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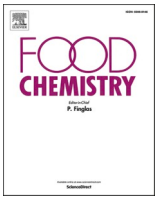

Effect of microbial biostimulants on the antioxidant profile, antioxidant capacity and activity of enzymes influencing the quality level of raspberries (*Rubus idaeus* L.)

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ABSTRACT

The influence of four microbial biostimulants containing various strains of *Bacillus subtilis* and/or *Paenibacillus* sp. on the quality of raspberries cv. Delniwa, Poemat, and Enrosadira cultivated in two consecutive seasons was investigated. The biostimulants influenced the antioxidant level, antioxidant capacity, phenolic acids and flavonoids profiles, enzymatic activity, and the degree of methylation and acetylation of the pectin in the raspberry fruits. The biostimulants had the greatest effect on the antioxidant content (16% - 20% increase) and capacity in the Delniwa raspberry fruits from the first season. A positive correlation was found between the activity of the β-galactosidase enzyme and ferric reducing power. In the second season, a decrease in the activity of pectin esterase and α-L-arabinofuranosidase and an increase in the degree of methylation of pectin were noted. Our results suggest that the changes in raspberry quality were related to the type of biostimulant applied.

1. Introduction

Raspberry (*Rubus idaeus* L.) is one of the most popular fruits in Europe. It is cultivated for its health-promoting properties (Valentinuzzi et al., 2018), among other reasons. Raspberry fruits contain abundant antioxidant compounds, including flavonols, catechins, ascorbic acid and ellagic acid derivatives (Arnold et al., 2022). These compounds not only scavenge free radicals and prevent diseases, including cancer (Iqbal et al., 2022; Qi et al., 2024), but also affect the shelf life and quality of the fruit following harvest (Le Bourvellec et al., 2009; Siemińska-Kuczer et al., 2022). The rapid loss of firmness and the rotting of raspberries after harvesting limits their freshness and is a significant problem for fruit producers (Valentinuzzi et al., 2018). The relatively short shelf-life of raspberry is the effect of cell wall solubilization induced by the hydrolytic reduction of polysaccharides (Stewart et al., 2001; Vicente et al., 2007). This affects both the availability and price of the fruit. For this reason, agronomic strategies are being sought to extend the shelf life of raspberries. Moreover, the European Union promotes organic farming by banning synthetic pesticides and fertilizers to protect plants (Sangiorgio et al., 2021) Biostimulants are considered as an alternative to plant protection products used to date.

Using biostimulants is an environmentally safe and effective way of improving the quality of fruit crops. Biostimulants based on microorganisms work similarly to other popular biostimulants (e.g. humic and fulvic acids, protein hydrolysates, and seaweed extracts) and ensure the increased uptake and rational use of nutrients (Drobek et al., 2021). A wide range of microbial biostimulants has been shown to improve crop yields by increasing their resistance to biotic and abiotic stresses. Among many other living microorganisms, *Bacillus* sp. and *Paenibacillus* sp. have been noted for their beneficial properties of inducing protective mechanisms and promoting plant development (Etesami et al., 2023). It has been observed that *Bacillus* sp. supports the yield and sugar content of sugar beets and increases barley yield (Çakmakçi et al., 1999). Spraying beans with a biostimulant containing *Bacillus licheniformis* and yeast increased the length, fresh and dry weights of the roots and the macronutrient (K, N, Ca, Mg) content of the roots (Akhtar et al., 2020). It has also been shown that *Bacillus* sp. protects rice against pathogenic microorganisms (Wozniak et al., 2020). In the case of raspberries, the positive effect of *Bacillus* sp. on both the growth rate and raspberry yield has been reported (Karakurt et al., 2011). *Paenibacillus* is a bacterium that antagonizes pathogens such as *Verticillium dahliae* and *Thielaviopsis basicola*. The protection provided by *Paenibacillus* sp. against pathogens is based on the formation of a biofilm around the roots, which shields the plant from disease. *Paenibacillus* sp. also produces many antibiotics

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against Gram-negative bacteria (Rybakova et al., 2016).

This paper aimed to comprehensively investigate the effect of microbiological biostimulants containing various strains of *Bacillus* sp. and *Paenibacillus* sp. on the nutritional quality and durability of three raspberry cultivars (Delniwa, Poemat and Enrosadira). The antioxidant content, the phenolic acids and flavonoids profiles, the enzymatic activity and the degree of methylation and acetylation were elucidated as the crucial parameters of raspberry quality tested in two consecutive growing seasons.

2. Materials and methods

2.1. Test material

The research material consisted of red raspberries (*Rubus idaeus* L.) from a field experiment, that were grown in the presence of biostimulants. The following three popular European raspberry varieties were selected for the experiment: Delniwa, Poemat, and Enrosadira. The plants were grown in the presence of various combinations of organic biostimulants (Table 1). The strains were selected from the SYMBIO-BANK of the National Institute of Horticultural Research in Skierniewice, Poland. The experiment was conducted at an organic farm in the temperate climatic zone in Kańczuga in southeast Poland (49° 58′ 44.0″ N 22° 23′ 39.4″ E). Weather conditions during the experiment ate characterized in Table S1. The experiment was established in May 2020 using the complete randomization method with three replications. Each replicate included 21 plants. There were 63 plants per experimental treatment for each raspberry variety. Due to the fact that the experiment included 3 raspberry varieties and 5 treatments, it used 945 plants. Each year, biopreparations were applied 2–3 times during the growing season from May to July at 7 to 10-day intervals. The fruits were harvested once in the first season (October 7, 2020, Fig. S1) and twice in the second year of the experiment (August 9, 2021 and October 14, 2021). Quality parameters of raspberries such as dry weight, soluble solid content, pH and titratable acidity are presented in Table S2.

2.2. Total anthocyanin content

The total anthocyanin content (TAC) of the raspberries was determined according to the procedures described by Spayd and Morris (1981) and da Silva et al. (2007). The raspberries (100 g) were homogenized. A 2 g of the pulp sample was added to 18 ml of 0.5% HCl in methanol and incubated at 4 °C for 1 h in the dark. The mixture was centrifuged. The absorbance of the supernatant was measured at a wavelength of 520 nm (Genesys 10S UV–Vis, Thermo Scientific, USA). The total anthocyanin content was calculated in triplicate from the formula: A_{520} × dilution factor × MW_{PGN}/MEC; where MW_{PGN} (molecular weight of pelargonidin-3-glucoside) = 433.2 and MEC (molar extinction coefficient) = 2.908×10^4; and was expressed in mg 100 g^{-1} FW.

2.3. Total polyphenol content

The total polyphenol content (TPC) was determined according to Sim et al. (2010). A sample (100 g) of raspberries was weighed and the juice was extracted by squeezing. An aliquot (20 μl) of juice was added with 1.58 ml of water and 100 μl of Folin–Ciocalteu reagent. The mixture was mixed, and 300 μl of saturated sodium carbonate solution was immediately added. The solutions were incubated at 40 °C for 30 min and protected from light. The absorbance was measured at 765 nm (Genesys 10S UV–Vis, Thermo Scientific, USA). The total content of polyphenols was determined against standard solutions of gallic acid (0.05, 0.15, 0.25, 0.35, 0.5, and 1.0 mg ml^{-1}) in triplicate and expressed in terms of mg 100 g^{-1} FW.

2.4. Total vitamin C content

The total vitamin C content was determined according to the Polish standard (PN-A-04019:1998, 1998). First, 50 g of raspberries were mixed with a 2% oxalic acid solution and filtered through paper filters. Then, 10 ml of the filtrate was titrated with a 2,6-dichlorophenol solution until a color change occurred. The total vitamin C content was expressed in terms of the mean of the triplicate data in mg 100 g^{-1} FW.

2.5. Antioxidant capacity

2.5.1. DPPH assay

The DPPH assay was used to determine the radical scavenging capacity of raspberries according to the method used by Hangun-Balkir and McKenney (2012). The DPPH (2,2-diphenyl-1-picrylhydrazyl) reagent was prepared by dissolving 0.01 g DPPH in 100 ml of 80% ethanol. Next, 100 g of raspberries were homogenized. 20 ml of 80% ethanol was added to 5 g of the pulp. The mixture was stirred for 20 min, then centrifuged (1800 ×*g*, 15 min, 4 °C), and the supernatant was collected. After that, 2 ml of DPPH reagent was added to 2 ml of supernatant and 2 ml of 80% ethanol (control). The solution was incubated at room temperature for 30 min, and the absorbance was measured at 517 nm using the same spectrophotometer as in previous sections. The antioxidant capacity is expressed as a percentage of the DPPH radical scavenging capacity according to the following formula: DPPH [%] = (A control – A sample) / A control × 100. The antioxidant capacity is expressed as the mean of the triplicates in %.

2.5.2. Ferric reducing power (FRAP)

The ferric reducing power (FRAP) was determined according to Pulido et al. (2000). An average of 0.02 g of freeze-dried fruit pulp was

Table 1
Growing conditions of raspberries cv. Delniwa, Poemat, and Enrosadira.

1 (control)	without microbiological biostimulants with manure
2	preparation carrier P3 (10 g in the soil) preparation carrier P4 (1 g 10 ml^{-1}; 20 ml applied to the root) preparation carrier P1 (50 l ha^{-1}) + preparation carrier P2 (50 l ha^{-1}) + 300 ml water; foliar applied; 2–3 times during the growing season preparation carrier P1 (6.25 ml) + preparation carrier P2 (6.25 ml) + 37.5 ml water; soil application; 2–3 times during the growing season
3	P3 (10 g in the soil) P4 (1 g 10 ml^{-1}; 20 ml applied to the root) P1 (50 l ha^{-1}) + P2 (50 l ha^{-1}) + 300 ml water; foliar applied; 2–3 times during the growing season P1 (6.25 ml) + P2 (6.25 ml) + 37.5 ml water; soil application; 2–3 times during the growing season
4	P1 (50 l ha^{-1}) + P2 (50 l ha^{-1}) + 300 ml water; foliar applied; 2–3 times during the growing season P1 (6.25 ml) + P2 (6.25 ml) + 37.5 ml water; soil application; 2–3 times during the growing season
5	P3 (10 g in the soil) P4 (1 g 10 ml^{-1}; 20 ml applied to the root)

P1: liquid preparation containing *Bacillus subtilis* (B4/19-AF75AB2) and *Bacillus subtilis* (B7/19- Sp115AD), P2: liquid preparation containing *Bacillus subtilis* (B7/19-Sp115AD) and *Paenibacillus* sp. (B13/19-Sp116AC), P3: powder preparation containing *Bacillus subtilis* (B4/19-AF75AB2) and *Bacillus* sp. (B6/19-AF75BC), P4: gel preparation containing *Bacillus subtilis* (B4/19-AF75AB2), *Bacillus* sp. (B6/19-AF75BC) and *Bacillus subtilis* (B7/19-Sp115AD).

collected for each sample. The pulp was covered with ethanol, stirred for 1 h and centrifuged (12,000 ×*g*). Then, 0.5 ml of phosphate buffer (0.2 M, pH 6.6) and 0.5 ml of 1% potassium ferrocyanide (K 3 [Fe (CN 6)]) were added to 0.5 ml of the extract. The mixture was incubated at 50 °C for 20 min. Next, 0.5 ml of 10% trichloroacetic acid was added to the mixture, and 1 ml of the solution was taken and mixed with 1 ml of distilled water and 0.2 ml of 0.1% iron chloride ($FeCl_3$). The absorbance of the solutions was measured at 700 nm (Genesys 10S UV–Vis, Thermo Scientific, USA). The FRAP was calculated based on the concentrations of Trolox (EMD Millipore, USA) standard solutions and expressed in terms of μmol g^{-1} FW. The analysis was performed in triplicate for each variant of the experiment.

2.6. Biomarkers of oxidative stress

The content of malondialdehyde (MDA), a marker of oxidative stress, was determined according to Liu et al. (2018) and Yang et al. (2020). To determine this value, 100 g of raspberries were homogenized. Then, 0.9 ml of 0.1% glacial trichloracetic acid was added to 0.1 g of the pulp. The mixture was incubated in an ice bath for 10 min and then centrifuged (1800 ×*g*, 10 min, 4 °C). Next, 1 ml of a solution of 0.67% thiobarbituric acid in 10% trichloroacetic acid was added to 0.05 ml of the supernatant. The sample was incubated at 95 °C for 15 min, then cooled in an ice bath and centrifuged (1800 ×*g*, 10 min, 4 °C). The absorbance was measured at 430, 532, and 600 nm. The MDA content was calculated using the following formulas: C [μmol L^{-1}] = 6.45 × (A532 - A600) - 0.56 × A430, MDA content [μmol kg^{-1}] = C × V/Vs × W × 1000; where C is the MDA concentration in the reaction mixture, V is the total sample volume [ml], Vs is the volume of the sample extract solution taken for the reaction [ml], and W is the sample weight [kg]. The total MDA content is expressed as the mean of the triplicate data in μmol kg^{-1} FW.

2.7. Identification of polyphenols using HPLC

The individual polyphenol content was determined according to the method developed by Häkkinen et al. (1998) and Jakobek et al. (2007) with some modifications. First, 100 g of raspberries were homogenized and freeze-dried. Second, 2.5 ml of vitamin C solution (16 mg ml^{-1}), 12.5 ml of methanol, and 5 ml of HCl (37%) were added to 0.5 g of dried material, and water was then added to make up a volume of 25 ml. The mixture was incubated at 35 °C for 16 h. Then the mixture was centrifuged, and 20 ml of the supernatant was freeze-dried. The dried material was dissolved in 2 ml of methanol, diluted 20 times, filtered (0.45 μm Teflon filter) and placed in an HPLC vial.

Methanolic solutions of polyphenol standards (gallic acid, catechin, chlorogenic acid, epicatechin, caffeic acid, p-coumaric acid, hesperidin, rutin, ellagic acid, and quercetin) were prepared in the following concentrations: 0.01, 0.03, 0.05, 0.07,and 0.1 mg ml^{-1}. The solutions were filtered (0.45 μm Teflon filter) and placed in HPLC vials.

The samples were analyzed using an HPLC system consisting of an 1130 HPLC quaternary pump, an S 5300 sample injector, an S 4120 column oven, and an S 3350 PDA detector (Sykam GmbH, Gewerbering, Germany) equipped with a Bionacom Velocity STR (4.6 mm i.d. × 250 mm, 5 μm) analytical column. The analysis was performed under the following conditions: injection volume 20 μl and mobile phase: 0.5% formic acid (A) and methanol (B). The extracts were eluted with the following binary gradient: Initial: 60% A, 40% B, 10 min: 40% A, 60% B, 21 min: 10% A, 90% B, 23 min: 0% A, 100% B, 30 min: 40% A, 60% B, 35 min: 60% A, 40% B, 38 min: 60% A, 40% B. The flow rate was 1.0 ml min^{-1} at 30 °C, wavelength 270 nm.

The individual polyphenol contents were determined as the mean of three replicates and were expressed in terms of mg 100 g^{-1} FW.

2.8. Cell wall stability determination

2.8.1. Enzyme activity

The enzymatic activity in raspberries was determined using the method developed by Wei et al. (2010). First, 100 g of fruit was homogenized. Then, 6 ml of 12% polyethylene glycol containing 0.2% sodium bisulphate was added to 3 g of pulp, mixed, and centrifuged (21,000 ×*g*, 10 min, 4 °C). 6 ml of 0.2% sodium bisulfate was added to the residue, which was mixed randomly and centrifuged (21,000 ×*g*, 10 min, 4 °C). Then, 6 ml of the extraction solution (0.1 M sodium acetate/100 mM sodium chloride/2% mercaptoethanol/5% polyvinylpyrrolidone) was added to the residue. The mixture was incubated in the dark for 1 h at 4 °C and centrifuged (21,000 ×*g*, 10 min, 4 °C). The supernatant was collected as an enzyme extract for further analysis.

To determine the polygalacturonase (PG, EC 3.2.1.15) activity, 0.2 ml of the enzyme extract was taken, then 0.8 ml of 0.5% polygalacturonic acid (in 50 mM sodium acetate buffer, pH 5.2) was added. After incubation (37 °C, 2 h), 2 ml of 0.1 M borate buffer (pH 9) and 0.3 ml of 1 M cyanoacetamide were added to the mixture. After that, the mixture was placed in a water bath (100 °C, 10 min). After they were set aside to cool down, the absorbance of the solutions was measured at 276 nm. Galacturonic acid solutions were used as standards. The PG activity was expressed as the mean of triplicates in terms of μg g^{-1} FW min^{-1}.

To determine the activity of pectin esterase (pectin methylesterase, PME, EC 3.1.1.11), 1 ml of the enzyme extract was taken, and 4 ml of 1% citrus pectin was added. The pH was measured, and the mixture was incubated at 37 °C for 1 h. After incubation, the sample was titrated with 0.01 M sodium hydroxide to pH 7.4. The PME activity was expressed as the mean of the triplicates in terms of μmol g^{-1} FW min^{-1}.

To determine the activity of α-L-arabinofuranosidase (AF, EC 3.2.1.55) and β-galactosidase (β-Gal, EC 3.2.1.23), 0.5 ml of enzyme extract was taken for the determination of. 0.5 ml of the appropriate substrate (3 mM *p*-nitrophenyl-α-D-arabinofuranosidase or 3 mM *p*-nitrophenyl-β-D-galactopyranosidase) and 0.5 ml of 0.1 M sodium acetate solution after incubation (40 °C, 10 min) was added to the enzyme extract. The mixture was incubated at 37 °C for 30 min. Next, 2 ml of 0.5 M sodium carbonate was added to the mixture to stop the reaction. The absorbance was measured at 400 nm. The standards used were various concentrations of *p*-nitrophenyl. The enzymatic activity of AF and β-Gal was expressed as the mean of the triplicate samples in terms of μmol g^{-1} FW min^{-1}.

2.8.2. Pectin extraction

Pectin was extracted from raspberries using ammonium oxalate solutions following alcoholic extraction of cell wall polysaccharides (Drobek et al., 2020). First, fresh raspberry fruits were homogenized with 96% ethanol, mixed and then filtered using 0,45 μm nylon filters (Merck Milipore, Germany). The residue was again mixed with ethanol and filtered until the negative result of the Dubois test for the presence of sugars (Dubois et al., 1956) and finally rinsed with acetone and dried at 40 °C.

Alcohol insoluble residue was then mixed with 0.25% ammonium oxalate (1:20 *w/v*), incubated at 85 °C) for 1 h and centrifuged at 20,000 ×*g*. The supernatant was collected, mixed with 96% ethanol alcohol and incubated for 24 h at 4 °C to precipitate the pectin. The mixture was then centrifuged at 20,000 ×*g*, and the pellet was washed twice with 96% ethanol and freeze-dried.

2.8.3. Galacturonic acid content

The D-galacturonic acid (GalA) content was determined using a continuous flow analyzer (CFA) SanPlus (Skalar, The Netherlands) according to the procedure recommended by the manufacturer. 2 mg of raspberry pectin fraction was incubated with 2 M methanolic HCl at 80 °C for 72 h and then with 2 ml of 3 M trifluoroacetic acid (TFA) solution at 100 °C for 7 h. Mono-galacturonic acid solutions (10–100 μg/ml) were used as a standard calibration curve. The samples were diluted

10 times and analyzed on a CFA in triplicate.

2.8.4. Degree of methylation and acetylation of pectin

The saponification procedure was performed to determine the degree of methylation (DM) and the degree of acetylation (DA) of the samples as follows: 5 mg of raspberry pectin extracted with ammonium oxalate was weighed according to Yu et al. (2021). Then, 0.5 ml of 0.2 M NaOH was added to the pectin and incubated at 4 °C for 2 h. The mixture was neutralized by adding 0.5 ml of 0.2 M H_2SO_4 and then centrifuged (10 min, 2000 ×*g*). The sample was filtered through a nylon filter (0.22 μm). The DM and DA were determined using an HPLC system consisting of an 1130 HPLC quaternary pump, an S 5300 sample injector, an S 4120 column oven and an S 3590 RI Detector (Sykam GmbH, Gewerbering, Germany) equipped with a Bionacom Velocity LPH (4.6 mm i.d. × 250 mm, 5 μm) analytical column coupled with a Bionacom Ultra Filter column protector (0.5 μm titanium frit) according to the method developed by Levigne et al. (2002) with some modifications by Yu et al. (2021). The mobile phase was 4 mM H_2SO_4, with a flow rate of 0.8 ml min^{-1}. Standard solutions of methanol and acetic acid with concentrations of 0.1%, 0.3%, 0.5%, 0.7%, 1%, 2%, and 3% were prepared to establish the standard curve. The determination was performed in triplicate for each variant of the experiment. The DM and DA were expressed in % terms.

2.9. Principal component analysis

Principal component analysis (PCA) was performed using Statistica (v.12, StatSoft Inc., USA) to assess the relationship between the tested parameters.

2.10. Statistical analysis

The results were analyzed using Statistica (v.12, StatSoft Inc., USA) by applying two-way analysis of variance (ANOVA) followed by a Tukey's HSD test; the significant differences were determined at $P < 0.05$. All experiments were performed in triplicate.

3. Results and discussion

Three varieties of raspberries (Delniwa, Poemat and Enrosadira) we cultivated in the presence of microbiological biostimulants in two consecutive seasons in order to evaluate the effect of applied treatment on the nutritional quality expressed by the total antioxidant, phenolics and vitamin C content, antioxidant capacity and biomarkers of oxidative stress, polyphenols profile, activity of cell wall-degrading enzymes, galacturonic acid content and degree of methylation and acetylation of pectins.

The antioxidant content of fruit indicates its health-promoting properties and quality (Jin et al., 2012). The total content of anthocyanins, polyphenols, and vitamin C in raspberry cv. Delniwa, Poemat and Enrosadira is presented in Table 2. For the Delniwa variety in the first season of cultivation (2020), the average increase in the level of antioxidants of 18% was mainly determined by biostimulants 2 (acting on vitamin C), 3 (polyphenols, vitamin C), and 4 (anthocyanins). In the second season (2021), the antioxidant content in raspberry cv. Delniwa increased by an average of 16% in raspberries treated with biostimulants 2 (vitamin C), 3 (vitamin C), 4 (anthocyanins), and 5 (anthocyanins and polyphenols). Overall, the antioxidant content increased in the second season compared to the first season, excluding the raspberries treated with biostimulants 2 and 3, where the polyphenol content decreased from 162.4 mg 100 g^{-1} in the control to 125.4 and 147.5 mg 100 g^{-1}, respectively (Table 2). These values are consistent with the average content of polyphenols (150 mg 100 g^{-1}) in raspberries depending on the variety, harvest season and cultivation method (Ponder & Hallmann, 2019).

The Poemat variety was less prone to a change in the level of antioxidants than Delniwa. For Poemat, average increases in the polyphenol content after treatment with biostimulants 2, 4, and 5 of 17% and vitamin C after treatment with biostimulant 2 by 19% as compared to the control were recorded in the second season (2021). In the second season, the antioxidant content was higher than in the first season, except for vitamin C, which decreased by an average of 9.5% in raspberry cv. Poemat treated with biostimulant 4 (Table 2). For the Enrosadira raspberry, in the second season, biostimulants 2, 3, and 4 statistically increased the content of anthocyanins by 3%, and biostimulant 5 increased the content of polyphenols by 16%. In Enrosadira, as in Delniwa and Poemat, the anthocyanin content was 10.7 ± 0.8–58.9 ± 1.9 mg 100 g^{-1}. Significant differences in the total anthocyanin content may depend on the variety grown, the cultivation method applied, and the extraction method employed, among other factors, and it could be around 12–70 mg 100 g^{-1} (Sariburun et al., 2010). The common relationship between the tested raspberries was an increase in the antioxidant content in the second season compared to the first season for most of the applied treatments, especially for biostimulants 4 and

Table 2
The antioxidant content (anthocyanins, polyphenols, vitamin C) in raspberry cv. Delniwa, Poemat, and Enrosadira.

Variety	Biostimulant	Anthocyanin content [mg 100 g^{-1} FW]		Polyphenol content [mg 100 g^{-1} FW]		Vitamin C content [mg 100 g^{-1} FW]	
		2020	2021	2020	2021	2020	2021
Delniwa	1 (control)	21.37 ± 0.92^{c}	50.64 ± 0.44^{c}	141.10 ± 2.61^{bc}	162.40 ± 4.89^{bc}	19.69 ± 0.67^{ab}	31.91 ± 1.90^{b}
	2	17.90 ± 0.74^{b}	43.42 ± 0.08^{a}	149.65 ± 3.00^{c}	125.44 ± 7.10^{a}	24.33 ± 0.80^{c}	37.07 ± 0.64^{c}
	3	14.69 ± 1.10^{a}	45.93 ± 0.10^{b}	164.12 ± 4.25^{d}	147.54 ± 2.92^{b}	23.08 ± 0.78^{c}	39.38 ± 0.97^{c}
	4	24.85 ± 0.08^{d}	56.34 ± 0.52^{d}	136.63 ± 3.19^{b}	159.38 ± 9.00^{bc}	18.47 ± 0.75^{a}	26.70 ± 0.02^{a}
	5	17.21 ± 0.96^{b}	56.17 ± 1.16^{d}	119.15 ± 3.15^{a}	170.32 ± 4.49^{c}	20.61 ± 0.75^{b}	26.50 ± 1.28^{a}
Poemat	1 (control)	18.65 ± 0.78^{b}	57.43 ± 2.35^{a}	131.49 ± 2.10^{b}	139.05 ± 5.36^{a}	19.81 ± 0.74^{bc}	22.74 ± 1.26^{b}
	2	17.33 ± 1.51 ^{ab}	57.06 ± 1.01 ^{a}	133.65 ± 4.68^{b}	173.92 ± 4.98^{c}	16.17 ± 0.65^{a}	26.88 ± 1.25^{c}
	3	15.96 ± 1.39 ^{ab}	57.28 ± 1.38 ^{a}	131.20 ± 3.37^{b}	151.89 ± 3.84^{ab}	18.93 ± 0.03^{b}	19.50 ± 0.28^{a}
	4	14.80 ± 0.87 ^{a}	56.99 ± 2.15 ^{a}	118.86 ± 2.39^{a}	155.01 ± 8.46^{b}	23.16 ± 0.74^{d}	20.95 ± 0.76^{ab}
	5	15.55 ± 1.10 ^{ab}	58.87 ± 1.86 ^{a}	119.24 ± 2.30^{a}	158.28 ± 5.02^{b}	20.96 ± 0.75^{c}	20.69 ± 0.75^{ab}
Enrosadira	1 (control)	13.79 ± 0.27^{c}	39.26 ± 0.28^{b}	79.87 ± 5.17^{a}	121.77 ± 7.08^{a}	17.29 ± 0.03^{c}	33.94 ± 1.57^{c}
	2	10.71 ± 0.77^{a}	40.13 ± 0.22^{c}	92.92 ± 1.91^{b}	120.02 ± 3.79^{a}	17.72 ± 0.75^{c}	33.13 ± 1.50^{bc}
	3	12.38 ± 0.84^{abc}	41.02 ± 0.04^{d}	79.31 ± 2.01^{a}	121.60 ± 3.68^{a}	15.21 ± 0.74^{b}	35.49 ± 1.36^{c}
	4	12.69 ± 0.78^{bc}	39.95 ± 0.17^{c}	86.78 ± 1.24^{ab}	123.56 ± 6.53^{a}	11.97 ± 0.72^{a}	24.66 ± 0.66^{a}
	5	11.80 ± 0.60^{ab}	35.12 ± 0.05^{a}	87.80 ± 4.99^{ab}	141.73 ± 8.24^{b}	14.39 ± 0.69^{b}	30.11 ± 0.70^{b}

The data are means ± SD (n > 3). Different letters (a – d) indicate the differences between biostimulants in each variety ($P < 0.05$) as determined by the ANOVA and Tukey tests. 2020: first season, 2021: second season; 1 (control), 2–5: biostimulants. A statistically significant increase in the content of the tested antioxidants compared to the control is indicated by green shading. A statistically significant decrease in the content of the tested antioxidants compared to the control is indicated by red shading. A separate statistical analysis was performed for each season.

5 (Table 2). Because the raspberries were grown under the same conditions, it may be concluded that the cultivation conditions (biostimulants 2–5), apart from the type of cultivar grown, influenced the changes in the antioxidant level. It has been reported that using appropriate growing conditions increases antioxidant and antioxidant enzyme content, which increases the antioxidant capacity of raspberries. Increasing the antioxidant capacity increases tissue resistance to pathogen invasion, reduces susceptibility to fruit rot, and enhances the health-promoting properties of the fruit (Jin et al., 2012). A significant increase in antioxidant content, including anthocyanin and vitamin C was also observed for strawberries subjected to fortification with microbial biostimulants containing *Bacillus subtilis* and *Paenibacillus polymyxa* (Drobek et al., 2024). The positive effect of microbial biostimulants on strawberries was also shown for fruits inoculated with *Rhizobium* and *Phyllobacterium* which resulted in an increased content of ascorbic acid and selected anthocyanins and polyphenols (Flores-Félix et al., 2018).

The antioxidant activity is widely used to test the ability of food components to act as a free radical scavenger (Chandrasekaran et al., 2019). The antioxidant properties of the tested raspberry samples are presented in Table 3. The antioxidant capacity of the raspberry samples was tested using the DPPH and FRAP methods. Most of the fruit showed >50% DPPH activity. The antioxidant capacity of the raspberry samples ranged from 50.3% ± 1.5% to 78.8% ± 0.2% in both harvest seasons (Table 3). The results were only higher than those produced by the controls for raspberries treated with biostimulants 2, 4 (Delniwa), and 3 (Enrosadira) by an average of 9% in the first harvest season (2020). These values are comparable with other studies where the DPPH level in the raspberry extract ranged from 37.6% to 87% (Basu & Maier, 2016).

The FRAP values, expressed as the Trolox equivalent, of the three tested raspberry varieties ranged from 4.4 ± 0.5 to 13.3 ± 1.1 μmol TE g^{-1} FW (Table 3). After treatment 2 (Poemat), the FRAP increased by 23% in the first season, and for 3 (Delniwa), 2, and 4 (Enrosadira), it increased by an average of 31% in the second season. In other cases, the biostimulants used reduced the ability to reduce FRAP compared to the control in both seasons.

The MDA content of the raspberry samples increased after treatment with biostimulant 2 (Poemat) by 33% in the first season. Treatment with biostimulants 2 (Enrosadira), 4, and 5 (Poemat) produced increases of 38%, 18% and 28%, respectively, in the second season. In the remaining variants of the experiment, a reduction in MDA was observed, the most distinctly for Delniwa subjected to biostimulant 5. A MDA content lower than that of the control group indicates the alleviation of oxidative stress following the use of the tested biostimulants (2–5) (Song et al., 2020).

The expected result is an increase in antioxidant capacity as measured by the DPPH and FRAP tests and a decrease in MDA content, which is a positive effect of the selected biostimulant on the quality characteristics of the raspberries. However, the processes that alleviate oxidative stress are complex and depend on the expression of genes associated with oxidants (Song et al., 2020). A reduction of 31.38 and 33.52% in MDA content under the influence of microbial biostimulants containing *Trichoderma album* and *Bacillus megaterium*, respectively, was observed for onions in their bulb tissues. However, in this experiment, the MDA content in control samples (approximately 11 nmol g^{-1} FW) was considerably higher than that determined for raspberries, which confirms that plants encounter oxidative stress under field circumstances (Younes et al., 2023).

The relationship between the antioxidant (anthocyanins, polyphenols, vitamin C) content and the antioxidant capacity determined by DPPH and FRAP tests was analyzed using PCA (Fig. S2). The increase in the total content of anthocyanins and vitamin C contributed to the increase in DPPH (observed clearly for Delniwa treated with biostimulant 2), while the total anthocyanin and polyphenol contents increased with FRAP in Delniwa (biostimulant 5), Poemat (biostimulants 2 and 3), and Enrosadira raspberries (biostimulant 4) (Fig. S2). The presented relationship suggests that the tested antioxidants act as scavengers of free radicals. The ability of anthocyanins (Bobinaite et al., 2012), polyphenols (Weber et al., 2008) and vitamin C (Bobinaite et al., 2012) to scavenge free radicals in raspberries has been proven by numerous studies. However, evidence suggests that polyphenolic compounds and vitamin C are responsible for most of the antioxidant capacity of raspberries (Bobinaite et al., 2012).

The influence of the phenolic compound content on the antioxidant capacity of the tested raspberries was observed. The DPPH and MDA content and FRAP level were compared with the phenolic acids and flavonoid contents (Fig. S3). PCA showed that an increase in DPPH was, for the most part, associated with an increase in the phenolic acid (gallic acid, ellagic acid) and flavonoid (catechin and epicatechin) content in both seasons for the three tested raspberry varieties. Polyphenolic compounds can act as donors of hydrogen atoms and electrons and increase the antioxidant capacity of fruit (George et al., 2015). Moreover, a correlation between an increased ellagic acid content and an increased antioxidant capacity along with decreased MDA levels has been demonstrated (Kowalska et al., 2019). This was particularly notable for

Table 3
Antioxidant capacity (DPPH and FRAP) and malondialdehyde content in raspberry cv. Delniwa, Poemat, and Enrosadira in two harvest seasons 2020 and 2021.

Variety	Biostimulant	DPPH [%]		FRAP [μmol g^{-1} FW]	MDA [μmol kg^{-1} FW]		
		2020	2021	2020	2021	2020	2021
Delniwa	1 (control)	55.02 ± 1.51^{a}	74.20 ± 0.16^{c}	9.16 ± 0.59^{b}	11.31 ± 1.16^{ab}	4.11 ± 0.27^{c}	5.47 ± 0.20^{bc}
	2	59.80 ± 0.49^{bc}	74.47 ± 1.28^{c}	7.19 ± 0.32^{a}	9.30 ± 0.88^{a}	2.88 ± 0.16^{ab}	5.71 ± 0.15^{c}
	3	53.36 ± 0.46^{a}	74.67 ± 0.07^{c}	6.50 ± 0.55^{a}	12.58 ± 0.92^{b}	2.98 ± 0.19^{b}	5.25 ± 0.07^{b}
	4	62.41 ± 1.85^{c}	69.49 ± 0.32^{b}	7.72 ± 0.7^{a}	11.58 ± 0.24^{ab}	2.74 ± 0.24^{ab}	5.28 ± 0.26^{b}
	5	56.63 ± 1.56^{ab}	67.26 ± 0.25^{a}	7.13 ± 0.11^{a}	13.29 ± 1.09^{a}	2.37 ± 0.02^{a}	4.34 ± 0.17^{a}
Poemat	1 (control)	66.00 ± 0.78^{d}	66.99 ± 0.50^{c}	7.80 ± 0.55^{c}	10.28 ± 0.54^{a}	2.43 ± 0.19^{ab}	6.23 ± 0.28^{ab}
	2	51.52 ± 1.10^{ab}	68.10 ± 0.62^{c}	9.57 ± 0.36^{d}	10.64 ± 1.13^{ab}	3.23 ± 0.16^{c}	5.48 ± 0.27^{a}
	3	50.33 ± 1.45^{a}	67.91 ± 0.63^{c}	6.45 ± 0.46^{b}	12.45 ± 0.51^{b}	2.26 ± 0.11^{a}	6.08 ± 0.18^{ab}
	4	53.60 ± 1.49^{b}	64.73 ± 0.31^{b}	4.47 ± 0.31^{a}	11.11 ± 0.91^{ab}	2.44 ± 0.23^{ab}	7.35 ± 0.23^{b}
	5	61.13 ± 0.59^{c}	63.24 ± 0.25^{a}	6.74 ± 0.70^{bc}	10.56 ± 0.35^{ab}	2.84 ± 0.19^{bc}	7.97 ± 0.37^{c}
Enrosadira	1 (control)	75.01 ± 1.24^{ab}	78.37 ± 0.12^{b}	5.90 ± 0.49^{b}	6.76 ± 0.29^{b}	3.51 ± 0.06^{c}	5.11 ± 0.30^{ab}
	2	76.37 ± 1.24^{bc}	79.98 ± 0.39^{c}	4.44 ± 0.47^{a}	8.81 ± 0.73^{c}	2.59 ± 0.18^{b}	7.04 ± 0.39^{c}
	3	78.81 ± 0.25^{c}	77.19 ± 0.39^{a}	5.48 ± 0.33^{ab}	6.42 ± 0.39^{b}	2.33 ± 0.20^{ab}	4.49 ± 0.16^{a}
	4	72.25 ± 1.67^{a}	77.31 ± 0.34^{a}	5.12 ± 0.34^{ab}	9.83 ± 0.67^{c}	2.62 ± 0.17^{b}	5.68 ± 0.26^{b}
	5	76.80 ± 0.98^{bc}	77.76 ± 0.12^{ab}	5.07 ± 0.28^{ab}	4.94 ± 0.24^{a}	1.99 ± 0.13^{a}	5.10 ± 0.14^{ab}

The data are means ± SD (n > 3). Different letters (a – d) indicate the differences between biostimulants in each variety ($P < 0.05$) as determined by the ANOVA and Tukey tests. 2020: first season, 2021: second season; 1 (control), 2–5: biostimulants; DPPH: antioxidant capacity, MDA: malondialdehyde content, FRAP: ferric reducing power. A statistically significant increase in the tested parameters compared to the control is indicated by green shading. A statistically significant decrease in the tested antioxidants compared to the control is indicated by red shading. A separate statistical analysis was performed for each season.

the Delniwa raspberries in both harvesting seasons (Fig. S3D, S3K). The influence of the chemical structure of polyphenolic compounds on the antioxidant capacity was also reported. The antioxidant capacity of the flavonoids results from the presence of the 3′, 4′-dihydroxy group in the B ring, the 3OH in the C ring, the C2-C3 double bond in the C ring fused with the 4-keto group, 3OH in the C ring and 5OH in the A ring coupled with the 4-carbonyl group and the C2-C3 double bond (Sun & Powers, 2007). The flavonoids with the greatest antioxidant potential are quercetin (not identified in the tested raspberries) and catechin (found in the tested raspberries). Their high degree of activity is due to five hydroxyl groups; quercetin also contains a 2,3 double bond in the C-ring and a 4-oxo group (Rice-Evans et al., 1996). The phenolic acids with the greatest antioxidant activity include gallic acid and its derivative ellagic acid, which were detected in the tested raspberries. The effectiveness with which free radicals were scavenged by gallic acid and its derivatives resulted from the presence of the OH group in the para position to the carboxyl group (Lu et al., 2006).

Phenolic compounds play the main role in reducing the level of mutagenic and carcinogenic reactive oxygen species in plant and animal cells (Chandrasekaran et al., 2019). The content of the tested phenolic acids and flavonoids in raspberries depended on the biostimulants used (Table 4). The greatest increase in the polyphenolic compound content after applying the biostimulants (2 – 5) was noted for the Poemat variety in the first season (Table 4). Biostimulants 2–5 increased the ellagic acid content (by 22%–127%), gallic acid (by 18%–23%), catechin (by 61%–119%) and rutin (0.1–5.5 times; none in the control). However, biostimulants 3 and 4 mainly had the greatest effect on the content of caffeic acid (a 200%–700% increase) and chlorogenic acid (by 3%–67%), and biostimulants 3–5 contributed to an increase in hesperidin levels (by 100%–200%) in the Poemat variety in 2020. Among the tested phenolic acids and flavonoids, gallic acid and catechin were present in the highest quantities (9.6 ± 0.0–18.6 ± 0.3 mg 100 g^{-1} and 4.3 ± 0.2–27.3 ± 1.8 mg 100 g^{-1}, respectively), which corresponded to the literature data, where the content of gallic acid in raspberries has been reported to range from 7.7 to 19.7 mg 100 g^{-1} and the levels of catechin ranged from 6.5 to 42.43 mg 100 g^{-1} (Frum et al., 2017; Gevrenova et al., 2013; Okatan, 2020). PCA revealed a relationship between the compactness of the tested polyphenolic compounds (Fig. S3). According to the results, the increase in gallic acid was accompanied by an increase in catechin in all experiments.

Abiotic and biotic stresses induce increased production of phenolic compounds in fruit. The absence of herbicides, pesticides, and insecticides in cultivation typically accelerates the production of phenolic compounds (Häkkinen & Törrönen, 2000). Colonization of plants by beneficial microbes can also trigger phenolic compounds production as the result of more frequent interactions between plants and beneficial biotic agents (Munné-Bosch & Bermejo, 2024). The overall overproduction of phenolic acids and flavonoids in the first season and their decrease in the second season may suggest that the selected biostimulants (2 – 5) increased plant resistance to abiotic and biotic stresses in the second growing season, i.e., their impact is long-term.

It is assumed that changes in the antioxidant and phenolic compound content occur due to the biostimulants used, the main components of which are *Bacillus* sp. and *Paenibacillus* sp. The antioxidant and phenolic compound content plays a key role in the fight against pathogens and the reduction of reactive oxygen species. It has been shown that *Bacillus* sp. and *Paenibacillus* sp. increase the antioxidant capacity, act as a regulator of the synthesis of antioxidant compounds and reduce the risk of infection in tomatoes (Chandrasekaran et al., 2019), peaches (Wang et al., 2013), and grapes (Jiang et al., 2014), which confirms that the selection of *Bacillus* sp. and *Paenibacillus* sp. as a component of biostimulants is beneficial. Our results show that the effect of applied microbial biostimulants was strongly variety-dependent, however, in all tested raspberries biostimulants 4 and 5 showed the highest ability to enhance phenolic acids and flavonoids content.

Pectinolytic enzymes are responsible for relaxing the structure of the cell wall and may increase the release of antioxidants from cells (Szymanowska & Baraniak, 2019). The changes in enzymatic activity in the tested raspberries are shown in Fig. 1. A general downward trend in the PG, PME and AF activity was observed between the seasons. For the Delniwa variety, biostimulants 3, 4, and 5 significantly decreased the activity of PG and PME by an average of 27% (for PG) and 10% (for PME) in the second season compared to the first season. The decreased activity of these two enzymes due to the action of the biostimulants was most notable for the Delniwa and Enrosadira varieties. Moreover, in 2020 the biostimulants 3, 4, and 5 and 3 and 4 significantly reduced the PG activity for the Delniwa and Enrosadira varieties compared to the control by an average of 42% (for Delniwa) and 26% (for Enrosadira). A similar relationship was observed for PME; biostimulants 3, 4 and 5 lowered the activity of this enzyme by an average of 7% for Delniwa in the first season.

Biostimulants 2 (Poemat in 2021) and 4 (Poemat in 2020, Enrosadira 2021) were the most effective in reducing AF activity compared to the control. In contrast, the tested biostimulants increased the activity of β-Gal in the second season compared to the first season (Fig. 1). The biostimulants reduced the activity of β-Gal for Delniwa (2 in 2021, 3, 4 in 2020 and 2021), Poemat (2, 3 in 2021) and Enrosadira (2, 3, 4 in 2021). The results show a decrease in PG, PME, and AF activity between the seasons compared to the control after applying selected biostimulants.

PCA (Fig. S2) revealed that with increasing of β-Gal, the MDA content and FRAP in the first season for the three varieties of raspberries increased. The increase in β-Gal content may be related to the softening of the raspberry cell wall, which in turn caused an increase in oxidative stress, as defined by the level of MDA (Huang et al., 2023). Bennett et al. (2010) observed that in banana cell wall fractions after enzymatic and acid hydrolysis considerable increase in antioxidant capacity occurred. This effect was also indicated by an increase in the antioxidant potential (FRAP), which may have been activated to prevent the effects of oxidative stress (Tan et al., 2012).

The second noted relationship concerns the increased PG and AF of Delniwa, Poemat, and Enrosadira in the second season. The PG and AF activity increased in the early stages of fruit softening (Chea et al., 2019). *Bacillus* sp. and *Paenibacillus* sp. are capable of producing pectinolytic enzymes, which suggests that the tested biostimulants may modify the qualitative characteristics of raspberries (Ouattara et al., 2008; Soriano et al., 2005).

The DM and DA play a key role in pectin functionality. The demethylation of homogalacturonan is the key factor of the cell wall expansion mechanism participating in shaping plant cells (Haas et al., 2020). Changes in DM and DA result from the maturity and rotting phases of the fruit and are also directly related to the activity of pectinolytic enzymes (Willats et al., 2006). Overall, the pectin from the samples tested can be characterized for the most part as highly methylated (> 50%) (Fig. 2A, B, C) and poorly acetylated (<50%) (Fig. 2D, E, F). The increase in DM is mainly related to the effects of biostimulants 3 and 4, and to a lesser extent, 2 and 5. In the first season, a statistically significant increase in DM was observed for the samples treated with biostimulants 2 (Delniwa), 4, 5 (Poemat), and 3 (Enrosadira). In the second season, DM increased significantly compared to the control after treatment with biostimulants 3 (Delniwa, Poemat), 4 (Delniwa, Poemat), and 5 (Delniwa, Enrosadira). The increase in DM compared to the control was 9% to 44%, depending on the cultivar and preparation used. Biostimulants 3 (in part), 4, and 5 had the greatest effect on the increase in DA compared to the control. An increase in DA was observed for samples treated with biostimulants 4 and 5 (Delniwa), 3 (Poemat), and 5 (Enrosadira) in the first season and 4 (Poemat) and 5 (Enrosadira) in the second season compared to the control. These results are consistent with other studies where DM of 12%–53% and DA 1%–19% were found in raspberry juice (Will & Dietrich, 1994).

There was an overall upward trend for galacturonic acid in the second season compared to the first. Statistically significant increases in the

Table 4
The content of selected phenolic acids and flavonoids in raspberry cv. Delniwa, Poemat, and Enrosadira.

Variety	Biostimulant	Caffeic acid [mg 100 g^{-1} FW]		Chlorogenic acid [mg 100 g^{-1} FW]		Coumaric acid [mg 100 g^{-1} FW]		Ellagic acid [mg 100 g^{-1} FW]		Gallic acid [mg 100 g^{-1} FW]	
		2020	2021	2020	2021	2020	2021	2020	2021	2020	2021
Delniwa	1 (control)	1.11 ± 0.04^{b}	0.80 ± 0.01^{d}	0.36 ± 0.03^{c}	0.29 ± 0.03^{ab}	0.01 ± 0.00^{a}	0.02 ± 0.00^{a}	1.44 ± 0.07^{a}	1.32 ± 0.1^{b}	14.69 ± 0.07^{a}	14.11 ± 0.25^{c}
	2	1.67 ± 0.01^{d}	0.57 ± 0.03^{b}	0.26 ± 0.00^{ab}	0.31 ± 0.02^{b}	0.00 ± 0.00^{a}	0.02 ± 0.00^{a}	2.35 ± 0.03^{c}	1.34 ± 0.09^{b}	13.92 ± 0.23^{a}	11.75 ± 0.17^{b}
	3	1.35 ± 0.03^{c}	0.77 ± 0.02^{cd}	0.30 ± 0.01^{b}	0.31 ± 0.00^{b}	0.00 ± 0.00^{a}	0.01 ± 0.00^{a}	1.48 ± 0.01^{a}	1.31 ± 0.16^{b}	14.18 ± 0.11^{a}	15.73 ± 0.00^{d}
	4	0.18 ± 0.01^{a}	0.75 ± 0.01^{c}	0.24 ± 0.01^{a}	0.28 ± 0.01^{ab}	0.00 ± 0.00^{a}	0.01 ± 0.00^{a}	2.10 ± 0.03^{b}	1.32 ± 0.03^{b}	14.64 ± 0.10^{a}	11.01 ± 0.05^{a}
	5	0.16 ± 0.01^{a}	0.05 ± 0.00^{a}	0.30 ± 0.01^{b}	0.26 ± 0.01^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	2.53 ± 0.00^{d}	0.75 ± 0.02^{a}	14.14 ± 0.60^{a}	10.90 ± 0.06^{a}
Poemat	1 (control)	0.15 ± 0.00^{b}	0.05 ± 0.00^{a}	0.27 ± 0.00^{a}	0.27 ± 0.01^{b}	0.01 ± 0.00^{a}	0.00 ± 0.00^{a}	0.92 ± 0.02^{a}	0.62 ± 0.01^{a}	11.99 ± 0.14^{a}	9.61 ± 0.09^{a}
	2	0.03 ± 0.00^{a}	0.03 ± 0.00^{a}	0.33 ± 0.01^{b}	0.29 ± 0.00^{b}	0.11 ± 0.01^{c}	0.07 ± 0.00^{b}	2.40 ± 0.01^{e}	0.91 ± 0.09^{bc}	14.51 ± 0.25^{cd}	9.87 ± 0.06^{b}
	3	0.79 ± 0.04^{d}	0.47 ± 0.01^{d}	0.43 ± 0.01^{c}	0.23 ± 0.02^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	1.73 ± 0.01^{d}	0.78 ± 0.05^{b}	14.06 ± 0.08^{b}	11.26 ± 0.05^{c}
	4	0.27 ± 0.00^{c}	0.35 ± 0.01^{b}	0.48 ± 0.03^{d}	0.35 ± 0.03^{c}	0.03 ± 0.00^{b}	0.01 ± 0.00^{a}	1.08 ± 0.02^{b}	1.30 ± 0.01^{d}	14.84 ± 0.10^{d}	9.61 ± 0.13^{a}
	5	0.18 ± 0.01^{b}	0.44 ± 0.02^{c}	0.34 ± 0.01^{b}	0.22 ± 0.00^{a}	0.00 ± 0.00^{a}	0.01 ± 0.00^{a}	1.51 ± 0.04^{c}	0.97 ± 0.05^{c}	14.28 ± 0.07^{bc}	9.64 ± 0.02^{a}
Enrosadira	1 (control)	0.12 ± 0.02^{b}	0.22 ± 0.01^{b}	0.43 ± 0.03^{c}	0.64 ± 0.03^{c}	0.01 ± 0.00^{b}	0.01 ± 0.00^{b}	1.40 ± 0.03^{c}	1.54 ± 0.12^{c}	13.23 ± 0.34^{c}	18.57 ± 0.27^{d}
	2	0.11 ± 0.00^{b}	0.53 ± 0.0^{c}	0.35 ± 0.03^{b}	0.25 ± 0.01^{a}	0.00 ± 0.00^{a}	0.01 ± 0.00^{ab}	0.96 ± 0.09^{b}	1.02 ± 0.04^{b}	12.95 ± 1.17^{bc}	11.30 ± 0.24^{b}
	3	0.91 ± 0.04^{c}	0.52 ± 0.02^{c}	0.24 ± 0.00^{a}	0.24 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.79 ± 0.02^{ab}	0.76 ± 0.07^{a}	11.53 ± 0.17^{ab}	11.76 ± 0.06^{c}
	4	0.98 ± 0.01^{d}	0.71 ± 0.02^{d}	0.24 ± 0.00^{a}	0.24 ± 0.00^{a}	0.00 ± 0.00^{a}	0.01 ± 0.00^{ab}	0.74 ± 0.06^{a}	0.75 ± 0.04^{a}	13.10 ± 0.04^{c}	11.17 ± 0.03^{b}
	5	0.03 ± 0.00^{a}	0.04 ± 0.00^{a}	0.33 ± 0.01^{b}	0.49 ± 0.03^{b}	0.04 ± 0.00^{c}	0.05 ± 0.00^{c}	1.29 ± 0.09^{c}	0.77 ± 0.02^{a}	10.23 ± 0.02^{a}	10.66 ± 0.03^{a}

Flavonoids

Variety	**Biostimulant**	**Catechin [mg 100 g^{-1} FW]**		**Epicatechin [mg 100 g^{-1} FW]**		**Hesperidin [mg 100 g^{-1} FW]**		**Rutin [mg 100 g^{-1} FW]**	
		2020	2021	2020	2021	2020	2021	2020	2021
Delniwa	1 (control)	23.00 ± 0.22^{e}	21.71 ± 0.82^{c}	0.28 ± 0.02^{d}	0.38 ± 0.03^{b}	0.00 ± 0.00^{a}	0.16 ± 0.00^{d}	0.06 ± 0.01^{a}	0.00 ± 0.00^{a}
	2	16.64 ± 0.78^{c}	15.58 ± 1.43^{b}	0.35 ± 0.01^{e}	0.36 ± 0.04^{b}	0.18 ± 0.01^{d}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	3	8.90 ± 0.11^{a}	14.26 ± 0.35^{b}	0.11 ± 0.01^{a}	0.24 ± 0.02^{a}	0.22 ± 0.01^{e}	0.05 ± 0.01^{b}	0.08 ± 0.00^{a}	0.00 ± 0.00^{a}
	4	19.36 ± 0.73^{d}	14.18 ± 1.28^{b}	0.22 ± 0.02^{c}	0.39 ± 0.01^{b}	0.11 ± 0.01^{b}	0.11 ± 0.00^{c}	4.73 ± 0.07^{c}	0.07 ± 0.01^{c}
	5	14.26 ± 0.28^{b}	9.12 ± 0.06^{a}	0.17 ± 0.01^{b}	0.22 ± 0.01^{a}	0.15 ± 0.00^{c}	0.01 ± 0.00^{a}	0.42 ± 0.02^{b}	0.02 ±0.00^{b}
Poemat	1 (control)	10.86 ± 0.00^{b}	4.28 ± 0.16^{a}	0.38 ± 0.03^{b}	0.32 ± 0.01^{d}	0.14 ± 0.01^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	2	18.99 ± 0.48^{a}	5.70 ± 0.05^{a}	0.18 ± 0.00^{a}	0.03 ± 0.00^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	5.46 ± 0.03^{d}	0.00 ± 0.00^{a}
	3	18.02 ± 1.04^{a}	9.35 ± 0.00^{c}	0.26 ± 0.12^{ab}	0.20 ± 0.02^{b}	0.24 ± 0.02^{c}	0.08 ± 0.01^{b}	0.55 ± 0.05^{c}	0.00 ±0.00^{a}
	4	23.92 ± 1.06^{c}	7.98 ± 0.23^{b}	0.28 ± 0.02^{ab}	0.10 ± 0.01^{a}	0.34 ± 0.02^{d}	0.07 ± 0.00^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	5	17.60 ± 0.09^{a}	6.07 ± 0.36^{a}	0.18 ± 0.01^{ab}	0.17 ± 0.01^{b}	0.25 ± 0.02^{c}	0.22 ± 0.02^{c}	0.12 ± 0.01^{b}	0.00 ±0.00^{a}
Enrosadira	1 (control)	20.05 ± 0.90^{d}	27.31 ± 1.84^{d}	0.35 ± 0.02^{c}	0.42 ± 0.03^{b}	0.13 ± 0.00^{b}	0.00 ± 0.00^{a}	0.16 ± 0.01^{b}	0.00 ± 0.00^{a}
	2	10.88 ± 0.49^{c}	11.82 ± 0.99^{b}	0.25 ± 0.01^{b}	0.15 ± 0.00^{a}	0.01 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	3	8.27 ± 0.15^{b}	8.82 ± 0.13^{a}	0.10 ± 0.00^{a}	0.16 ± 0.00^{a}	0.01 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	4	11.14 ± 0.11^{c}	17.66 ± 0.47^{c}	0.12 ± 0.01^{a}	0.41 ± 0.00^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	5	6.55 ± 0.18^{a}	9.59 ± 0.19^{ab}	0.46 ± 0.03^{d}	1.61 ± 0.10^{c}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ±0.00^{a}

The data are mean ± SD (n > 3). Different letters (a – e) indicate the differences between the biostimulants in each variety ($P < 0.05$) as determined by the ANOVA and Tukey tests. 2020: first season, 2021: second season; 1 (control), 2–5: biostimulants. A statistically significant increase in the polyphenol and phenolic acid content compared to the control is indicated by green shading. A statistically significant decrease in the polyphenol and phenolic acid content compared to the control is indicated by red shading. A separate statistical analysis was performed for each season.

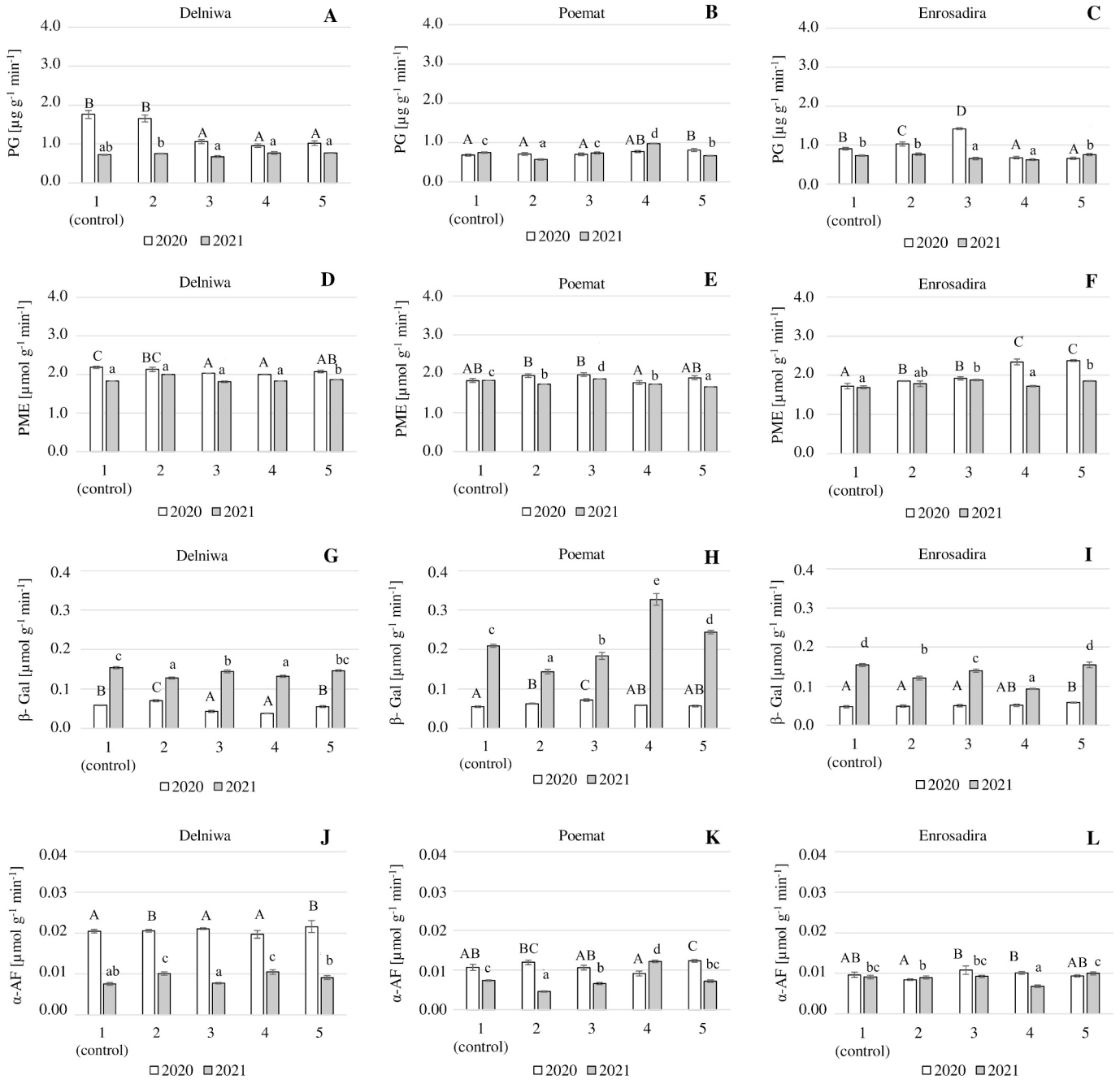


Fig. 1. Enzyme activity of raspberry cv. Delniwa, Poemat, and Enrosadira.
PG: polygalacturonase (A, B, C), PME: pectin methylesterase (D, E, F), β-Gal: β-galactosidase (G, H, I), α-AF: α-L-arabinofuranosidase (J, K, L). 2020: first season of cultivation, 2021: second season of cultivation; 1 (control), 2–5 – biostimulants. The data are means ± SD ($n > 3$). Different letters (A – D) and (a – d) indicate the differences between the biostimulants in each variety ($P < 0.05$) for 2020 and 2021 seaseon, respectively, as determined by ANOVA and Tukey tests. A separate statistical analysis was performed for each season.

content of galacturonic acid by 22%, 34%, and 22% relative to the control were noted in the first season for Enrosadira treated with biostimulant 2 and in the second season for Poemat treated with biostimulants 2 and 5 and for Enrosadira treated with biostimulant 3 (Fig. 2G, H, I). The total galacturonic acid content was within the range of 40%–65%, depending on the cultivar and the preparation used, consistent with other studies in which the galacturonic acid content of raspberries, depending on the cultivation method used, was about 60% (Ruiz-Torralba et al., 2021).

It is assumed that the greatest influence on DM changes is PME, which catalyzes the de-esterification of the pectin methoxy groups (Willats et al., 2006). This relationship was confirmed by PCA (Fig. S1) for the Delniwa (first season), Poemat, and Enrosadira (second season) raspberry cultivars; increased DM was accompanied by increased PME activity. PME is more effective with highly methylated pectin, increasing the enzyme activity (Dinu et al., 2007). The method used to remove methyl esters from pectin depends on the PME isoform applied (Ross et al., 2011). As demonstrated by PCA, demethylated pectin may be more susceptible to PG-catalysed degradation. The PG activity and DM increases were observed in Delniwa, Poemat, and Enrosadira for both seasons (Fig. S1). The results suggest that both enzymes have an affinity for highly methylated pectin.

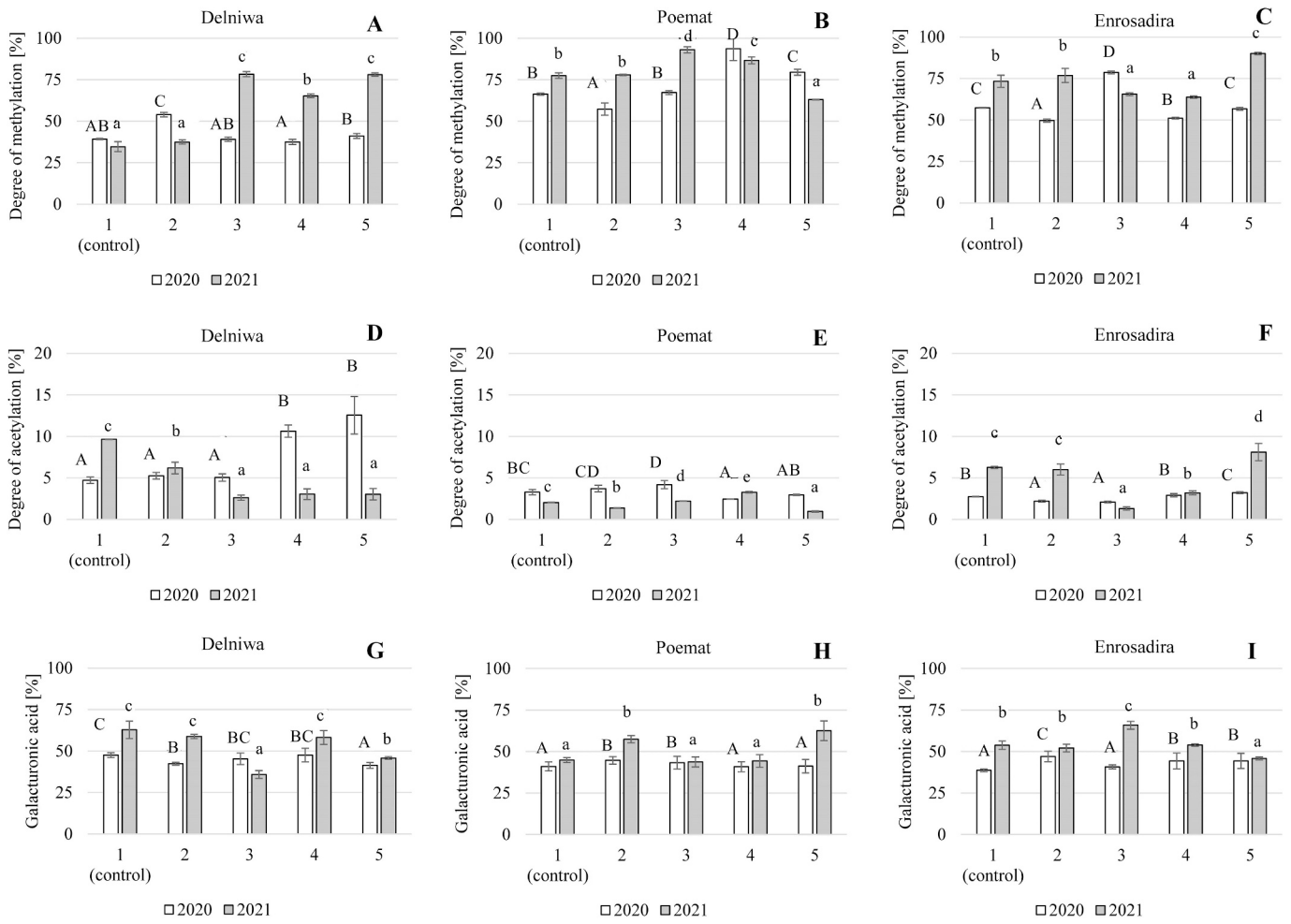


Fig. 2. Degree of methylation (A, B, C) and acetylation (D, E, F) and galacturonic acid content (G, H, I) in pectins extracted from raspberry cv. Delniwa, Poemat, and Enrosadira.
2020: first season of cultivation, 2021: second season of cultivation; 1 (control), 2–5: biostimulants. The data are means ± SD ($n > 3$). Different letters (A – D) and (a – d) indicate the differences between the biostimulants in each variety ($P < 0.05$) for 2020 and 2021 seaseon, respectively, as determined by ANOVA and Tukey tests. A separate statistical analysis was performed for each season.

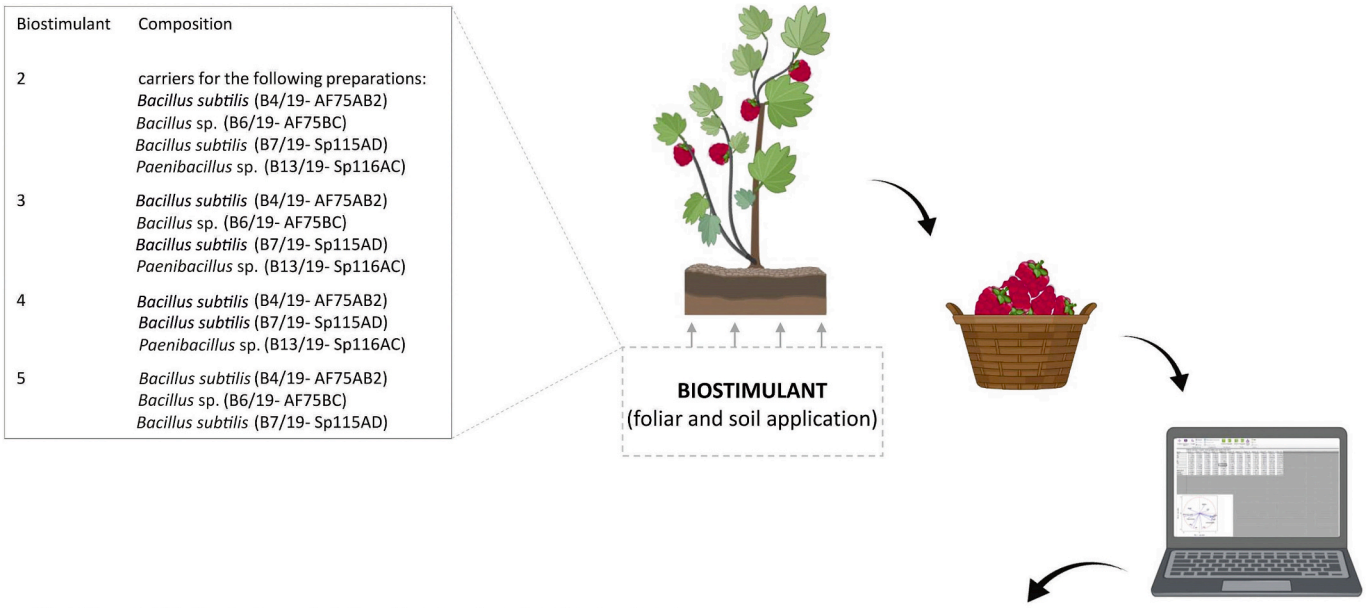
In addition, the pH influences the DM and enzymatic activity (Dinu et al., 2007). Thus, the activity of PME, which depends on the isoform and the reaction environment, may weaken the structure of the raspberry cell walls. Increased pectin DM increases the activity of PG and accelerates the processes of rotting and decay in the fruit, facilitating the invasion of bacteria and fungi (Ross et al., 2011). However, pectin demethylesterification by PME which occurs after deacetylation can cause the formation of blocks of non-methyl-esterified galacturonic acid residues and improve the gelling properties of fruit pectin, which is desirable in the processing sector (Remoroza et al., 2015). The increased PME activity could also be the result of enzyme production by the *Bacillus* sp. (Soares et al., 2001) and *Paenibacillus* sp. (Zhong et al., 2021) contained in the biostimulants, which may have caused a significant increase in DM in some raspberry samples.

PCA revealed that for both seasons, the increase in DM was accompanied by a decrease in galacturonic acid content in Delniwa, Poemat and Enrosadira raspberries (Fig. S1). Thus, the degree of methylesterification affected hydrolysis; an increase in DM made the pectin chains resistant to hydrolysis, decreasing the galacturonic acid content. It has been confirmed that PG first hydrolyzes the unmethylated pectin region (Bonnin et al., 2002).

The increase of DM and GalA content in pectins extracted from raspberries in the second season of cultivation under the influence of applied biostimulants may determine their suitability for processing. The mechanism of gelation of low methylated pectins (DM < 50%) and high methylated pectins (DM > 50%) are totally different. Low methylated pectins undergo gelation at wide-range of pH values (2.6–7), preferably in the presence of divalent metal ions, whereas high methylated pectin form gels at pH below 3.5 and at high sugars or other co-solutes content (55–75%) (Gallery et al., 2024). The increase in DM of pectins extracted from raspberries treated biostimulants indicates that they become suitable for the preparation of elastic, thermo-irreversible gel without the effect of syneresis (Einhorn-Stoll, 2018). Moreover, the increase of GalA content above 65% for Enrosadira treated with biostimulant 3 makes this raspberry a good source of pectin as a food additive (Mierczyńska et al., 2017).

The results confirm the influence of the selected biostimulants, the main components of which are *Bacillus* sp. and *Paenibacillus* sp., on the content of antioxidants, antioxidant activity, phenolic compound profile, enzymatic activity, and the DM and DA in raspberries cv. Delniwa, Poemat, and Enrosadira.

Applying selected biostimulants improved the quality of the raspberry fruit and generally enhanced benefits (Fig. 3). The increased antioxidant, phenolic acid, and flavonoid content in the two seasons studied and the larger increase observed in the second season compared to the first season were associated, for the most part, with the treatment of the raspberries with biostimulants 4 and 5. Biostimulants 3, 4, 5, and, less frequently, 2 increased the phenolic acid and flavonoid content, the



The influence of biostimulants on selected quality parameters of raspberry - summary.

GROUP OF PARAMETERS	*Parameter*	*Biostimulant*	*Variety*	*Increase / decrease in the first season (2020) against control [%]*	*Increase / decrease in the second season (2021) compared to the first (2020) [%]*
ANTIOXIDANTS	anthocyanins	4	Delniwa	16	126
	anthocyanins	5	Delniwa	20	227
	polyphenols	3	Delniwa	16	10
	vitamin C	2, 3	Delniwa	20	61
MALONDIALDEHYDE CONTENT AND FERRIC REDUCING POWER	malonodialdehyde	2	Delniwa	29	97
	ferric reduction power	2	Enrosadira	25	100
PHENOLIC ACIDS AND FLAVONOIDS	caffeic acid	3	Delniwa	27	43
	ellagic acid	4	Poemat	22	18
	ellagic acid	5	Delniwa	79	72
	gallic acid	2	Delniwa	5	15
	catechin	2, 4, 5	Delniwa	27	23
	epicatechin	5	Enrosadira	25	220
ENZYME ACTIVITY	polygalacturonase	4	Poemat	12	26
	polygalacturonase	4	Enrosadira	25	8
	β- galactosidase	2	Delniwa	20	82
	α-L-arabinofuranosidase	4	Poemat	15	34
DEGREE OF METHYLATION AND ACETYLATION	degree of methylation	5	Poemat	13	16
	degree of acetylation	4, 5	Delniwa	7	9
	degree of acetylation	4	Poemat	0,8	0,8

2, 3, 4, 5- tested biostimulants, Delniwa, Poemat, Enrosadira- variety of raspberry, 2020- first season, 2021- second season.

Fig. 3. Illustration of the biostimulant effect on the quality parameters of raspberries cv. Delniwa, Poemat, and Enrosadira. The parameters with statistically significant increases or decreases compared to the control in the two seasons studied were analyzed. The figure was created using BioRender (https://biorender.com/).

enzymatic activity of polygalacturonase and pectin methylesterase, and DM and DA in the two seasons studied compared to the control. A decrease in these parameters was noted between the seasons. Biostimulants 2 and 3 significantly decreased the MDA and FRAP in the first season and increased them in the second season. The values of the individual parameters differed; they were influenced not only by the biostimulant used but also by the raspberry variety cultivated.

4. Conclusion

This paper describes the influence of four biostimulants (2–5) on the antioxidant content (anthocyanins, polyphenols, vitamin C), antioxidant capacity (DPPH, FRAP, MDA), phenolic compound profile (phenolic acids, flavonoids), pectinolytic enzyme activity, degrees of methylation and acetylation, and the relationships between them are also established. The results of these studies indicate that the fruit of *R. idaeus* L. is a valuable source of pro-healthy compounds, such as anthocyanins, ascorbic acid, and polyphenolic compounds. The tested biostimulants had the greatest effect on the antioxidant levels of the Delniwa raspberry. In the first season, the greatest influence on the antioxidant content was found for biostimulants 2, 3, and 4, while in the second season, it was for biostimulants 2, 3, 4, and 5. For the raspberry cultivars tested (Delniwa, Poemat, and Enrosadira), the anthocyanin and vitamin C content increased with an increase in DPPH. The increase in anthocyanins and polyphenols was correlated with an increase in FRAP, which indicates the influence of the tested biostimulants on the antioxidant properties of raspberries. DPPH increased in the first season in the Delniwa and Enrosadira raspberries, while FRAP increased in the second season in the Poemat and Enrosadira raspberries treated with selected biostimulants. The activity of the pectinolytic enzymes responsible for, inter alia, fruit rotting processes under the influence of selected microbiological biostimulants was reduced compared to the control and

between the seasons.

Therefore, the tested biostimulants allow for modifying the antioxidant content and the pectinolytic enzyme activity, which affect the health-promoting properties and durability of raspberries. Further research is required to obtain a high-spectrum biostimulant formulation most suitable for all raspberry varieties. Moreover, further research will extend the knowledge of microbiological biostimulants and their influence on the content of valuable bioactive compounds and pectinolytic enzymes in fruit, directly affecting their quality.

CRediT authorship contribution statement

Justyna Cybulska: Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Formal analysis, Data curation, Conceptualization. **Artur Zdunek:** Writing – review & editing, Resources, Formal analysis. **Lidia Sas-Paszt:** Resources. **Magdalena Frąc:** Writing – original draft, Visualization, Methodology, Investigation.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Magdalena Frac reports financial support was provided by National Centre for Research and Development Poland. Magdalena Frac has patent #Method for obtaining a bacterial biopreparation and a bacterial biopreparation for maintenance and/or improving soil microbial biodiversity while controlling pathogens: *Botrytis* sp., *Colletotrichum* sp., *Phytophthora* sp., *Verticillium* sp. in soft fruit cultivation (in Polish), P.445051 pending to Institute of Agrophysics Polish Academy of Sciences. Magdalena Frac has patent #Method for obtaining a microbial fertilizing product and a microbial fertilizing product for conditioning seedlings, maintaining and/or improving the microbiological quality of the soil, while allowing for the control of phytopathogens in the cultivation of soft fruit (in Polish), P.445052 pending to Institute of Agrophysics Polish Academy of Sciences. Magdalena Frac has patent #A method of obtaining a microbiological fertilizing product and a microbiological fertilizing product for maintaining and/or improving the microbiological quality of the soil, allowing at the same time to control the phytopathogens *Botrytis* sp., *Colletotrichum* sp., *Phytophthora* sp., *Verticillium* sp. in the cultivation of soft fruit (in Polish), P.445053 pending to Institute of Agrophysics Polish Academy of Sciences. Magdalena Frac has patent #Method for obtaining a microbial fertilizing product and a microbial fertilizing product for soil conditioning and improving its biological properties while controlling pathogens *Botrytis* sp., *Colletotrichum* sp., *Phytophthora* sp., *Verticillium* sp. in soft fruit cultivation (in Polish), P.445054 pending to Institute of Agrophysics Polish Academy of Sciences.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.139746.

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