin extraction

The melanin extract was obtained from the preliminary washed and dried black grape pomace (sourced from the Armavir Wine Factory, Armavir, Republic of Armenia [RA]) by extraction with a 0.4 M concentration of NaOH solution at extraction temperature 60 °C for 2.5 h and a solid to liquid ratio of 1:14. The extract was separated from the insoluble mass by centrifugation at 5000g for 30 min. The melanin content in the obtained extracts was determined using two methods: The gravimetric method, which involves the isolation of purified melanin from an alkaline extract, and the spectroscopic method, as described by Aghajanyan et al. (2022; 2024b). The following equipment was used for melanin extraction from grape pomace: EuroEA 3000 elemental analyzer (EuroVector, Pavia, Italy); pH/mV/ion/TempMeter BT-675 (BOECO, Hamburg, Germany); 20L double layer stainless steel chemical reactor (LSR-20L, LABOAO, Zhengzhou, China); and laboratory centrifuge (GL-10MD, Cence, Changsha, China).

Spectral analysis

The absorption spectra of the grape melanin-containing fraction were recorded using a UV-1800 spectrophotometer (Nanbei Instrument Equipment, Zhengzhou, China). The infrared spectra were recorded using a Nexus Nicolet FT-IR spectrometer (Thermo Fisher Scientific, Wilmington, Delaware, USA) with a ZnSe prism (4000-650 cm^{-1}) and single reflection. The measurements were conducted with 32 scans and a resolution of 4 cm^{-1}.

Experimental sites and design

The experiments were conducted over 3 yr (2021-2024), covering the 2021-2022, 2022-2023, and 2023-2024 growing seasons. The study was conducted at the Agrobiotechnology Scientific Center of the Armenian National Agrarian University (ANAU), located in the Vagharshapat community (40°15’61.78’’ N; 44°29’18.23’’ E; 862.92 m a.s.l.), in the Etchmiadzin region of Armavir Marz, RA. The research was carried out on irrigated brown semi-desert soils (classified as Aridisol according to the USDA soil classification) under irrigated farming conditions. The climate is semi-arid, characterized by hot, dry, and clear summers, and short, freezing, snowy winters with partial cloud cover. Throughout the year, temperatures typically range from -6.7 to 34.4 °C, rarely falling below -12.8 °C or rising above 38.3 °C. Annual precipitation averages between 250 and 320 mm.

Soil samples from the experimental plot were collected in 2020 and analyzed at the Soil Testing Laboratory of the Scientific Center of Vegetable and Industrial Crops under the Ministry of Economy RA. The analysis was conducted to assess the mechanical and chemical composition of the soil. Humus content and easily hydrolysable N were determined using Tyurin’s method, total N by the Kjeldahl method, available P by the Machigin soil extraction method, and exchangeable K by the Maslova method (Yagodin et al., 2023).

The soils of the experimental plots exhibited low N content (3.0-3.5 mg 100 g^{-1} soil), moderate P levels, and high K content. Humus content ranged from 2.05% to 2.35%, with a soil pH of 7.1-7.2. Over the course of the study, the experimental plots had been previously cultivated with corn, soybeans, and potatoes. The multi-row winter barley (*Hordeum vulgare* L.) ‘Marina’, sourced from the grain crop collection at the Scientific Center for Agrobiotechnology of ANAU, was selected for the study. The biostimulatory effects of melanin on winter barley were evaluated through both laboratory and field experiments.

In laboratory experiments, barley seeds were pre-treated by soaking them in melanin extract at concentrations of 0.041%, 0.028%, and 0.016% for 40-50 min, using a 1:4 extract-to-seed ratio. A total of 50 seeds per dish, with four replicates, were placed on moistened filter paper in Petri dishes and incubated at 20 °C. Germination percentage, rooting, and sprout formation were recorded after 9 d (Fiodor et al., 2023).

Following the harvest of the preceding crop, fertilization was carried out at rates of $P_{100}K_{60}$, with subsequent deep tillage to a depth of 30 cm. The fertilizers applied included triple superphosphate ($Ca(H_2PO_4)_2{\cdot}H_2O$, containing 46% diphosphorus pentoxide, P_2O_5) and potassium sulfate (K_2SO_4, with 41% K_2O content). Due to the soil’s insufficient N levels, co-fertilization was performed at sowing in the spring, applying N_{60} using urea ($CO(NH_2)_2$, a N-rich fertilizer with 46% N content). The remaining N, N_{35}, was applied during the tillering phase of barley growth.

Winter barley was cultivated in 5×2 m plots, with 10 rows per plot and a sowing rate of five million germinated seeds per hectare. The seeds were manually sown with 20 cm inter-row spacing, with 50 cm between variants and 100 cm between replicates. Each variant was replicated three times, and results were averaged across 12 plots. Due to the variety's high resistance to pests, no pesticides were applied. Field germination was assessed on the 10^{th} day using standard methods. Foliar applications of melanin solutions at concentrations of 0.024%, 0.014%, and 0.009% were conducted three times, beginning at the stem elongation stage and continuing through the heading stage. The pH of the applied solutions ranged from 7.0 to 7.5. A KNAPSAK-15 battery-powered sprayer was used, with an application volume of 300 L ha^{-1}. All trials were conducted, and parameters recorded, in accordance with the CPVO DUS testing protocol for barley, following a completely randomized design (CRD).

Analysis of winter barley productivity

At physiological maturity, barley was manually harvested by selecting 100 ears from each plot. Morphological characteristics and productivity were analyzed using established methodologies (Lyon and Klein, 2024). Seed sowing quality, germination, plant survival, growth duration, growth rate, and yield structure were assessed using the metric method, with three replicates per treatment.

Statistical analysis

Statistical analysis was performed using one-way ANOVA to compare the means across various experimental variants. The results are presented as mean values based on three replicates. Statistical significance was determined using the least significant difference (LSD) test at $p < 0.05$. The effects of melanin treatments were considered statistically significant at $p < 0.05$, ensuring accurate differentiation between means while minimizing the risk of Type I error. Error bars in the graphs represent standard deviation and were generated using Microsoft Excel.

RESULTS

The results are presented in two parts: The first focuses on melanin extraction from grape pomace and the identification of the extracted melanin samples, while the second explores the optimal concentrations and their application in winter barley production.

Technological parameters for obtaining melanin extract and identification of the obtained samples.

The experimental data on the extraction of melanin from grape pomace demonstrated that the yield of melanin with biostimulating properties is influenced by the concentration of the alkaline solution, the extraction duration, and the temperature of the extractant.

The data illustrated in Figure 1 shows that melanin yield from black grape pomace, obtained through extraction with a 0.2 M concentration of NaOH solution at extraction temperature of 60 °C for 2.5 h, was approximately 14.05%. In contrast, the yields with 0.3, 0.4, 0.6, 0.7, and 1.0 M concentrations of NaOH solution were approximately 15.6%, 16.3%, 17.0%, 17.8%, and 19.1%, respectively.

The studies have shown that the optimal ratio of solid (extract) to liquid (alkaline solution) phases in the extraction process is 1:14.

The research revealed that when extraction was carried out with a 0.4 M concentration of NaOH solution at extraction temperature of 25, 40, 60, 80, and 120 °C, the melanin yield from grape pomace was approximately 7.8%, 14.0%, 17.1%, 18.2%, and 19.4%, respectively (Figure 2).

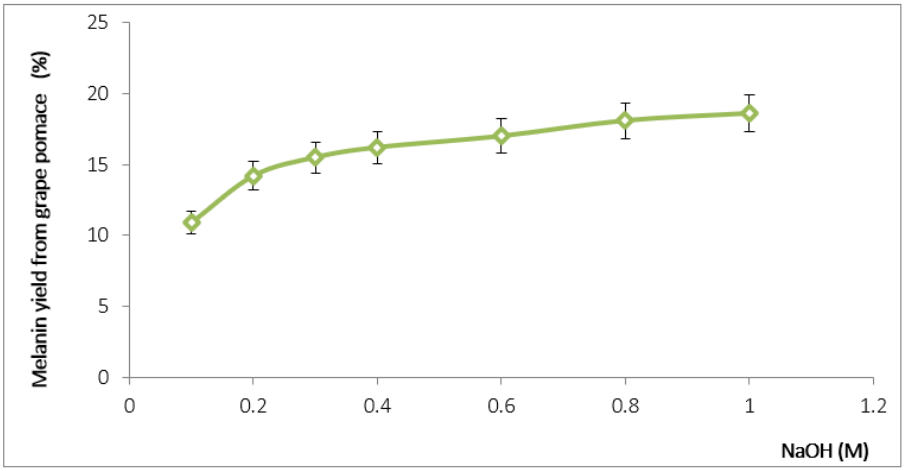

Figure 1. Dependence of melanin yield obtained from grape pomace through extraction on the concentration of NaOH used, τ-(extraction time) 2.5 h, t = 60 °C. Vertical bars correspond to standard error.

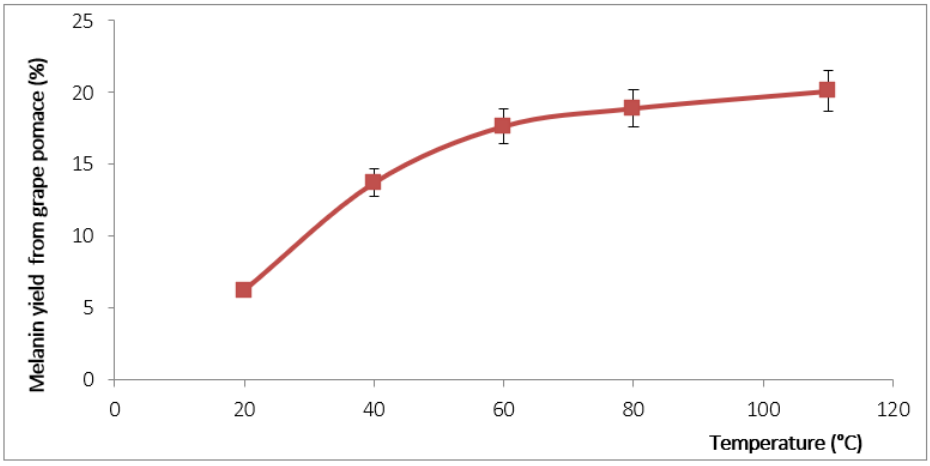

Figure 2. Dependence of the melanin yield obtained from grape pomace through extraction with a 0.4 M NaOH solution on the extractant temperature, τ- (extraction time) 2.5 h. Vertical bars correspond to standard error.

Figure 3 illustrates the influence of extraction duration on melanin yield, showing that the contact time between the solid and liquid phases significantly affects the extraction parameters. Therefore, it is recommended to conduct the extraction for at least 2.0-2.5 h.

The optimal parameters for efficient melanin extraction from black grape pomace are a solid-liquid ratio of 1:14, 0.4 M alkaline solution, an extraction temperature of 60 °C, and a duration of 2.5 h. Under these conditions, the melanin yield reached 90% relative to its initial content in the raw material. In the DM of the extract, melanin accounts for approximately 56%. Other major components include phenolates (36%), flavonoids (3.5%), and sodium chloride (0.5%-0.7%). In the extract, phenols are completely converted to phenolates. Minor constituents derived from grape skins-such as gallic acid, catechin, epicatechin, lignin, and related compounds-are also present. The residual sodium hydroxide (NaOH) content is low, as indicated by the extract's pH range of 8.0 to 8.5. Any remaining NaOH is neutralized with hydrochloric acid (HCl).

The infrared (IR) and ultraviolet (UV) absorption spectra of the obtained samples were recorded to identify the melanin. As can be seen from Figure 4, when using different technological parameters at the stage of melanin extraction from grape pomace, the IR spectrum of all obtained melanin samples (nine samples) has the same wavelength fluctuations. The spectrum contains bands that are identifying for melanins (Aghajanyan et al., 2022).

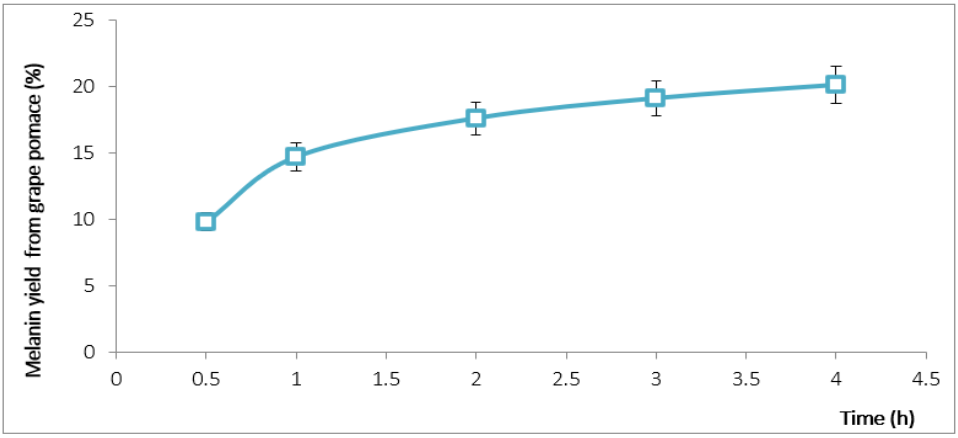

Figure 3. Dependence of melanin yield on the contact time of grape pomace with a 0.4 M NaOH solution during extraction, t = 60 °C. Vertical bars correspond to standard error.

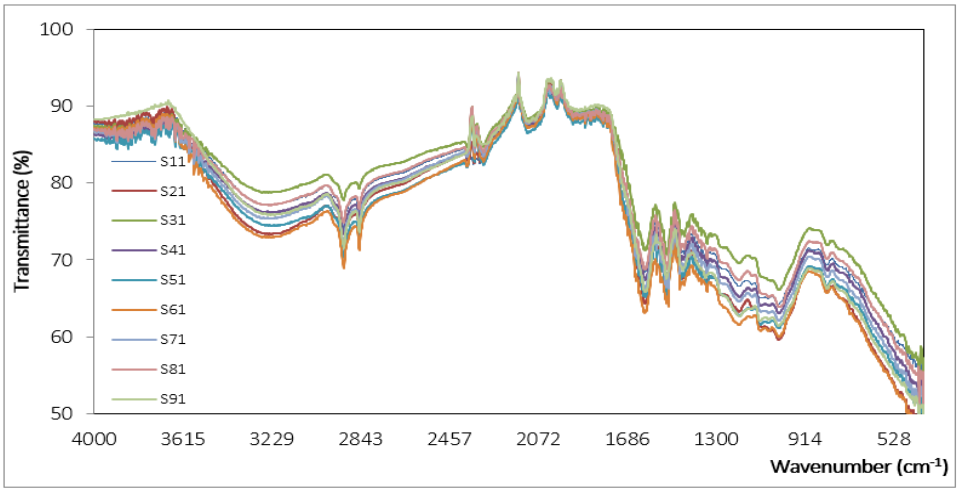

Figure 4. Infrared spectra of the obtained nine samples (S11-S91) of melanin. Extraction parameters of the obtained samples: S11 0.8 N NaOH, τ (extraction time) 2 h, t 60 °C; S21 0.7 N NaOH, τ 2 h, t 60 °C; S31 0.6 N NaOH, τ 2 h, t 60 °C; S41 0.4 N NaOH, τ 2 h, t 60 °C; S51 0.4 N NaOH, τ 2 h, t 40 °C; S61 0.4 N NaOH, τ 2 h, t 20 °C; S71 0.4 N NaOH, τ 1 h, t 60 °C; S81 0.4 N NaOH, τ 2.5 h, t 60 °C; S91 0.4 N NaOH, τ 4 h, t 60 °C.

The bands in the region of 3330 cm^{-1} are due to stretching vibrations of -OH and -NH groups, 2920 and 2850 cm^{-1} correspond to aliphatic $–CH_2$, $–CH_3$ groups, the characteristic absorption in 1610 cm^{-1} corresponds to stretching vibrations of conjugated double bonds C=C and C=O in the composition of secondary amides, in the range of 1520-1450 cm^{-1} to CO bonds of phenols. In the range 1200-1030 cm^{-1}, there are bands assigned to various stretching vibrations of the ether C–O–C and hydroxyl C–O groups. Comparison of the IR spectra of melanin pigments isolated from various plant raw materials with synthetic and microbial ones shows the similarity of the main absorption bands.

When comparing the IR spectra of the obtained melanin pigments with the spectrum of melanins obtained from various plant objects and with synthetic and microbial melanin (Aghajanyan et al., 2022), a similarity is found in relation to the main absorption bands.

To identify the nine melanin samples, their absorption spectra were also recorded in the ultraviolet (UV) region. As shown in Figure 5, the absorption exhibits flat curves with a gradual decline in optical density as the wavelength increases from 200 to 600 nm. This pattern is typical of all melanins, regardless of their origin, consistent with the findings of Aghajanyan et al. (2022). Maximum absorption is observed at a wavelength of 200 nm. A small absorption peak is observed in the spectrum at 260-280 nm, which is apparently due to the presence of protein in the pigment molecule. Notably, the UV absorption spectra of the nine melanin samples demonstrate high similarity, as illustrated in Figure 5.

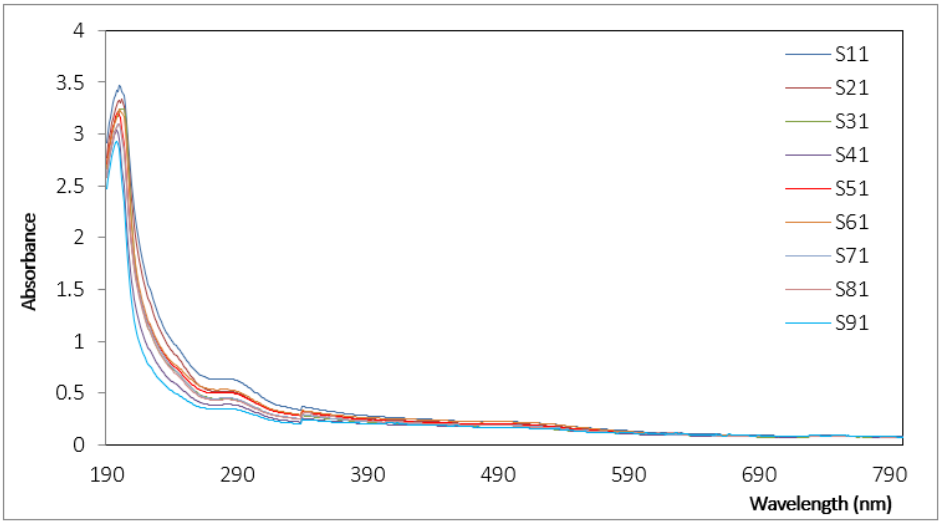

Figure 5. Absorption spectra of melanin samples in the UV region. Extraction parameters of the obtained samples: S11 0.8 N NaOH, τ (extraction time) 2 h, t 60 °C; S21 0.7 N NaOH, τ 2 h, t 60 °C; S31 0.6 N NaOH, τ 2 h, t 60 °C; S41 0.4 N NaOH, τ 2 h, t 60 °C; S51 0.4 N NaOH, τ 2 h, t 40 °C; S61 0.4 N NaOH, τ 2 h, t 20 °C; S71 0.4 N NaOH, τ 1 h, t 60 °C; S81 0.4 N NaOH, τ 2.5 h, t 60 °C; S91 0.4 N NaOH, τ 4 h, t 60 °C.

To identify the obtained melanin, qualitative reactions with oxidants (H_2O_2, $KMnO_4$, HNO_3) and FCl_3 characteristic of melanins were also used, and the presence of phenolic and quinone groups in the melanin structure was shown (Aghajanyan et al., 2024a).

To establish the belonging of the obtained pigment to melanins, qualitative reactions with oxidizing agents (H_2O_2, $KMnO_4$, and $FeCl_3$) were used. The experiments showed that in the presence of a small amount of $FeCl_3$ a brown, flocculent residue precipitated from the aqueous pigment solutions, which disappeared when an excess of $FeCl_3$ was added, indicating the presence of polyphenols in the melanin solutions. When $KMnO_4$ was added to the aqueous solutions of melanin, the solutions decolorized and a brown-black precipitate was formed. Discoloration of the melanin solutions also occurred when H_2O_2 solution was added to the melanin solutions.

Biostimulating effect of melanin extract in winter barley production

Adhering to established agrotechnical practices is crucial for optimizing the cultivation of agricultural crops. By diligently following these practices, farmers can enhance grain crop productivity, leading to improved food security and economic stability.

In our experiments, conducted in accordance with established agrotechnical practices for winter barley cultivation, melanin extract was applied in two key stages: Pre-sowing seed treatment and foliar applications during the vegetation period. Pre-sowing seed treatment was performed using melanin solutions at concentrations of 0.041%, 0.028%, and 0.016%. Among these, the most effective concentration was identified as 0.028% for pre-sowing treatment.

During laboratory experiments, biometric measurements were taken to assess the germination rates of embryonic roots and sprout formation, including the number of radicles and sprout length, in both control and melanin-treated variants. Additionally, seed germination energy and duration were analyzed.

As shown in Table 1, pre-sowing seed treatment with melanin solution (0.028%) resulted in intensive radicle formation, with treated seeds producing 1.7 more roots (average of 5.1 roots) than the control. Radicle length also increased in treated seeds. Sprout length was 1.8 cm longer in treated seeds (4.2 cm) compared to the control (2.4 cm). Overall, the length of the sprouts increased by 42%, and the length of the embryonic roots by approximately 44%, compared to the control variant. These improvements led to better-developed sprouts, ensuring higher germination, adaptation, and survival of winter barley. Laboratory germination for treated seeds was 98.8%, 5.7% higher than the control. These results suggest melanin extract enhances radicle formation and sprout development, acting as a biostimulant for early barley growth.

Table 1. Influence of melanin extract on the winter barley sowing quality indicators, root growth capacity and germination.

	Lab experiments				Field experiments	
Version	Number of radicles	Length of radicles	Length of sprouts	Germination	Number of sprouts	Field germination
	nr	cm	cm	%	nr m^{-2}	%
Control	3.4	2.2	2.4	93.1	451.0	90.2
Treated	5.1	3.5	4.2	98.8	489.0	97.8
LSD_{05}	0.6	0.4	0.4	-	8.2	-

After laboratory studies, both treated and untreated barley seeds were sown in the experimental field at a rate of 500 seeds per m². Regular measurements were taken to monitor seed germination, rooting, and sprout formation (Table 1). The results showed that the melanin extract enhanced sprout formation and field germination rates in winter barley. Field germination in the treated variant increased by 7.6%, reaching 97.8%, which positively influenced plant density by 15.8% and improved winter survival rates. Table 2 presents an 18.6% increase in plant density (from 357.7 to 424.2 plants m^{-2}) and a 2.4% higher survival rate (90.7%) in the experimental variant compared to the control.

Table 2. Effect of melanin extract on the survival and growth capacity of barley plants.

			Tillering rate		Plant height during the growth stages		
Version	Number of plants	Number of plants after the winter	Total	Productive	Stem extension	Heading	Ripening
	nr m^{-2}	nr m^{-2}			cm		
Control	405.1	357.7	4.4	3.8	54.7	77.1	82.4
Treated	469.1	424.2	6.8	4.2	61.3	85.5	95.7
LSD_{05}	15.4	17.9	0.6	0.4	5.1	6.4	4.6

Grain crop yield is significantly influenced by the average tiller rate. The melanin-treated version showed a notable improvement in tillering, surpassing the control by 2.4 for total tillers and 0.4 for productive tillers, positively impacting grain yield.

The stimulatory effect of melanin extract on barley plants gradually decreased as growth progressed (Figure 6). The average field germination difference between the control and treated variants was 7.6%, which decreased to 6.1% before wintering. These differences were significant, as indicated by the error bars representing the standard deviation. However, the difference in plant counts per square meter reduced to 2.1% after wintering, which was nonsignificant.

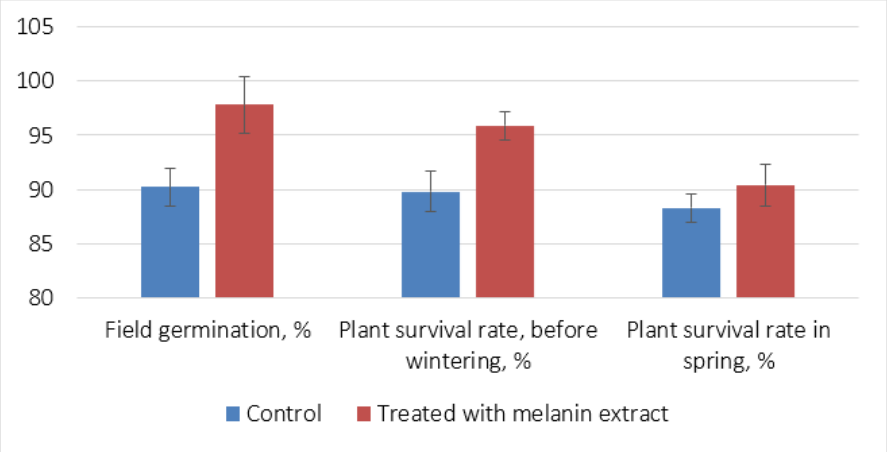

Figure 6. Stimulatory effect of melanin extract on barley plants during early vegetation. Vertical bars correspond to standard error.

To sustain the effects of the melanin extract, foliar applications were made three times at weekly intervals, beginning at Feekes 6-10 and continuing through Feekes 10.1, using melanin concentrations of 0.024%, 0.014%, and 0.009% (pH of the applied solutions ranged from 7.0 to 7.5). Among these, the most effective treatment was identified as 0.014% for foliar applications. The melanin extract notably enhanced barley growth, resulting in taller and more vigorous plants. At maturity, melanin-treated plants reached 91.7 cm, 13.3 cm taller than the control.

These plants sprouted 3 d earlier and began tillering 6 d sooner than the control, with faster growth observed at subsequent stages. By the heading stage, melanin-treated plants had advanced, while control plants remained in later stem extension stages, leading to an extended vegetation period (Table 3). At full ripening, melanin-treated plants were 12 d ahead of the control. Given that barley is a crop with a short growing season, the use of the melanin extract can further shorten its growing period, making it a more suitable predecessor for double cropping. This improves land use efficiency, enables earlier planting of subsequent crops, and enhances crop rotation, ultimately boosting overall productivity.

In melanin-treated version, lush growth was observed, along with notable improvements in key efficiency parameters, particularly in the spike's structural elements.

Key indicators of high grain yield -such as spike length, number and weight of grains per spike, and grain size (represented by the mass of 1000 grains), as shown in Table 4 -are critical for assessing a crop's yield potential and the effectiveness of treatments like melanin extract in enhancing productivity. In the test versions, the number of grains per spike exceeded the control by 24 grains (1.1 g), averaging 88.3 grains or 3.9 g per spike. Melanin extract also increased the 1000-grain mass by 1.1 g. Consequently, the final yield reached 6.85 t ha^{-1}, surpassing the control by 2.41 t. The average results from field experiments exhibited minimal variation across years, primarily influenced by annual weather conditions and preceding crops.

The effects of melanin treatment were statistically significant for all evaluated parameters (Tables 1, 2 and 4), with $p < 0.05$, indicating a clear differentiation between means and a minimized risk of Type I error.

Table 3. Effect of melanin on the transition periods of growth and development phases and the duration of the vegetation period in winter barley.

		Growth and development phases								
							Ripening			
	Version	Germination	Tillering	Spring re-growing	Stem extension	Heading	Milky	Waxy	Full	Duration of vegetation period (d)
2021-2022	Control	25.10	08.11	10.03	17.04	09.05	31.05	11.06	17.06	234
	Treated	23.10	03.11	03.03	09.04	01.05	21.05	31.05	04.06	223
2022-2023	Control	23.10	10.11	09.03	16.04	11.05	30.05	10.06	16.06	233
	Treated	20.10	05.11	02.03	08.04	01.05	20.05	31.05	04.06	226
2023-2024	Control	24.10	09.11	08.03	15.04	10.01	31.05	09.06	15.06	233
	Treated	21.10	03.11	01.03	07.04	30.04	20.05	30.05	03.06	224

Table 4. Effect of melanin extract on the structural elements and biological yield of winter barley.

	Per spike			1000 grains weight	Biological yield
Version	Number of spikelets	Number of grains	Weight of grains		
	nr	nr	g	g	t ha^{-1}
Control	21.3	64.3	2.8	43.1	4.44
Treated	29.4	88.3	3.9	44.2	6.85
LSD_{05}	2.7	5.7	0.4	1.7	0.6

DISCUSSION

Melanin, a versatile biomolecule, is crucial in biological systems and industrial applications due to its unique properties (Choi, 2021). A sustainable method for melanin production involves its extraction from plant waste via fermentation, converting agricultural residues into bioactive compounds like melanin (Meraj, 2023; Tsouko et al., 2023). Melanin derived from grape waste has shown promise as a biostimulant in agriculture and biotechnology (Razikova et al., 2024). Grape pomace contains phenolic acids, with concentrations varying by grape variety and processing method. For instance, white grape pomace contains 18.31-19.80 mg 100 g^{-1} syringic acid, while red varieties like 'Merlot' show higher levels (Onache et al., 2022). Grape skins also contain bioactive compounds with antioxidant and antimicrobial properties, increasing their value for sustainable applications (Andrade et al., 2021).

Fermentation-based melanin production is strain-dependent, requiring optimization of factors such as growth medium composition, temperature, pH, and precursor supplementation (Tsouko et al., 2023). The ratio of solid to liquid phases during extraction also significantly influences the yield and purity of melanin. Key factors include the melanin source (microbial or plant-based), the type of alkaline solution, and the extraction method (Tran-Ly et al., 2020).

Our study optimized melanin extraction from black grape pomace using a NaOH solution under low energy consumption conditions. From a technological perspective, the optimal yield was achieved with a 1:14 solid-to-liquid ratio, a 0.4 M concentration of NaOH solutions, an extraction temperature of 60 °C, and an extraction duration of 2.5 h. In contrast, Ma et al. (2019) optimized melanin extraction from rapeseed meal, obtaining a 9.0% crude melanin yield. Similarly, studies by Liu et al. (2019a) and Zheng et al. (2019) highlighted the importance of optimizing extraction parameters for diverse sources, such as *Auricularia polytricha* and *A. auricula*, and banana peel, achieving significant yields with tailored processes. These studies highlight the need for specific optimizations in melanin extraction from different plant materials, enhancing its applications in sustainable agriculture and biotechnology.

The extraction of melanin from grape pomace using highly concentrated alkaline solutions at elevated temperatures is not considered favorable. Under such conditions, the raw material tends to dissolve excessively, and non-melanin components may also solubilize, potentially leading to the formation of unwanted by-products and complicating the neutralization of residual NaOH in the final extract. For instance, increasing the NaOH concentration from 0.4 M to 0.8 M resulted in only a modest improvement in melanin yield-from 16.2% to 18.5%. Specifically, 1.944 g melanin was recovered using 0.4 M NaOH, while 2.42 g were obtained with 0.8 M NaOH, a net gain of only 0.476 g. Despite similar energy consumption in both extraction processes, the higher NaOH concentration (19.2 g used at 0.8 M) makes this approach less economically viable due to increased chemical usage and waste treatment requirements.

To verify the identity of the extracted pigments, we performed a comprehensive spectral analysis of all nine melanin samples obtained under varying extraction conditions. Despite differences in process parameters, the samples displayed consistent infrared (IR) spectral profiles, featuring absorption bands characteristic of melanin. These spectral features-widely recognized as markers for melanin compounds (Solano, 2014)-were present across all samples, indicating that the structural integrity of the melanin was preserved regardless of the extraction method used.

Melanin has proven to be an effective biostimulant, enhancing seed germination, early plant growth, and crop yields. In our experiments, winter barley seeds soaked in a 0.028% melanin extract for 40-50 min showed improved radicle elongation and sprout development, highlighting its phytostimulatory effects. Foliar applications of a 0.014% melanin extract solution further boosted winter barley yields. Furthermore, the UV-Vis spectra displayed broad absorbance with a gradual decrease toward the visible range, which is characteristic of melanins due to their complex, conjugated polymeric structures (Solano, 2014; Liu et al., 2019b). This spectral behavior aligns with earlier findings by Yao et al. (2012), who demonstrated similar profiles in natural and synthetic allomelanins. Our data also support the observations of Liu et al. (2019b), who emphasized the necessity of a multi-technique analytical framework-including UV-Vis, FT-IR, and electron spin resonance (ESR)-for the reliable identification of melanin.

As previously noted, the research was conducted under irrigated conditions within a semi-arid climate. During the study period (2022-2024), annual precipitation ranged from 348.1 to 361.4 mm- slightly above the long-term regional average -likely due to seasonal variability. Average annual air temperatures during these years remained relatively stable, ranging from 10.7 to 12.3 °C, with nonsignificant interannual fluctuations.

A consistently lower thermal background during the growing seasons contributed to stable yield performance across the study years. Winter barley grain yields showed minimal interannual variation, with recorded values of 6.95, 6.88, and 6.72 t ha^{-1} from 2022 to 2024. Deviations from the multi-year average yield were minor, ranging from ± 0.10 to ± 0.03 t ha^{-1} in 2022 and 2023. However, in 2024, a slight decline of 0.13 t ha^{-1} below the average yield was observed, likely due to reduced in-season precipitation and lower relative humidity levels.

Furthermore, trials evaluating the impact of melanin application revealed a significant enhancement in winter barley productivity. Control plots yielded an average of 4.44 t ha^{-1}, while plots treated with a melanin solution (0.028% for pre-sowing treatment and 0.014% for three foliar applications) achieved a notably higher mean yield of 6.85 t ha^{-1}. These results highlight the potential of melanin as an effective biostimulant for improving crop performance under semi-arid conditions.

These findings are consistent with previous research demonstrating the multifaceted physiological and agronomic benefits of melanin application in plants. Melanin has been shown to enhance key developmental processes such as seed germination and root and shoot growth, while significantly improving tolerance to

abiotic stresses through its potent antioxidant activity. Its ability to scavenge reactive oxygen species (ROS) contributes to greater cellular stability during critical phenological stages, including germination, flowering, and grain filling. In addition, melanin interacts with plant hormonal and enzymatic pathways, modulating the abscisic acid (ABA) signaling network to regulate stomatal conductance and osmotic balance under water-deficit conditions. It also promotes the activity of enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), thereby reinforcing the plant's defense mechanisms (Glagoleva et al., 2020; Muñoz-Torres et al., 2024).

Melanin's high thermal and photostability, along with its functional resilience under a wide range of environmental conditions, further support its suitability as an agricultural biostimulant. Its solubility and biological activity are pH-dependent, with increased stability observed in neutral to slightly alkaline environments. These properties underscore melanin's promise as a versatile tool for improving crop productivity and resilience in semi-arid and stress-prone agroecosystems (Glagoleva et al., 2020; Muñoz-Torres et al., 2024).

Beyond its intracellular effects on oxidative stress regulation and hormonal balance, melanin—derived from microbial, fungal, and plant sources—has demonstrated additional benefits as a sustainable biostimulant with broader agroecological significance. These advantages are reflected in structural and functional improvements that align with enhanced plant health and increased yield potential across various species (Zhao et al., 2017).

Empirical evidence from multiple crop systems—including potatoes, tomatoes, legumes, cereals, and vegetables—further supports the efficacy of melanin-based treatments. Approaches such as seed soaking and foliar spraying have led to significant increases in growth rate, stress tolerance, and final yield outputs (Margaryan et al., 2019; Garude et al., 2023; Aghajanyan et al., 2024a; 2024b). These effects are attributed not only to melanin's inherent antioxidant capacity but also to its role in modulating plant-microbe interactions and nutrient acquisition processes. Of particular interest is grape pomace-derived melanin, which combines antioxidant, antimicrobial, and biostimulant properties due to its rich matrix of phenolic acids and bioactive compounds. The composition of these compounds varies depending on grape cultivar and processing conditions, suggesting that the biostimulant potential of melanin can be enhanced or tailored through valorization of agro-industrial by-products-a promising step toward circular bioeconomy models in agriculture (Andrade et al., 2021; Onache et al., 2022).

However, the effects of melanin are dose-dependent, following a hormetic response curve. At low concentrations (e.g., 0.028%), melanin has been shown to stimulate beneficial physiological processes such as increased root number and shoot elongation in crops like potatoes (Margaryan et al., 2019). In contrast, higher concentrations can inhibit growth, indicating the need for precise dosage optimization to avoid phytotoxic effects and ensure consistent efficacy (Garude et al., 2023). Similar hormetic effects have also been confirmed in tomatoes and legumes, where melanin applications improved nutrient uptake and tolerance to abiotic stress (Muñoz-Torres et al., 2024).

Despite these promising results, several critical challenges remain. The stability and bioavailability of melanin under open-field conditions can be significantly affected by environmental variables, including UV radiation, soil pH, and microbial degradation, all of which may reduce its efficacy or alter its activity profile (Cordero and Casadevall, 2017).

A major technical limitation is the alkaline nature of melanin extracts, particularly those derived through alkaline hydrolysis. These extracts often retain residual NaOH and exhibit high pH values (typically 8.0-8.5), which may pose phytotoxic risks if not adequately neutralized. This is especially relevant for sensitive plant species or soils with low buffering capacity. Improperly adjusted pH levels can negatively impact germination, root development, and microbial communities in the rhizosphere (El-Naggar and Saber, 2022).

Therefore, while melanin has demonstrated substantial potential as a biostimulant that enhances crop performance and stress resilience, its effective deployment in sustainable agriculture will require advancements in formulation technology, standardization of application protocols, and large-scale validation across diverse agroecological contexts. Continued interdisciplinary research is essential to refine delivery systems, mitigate risks, and unlock melanin's full potential as a multifunctional tool for climate-resilient and resource-efficient crop production.

CONCLUSIONS

This study proposes a novel, cost-effective method for extracting melanin from grape pomace a low-concentration NaOH solution. This method not only ensures efficient melanin recovery but also valorizes winery by-products as sustainable and economically viable agricultural inputs.

In winter barley production, melanin extract exhibited pronounced biostimulatory effects, enhancing seed germination, accelerating plant development, shortening the growth period, promoting earlier maturation, and increasing grain yield. These results suggest that similar biostimulant benefits could be observed in other spiked cereals, due to their comparable physiological characteristics.

Author contributions

Conceptualization: G.A., G.H., K.Y., H.M. Methodology: A.A., G.A., G.H., H.M. Software: G.A., K.Y. Validation: A.A., G.A., H.M. Formal analysis: G.H., K.Y., N.G., H.M. Investigation: G.H., K.Y., N.G., A.T. Resources: G.H., K.Y., N.G., A.T. Data curation: A.A., G.A., A.T., H.M. Writing-original draft: A.A., G.A., H.M. Writing-review & editing: A.A., G.A., H.M. Visualization: G.H., K.Y., H.M. Supervision: A.A., H.M. Project administration: A.T. Funding acquisition: A.A., A.T. All co-authors reviewed the final version and approved the manuscript before submission.

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