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Alteration of the Content of Primary and Secondary Metabolites in Strawberry Fruit by *Colletotrichum nymphaeae* Infection

Maja Mikulic-Petkovsek,*[,†] Valentina Schmitzer,[†] Ana Slatnar,[†] Nika Weber,[†] Robert Veberic,[†] Franci Stampar,[†] Alenka Munda,[‡] and Darinka Koron[‡]

[†]Chair for Fruit, Wine and Vegetable Growing, Department of Agronomy, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, SI-1000 Ljubljana, Slovenia

[‡]Agricultural Institute of Slovenia, Hacquetova ulica 17, SI-1000 Ljubljana, Slovenia

ABSTRACT: The effects of infection with *Colletotrichum nymphaeae*, the causative agent of strawberry black spot, were studied on two strawberry cultivars: susceptible 'Elsanta' and tolerant 'Honeoye' cultivars. Four treatments were established: (1) artificial inoculation; (2) spray application of pyraclostrobin + boscalid (Signum); (3) foliar spraying with calcium (Stopit); and (4) control (spraying with water). Primary metabolites (sugars and organic acids) and secondary metabolites (phenolic compounds) were determined in strawberry fruit with the use of HPLC-MS^n. Infected fruit accumulated large amounts of total sugars and low levels of organic acids. The sugar/acid ratio was higher in the infected and in Ca-treated strawberries. The contents of ellagic acid derivatives, flavonols, oligomeric procyanidins, flavan-3-ols, and total phenolics were highest in inoculated strawberry fruit. Results indicated that fungicide and calcium sprayings did not alter polyphenolic levels in plant tissue.

KEYWORDS: *Fragaria* × *ananassa, strawberry anthracnose, fungus, inoculation, sugars, organic acids, phenolics, resistance*

INTRODUCTION

Strawberry anthracnose, also known as strawberry black spot, is a severe disease in commercial strawberry production all over the world, second only to gray mold. The disease predominantly infects fruit, stolons, and crown but can also affect strawberry roots.[1] The heaviest economic losses result from fruit infection, which can occur on immature fruit preharvest, on mature fruit at harvest, or in the postharvest storage stage. In Europe, *Colletotrichum acutatum* has been recognized as predominant and most important causative agent of strawberry anthracnose.[2] The species has been subjected to thorough taxonomic reassessment and is now recognized as a species complex, comprising 31 species. Several species within the *C. acutatum* species complex cause strawberry anthracnose; however, *Collectotrichum nymphaeae* (formerly known as *C. acutatum* molecular group A2 or *C. simmondsii*) is the most important.[3] The best way to control strawberry anthracnose is to prevent the introduction of the pathogen into the field by using anthracnose-free transplants. Different key management strategies, including methods for reduction of pathogen occurrence and spread, cultural practices (cultivation in plastic tunnels), and chemical and biological control measures have been suggested against *C. nymphaeae* infection. However, no chemical methods are effectively controlling the disease. Among cultural techniques, drip irrigation and the use of plastic tunnels considerably limit inoculum dispersal and therefore greatly reduce fruit losses.[1]

Strawberry is an important fruit crop in temperate regions including Central Europe. Attractive fruits are favored for their excellent taste and health-promoting properties due to their richness in vitamins, minerals, and antioxidative compounds.[4] Sugars and organic acids are regarded as significant quality factors defining strawberry fruit taste. Ripeness stage, plant vitality, pedoclimatic conditions, and genotype are known to affect the quantitative variations in sugars and organic acids in strawberry fruit.[5] Some organic acids also have an inhibitory effect on pathogenic organisms. Namely, a low pH level resulting from high content of organic acids produced by plants may require high energy inputs of pathogenic organism to maintain a favorable intracellular pH.[6]

Resistance to diseases may be related to the content and diversity of phenolic compounds in plant cells, as has been reported for some economically important pests and diseases of plants in general and also for several fruit species.[7,8] Phenolic compounds are toxic to the pathogens, and many of them, such as flavanols and hydroxycinnamic acids, can act as passive or inducible barriers against herbivores or microbes. In response to the pathogen attack, the content and composition of polyphenols can change, playing an active role in induced resistance to the pathogens.[8,9]

The most frequently identified phenolic groups in strawberries are hydroxybenzoic and hydroxycinnamic acids, hydrolyzable tannins, flavonols, flavan-3-ols, and anthocyanins. In strawberry fruit high content levels of flavan-3-ols, epicatechin, and procyanidin derivatives have been determined.[10,11] These compounds are beneficial as they increase plant antioxidative capacity and presumably contribute to the restriction of infection caused by plant pathogens such as *Botrytis cinerea*.[12,13] However, the presence of procyanidins in fruits results in an astringent and unfavorable taste.

Only two fungicides can be used against strawberry anthracnose in Slovenian production orchards.[14] Preventive control measures (healthy plant material), site selection,

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optimal nutrition, and air circulation are therefore very important. One possible way to effectively protect against strawberry anthracnose is the use of resistant or tolerant strawberry cultivars. Therefore, the aim of the study was to determine possible connections between the infection and content levels of primary and secondary metabolites in strawberry plants. The two studied cultivars ('Honeoye' and 'Elsanta') also differ in their susceptibility to *C. nymphaeae* infection.[15,16] Several papers indicate that fungicide sprayings alter polyphenolic levels in plant cells,[17] but other studies[18] suggest that no modification occurs. Therefore, the effects of Signum fungicide application on polyphenolic composition has been studied to determine the effect on strawberry phenolic profile. In the same way, calcium application has been evaluated. Finally, inoculation with *C. nymphaeae* can cause changes in the synthesis of primary and secondary metabolites as some studies have reported for different fungus−plant interactions.[8] The present study highlights individual phenolic compounds that could be involved in defense mechanisms against specific fungal pathogens in addition to other plant strategies, for example, morphological, biochemical, physical, and other mechanisms. With this knowledge breeding strategies can focus on providing plants with high levels of various compounds and, consequently, new anthracnose-resistant strawberry cultivars could be developed. Economic losses in strawberry production due to strawberry anthracnose can potentially be diminished and negative environmental impacts of fungicide treatments greatly reduced.

MATERIALS AND METHODS

Fungal Material. Samples of diseased strawberries showing symptoms of anthracnose were collected in 2009 from strawberry plantations near Ljubljana, central Slovenia. Fungal isolates were obtained by culturing pieces of necrotic tissue from rotten strawberry fruit on potato dextrose agar (PDA). They were identified as *C. nymphaeae* (Pass.) Aa on the basis of cultural characteristics and sequence analysis of ITS and TUB2 according to the method of Damm et al.[3] Single-spore isolate was prepared by spreading spore suspension on PDA plates and isolating individual germinating conidia. Prior to plant inoculation, the single-spore isolate was subcultured on PDA plates and incubated at 24 °C in the dark for 10 days. Spores were then scraped from the plates and dispersed in sterile distilled water. Spore suspension was adjusted to 10^6 spores/mL.

Plant Material and Growing Conditions. The open field trial was conducted in 2011 and 2012 at the experimental station of the Agricultural Institute of Slovenia, located at Brdo pri Lukovici (latitude, 46° 10′ N; longitude, 14° 41′ E). The soil texture is silty loam, rich in potassium and nitrogen and poor in phosphorus. The organic matter is high. Frigo plants of susceptible 'Elsanta' cultivar and tolerant 'Honeoye' cultivars[15,16] were planted on August 3, 2011, on slightly elevated beds covered with black polyethylene at a spacing of 0.25 m × 0.25 m in double rows. The experimental site was equipped with a drip irrigation system.

Six blocks with four treatments were established; each treatment per block included 10 plants: (1) artificial inoculation with *C. nymphaeae*; (2) spray application of pyraclostrobin + boscalid (Signum, BASF); (3) foliar spraying with calcium (Stopit, Yara); and (4) control (spraying with water). The first artificial inoculation was performed on May 21 and the second one on May 28. Spore suspension was applied to the plants by spraying till runoff with a hand sprayer. Immediately after inoculation, the plants were covered with a transparent polyethylene cover to maintain 100% relative humidity. After the first inoculation, no infection occurred due to very dry weather, and therefore a second inoculation was performed. Plants were sprayed with Signum after the second harvest on May 17 and with Stopit (concentration = 14 mL/L) on May 15 and 21. The control treatment plants were sprayed with water.

All plants were treated against diseases with cuprum (Champion, Nufarm) on March 20, 2012. Plant vigor was assessed in spring, and no differences were recorded between cultivars or blocks. The first flowers opened early in April and froze due to low temperatures. On April 20, 2012, all plants were covered with polyethylene to prevent further damage. The weather in the period between full flowering (May 3) and the beginning of harvest (May 17) was very hot and dry. Fruits were harvested two to three times a week, and fruit yield (mass and quantity) and number of infected fruits and runners per plant were evaluated each time. Ninety fruits were sampled per treatment (15 in each block), except for the inoculation treatment for which all fruits with visible symptoms were used. Fruits were recorded as diseased when lesions were visible. Disease incidence was determined as the percentage of the total number of ripe fruits harvested throughout the fruiting season. These percentages were calculated for each section and genotype. The fruits were immediately frozen in liquid nitrogen and stored for up to 1 month at −20 °C until chemical analyses.

Chemicals. The following standards were used for the determination of sugars and organic acids: sucrose, fructose, and glucose and citric, malic, and fumaric acid from Fluka Chemie (Buchs, Switzerland); shikimic acid from Sigma-Aldrich (Steinheim, Germany). For the quantitation of phenolic compounds the following standards were used: ellagic acid, cyanidin-3-glucoside, and pelargonidin-3-glucoside from Sigma-Aldrich Chemicals (St. Louis, MO, USA); quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-glucuronide, kaempferol-3-glucoside, (−)-epicatechin, *p*-coumaric acid, and procyanidin B2 from Fluka Chemie. Methanol for the extraction of phenolics was acquired from Sigma-Aldrich Chemicals. The chemicals for the mobile phases were HPLC-MS grade acetonitrile and formic acid from Fluka Chemie GmBH. Water for the mobile phase was double-distilled and purified with a Milli-Q system (Millipore, Bedford, MA, USA). For total phenolic content, Folin−Ciocalteu phenol reagent (Fluka Chemie GmBH), sodium carbonate (Merck, Darmstadt, Germany), and gallic acid and ethanol (Sigma-Aldrich) were used.

Extraction and Determination of Sugars and Organic Acids. Primary metabolites (sugars and organic acids) were analyzed in whole strawberry fruit. For each treatment, six repetitions were carried out (n = 6); each repetition included several fruits. For the extraction of primary metabolites, 5 g of fruit was homogenized in 25 mL of double-distilled water using an Ultra-Turrax T-25 (Ika-Labortechnik) and left for 30 min at room temperature as reported by Mikulic-Petkovsek et al.[19] After the extraction, the homogenate was centrifuged (Eppendorf Centrifuge 5810 R) at 12000 rpm for 7 min at 10 °C. The supernatant was filtered through a 0.20 μm cellulose ester filter (Macherey-Nagel) and transferred into a vial, and 20 μL of the sample was used for the analysis. The analysis of primary metabolites was carried out using a high-performance liquid chromatograph (HPLC) of Thermo Separation Products (San Jose, CA, USA). The separation of sugars was carried out using a 300 mm × 7.8 mm i.d. Rezex RCM-monosaccharide Ca+ 2% column from Phenomenex operated at 65 °C. The mobile phase was double-distilled water, and the flow rate was 0.6 mL/min; the total run time was 30 min, and a refractive index (RI) detector was used to monitor the eluted carbohydrates as described by Mikulic-Petkovsek et al.[19] Organic acids were analyzed with the same HPLC system, equipped with a UV detector set at 210 nm, using a 300 mm × 7.8 mm i.d. Rezex ROA-organic acid H^+ (8%) column from Phenomenex, as described by Mikulic-Petkovsek et al.[19] The column temperature was set at 65 °C. The elution solvent was 4 mM sulfuric acid in double-distilled water at a flow rate of 0.6 mL/min. The duration of the analysis was 30 min. The sugars and organic acids in strawberry extracts were identified by their retention time characteristics; the concentrations were calculated with the help of the corresponding external standard and expressed as grams per kilogram fresh weight (FW) for sugars and grams per kilogram or milligrams per kilogram FW for organic acids, respectively. The content of all analyzed sugars was summed and presented as total analyzed sugars. In a similar way total analyzed organic acids were calculated. Both values were used for the determination of the sugar/organic acid ratio.

Table 1. Two-Way ANOVA for Yield per Plant, Number of Fruits per Plant, Number of Infected Fruits, and Number of Infected Runners per Plant of Two Cultivars, Four Treatments, and the Interaction Cultivar × Treatment[a]

		yield			
cultivar	treatment	mass per plant (g)	no. of fruits per plant	no. of infected fruits per plant	no. of infected runners per plant
'Elsanta'	control	150.1 ± 14.4 abc	12.6 ± 1.3	0 ± 0.00 d	0.23 ± 0.06
	Signum	144.7 ± 16.1 abc	12.7 ± 1.8	0.05 ± 0.02 d	0.09 ± 0.02
	Ca	158.4 ± 21.9 ab	13.2 ± 1.5	0 ± 0.00 d	0.15 ± 0.03
	infected	86.7 ± 6.7 d	8.1 ± 0.9	0.54 ± 0.11 b	0.33 ± 0.01
'Honeoye'	control	78.9 ± 9.9 d	9.3 ± 1.3	0 ± 0.00 d	0.05 ± 0.02
	Signum	176.9 ± 17.2 a	13.7 ± 1.2	0.08 ± 0.01 cd	0.04 ± 0.03
	Ca	105.1 ± 9.12 bc	9.8 ± 1.1	0 ± 0.00 d	0.03 ± 0.01
	infected	98.3 ± 12.3 cd	8.9 ± 1.1	0.23 ± 0.03 c	0.17 ± 0.01
cultivar × treatment[b]		*	NS	*	NS
cultivar[b]		NS	NS	NS	*
treatment[b]		**	*	***	*

[a]Different letters in a column denote significant differences (Duncan's test, $p < 0.05$). [b]*, statistically significant differences at P value <0.05; **, statistically significant differences at P value <0.01; ***, statistically significant differences at P value <0.001.

Extraction of Phenolic Compounds. The extraction of fruit samples was performed as described by Mikulic-Petkovsek et al.,[20] with some modification. Phenolic compounds (flavonoids and phenolic acids) were analyzed in whole strawberry fruit. For each treatment, six repetitions were carried out ($n = 6$); each repetition included several fruits. Frozen fruits were ground to a fine powder in a mortar chilled with liquid nitrogen, and 5 g was extracted with 10 mL of methanol containing 3% (v/v) formic acid and 1% (w/v) 2,6-di-*tert*-butyl-4-methylphenol (BHT) in a cooled ultrasonic bath for 1 h. BHT was added to the samples to prevent oxidation.

After extraction, the fruit extracts were centrifuged for 10 min at 10000 rpm. Each supernatant was filtered through a Chromafil AO-20/25 polyamide filter produced by Macherey-Nagel (Düren, Germany) and transferred to a vial prior to injection into the high-performance liquid chromatography (HPLC) system.

Determination of Individual Phenolic Compounds Using HPLC-DAD-MS^n Analysis. Phenolic compounds were analyzed on a Thermo Finnigan Surveyor HPLC system (Thermo Scientific) with a diode array detector at 280 nm (flavan-3-ols, cinnamic acid derivatives), 350 nm (flavonols), and 530 nm (anthocyanins). Spectra of the compounds were recorded between 200 and 600 nm. The column was a 150 × 4.6 mm i.d., 3 μm, Gemini C_{18} (Phenomenex, Torrance, CA, USA) operated at 25 °C. The elution solvents were aqueous 0.1% formic acid in double-distilled water (A) and 0.1% formic acid in acetonitrile (B). Samples were eluted according to the linear gradient from 5 to 20% B in the first 15 min, followed by a linear gradient from 20 to 30% B for 5 min, then an isocratic mixture for 5 min, followed by a linear gradient from 30 to 90% B for 5 min, and then an isocratic mixture for 15 min before returning to the initial conditions.[21] The injection amount was 20 μL and flow rate, 0.6 mL/min.

All phenolic compounds were identified using a mass spectrometer (Thermo Scientific, LCQ Deca XP MAX) with electrospray ionization (ESI) operating in negative ion mode (all phenolic groups except for anthocyanins) and positive ion mode (anthocyanins). The analyses were carried out using full scan data-dependent MS^n scanning from m/z 115 to 1500. The injection volume was 10 μL, and the flow rate was maintained at 0.6 mL/min. The capillary temperature was 250 °C, the sheath gas and auxiliary gas were 20 and 8 units, respectively, and the source voltage was 4 kV for negative ionization and 0.1 kV for positive ionization. Spectrometric data were elaborated using Excalibur software (Thermo Scientific). The identification of compounds was confirmed by comparing retention times and their spectra as well as by adding the standard solution to the sample and by fragmentation.

Concentrations of phenolic compounds were calculated from peak areas of the sample and the corresponding standards and expressed in milligrams per kilogram FW of strawberry fruit. For compounds lacking standards, quantitation was carried out using similar compounds as standards. Thus, glycosides of kaempferol were quantitated in equivalents of kaempferol-3-glucoside and all procyanidin dimers and trimers in equivalents of procyanidin B2; *p*-coumaroyl glucoside was quantitated in equivalents of *p*-coumaric acid and pelargonidin-3-malonylglucoside in equivalents of pelargonidin-3-glucoside; all ellagic acid derivatives were quantitated in equivalents of ellagic acid.

Determination of Total Phenolic Content. The extraction of samples for the determination of total phenolics was made according to the same protocol as for phenolics, with the difference that no BHT was added. Total phenolic content (TPC) of extracts was assessed according to the Folin−Ciocalteu phenol reagent method.[22] To 100 μL of the sample extracts (diluted 1: 4 (v/v) with MeOH) were added 6 mL of double-distilled water and 500 μL of Folin−Ciocalteu reagent; after between 8 s and 8 min at room temperature, 1.5 mL of sodium carbonate (20% w/v) was added. The extracts were mixed and kept for 30 min at 40 °C before the absorbance was measured on a UV−vis Lambda Bio spectrophotometer (Perkin-Elmer, Waltham, MA, USA) at 765 nm. A mixture of water and reagents was used as a blank. TPC was expressed as gallic acid equivalents (GAE) in milligrams per kilogram FA of tissue. Absorption was measured in three replications.

Statistical Analysis. The data were analyzed using the Statgraphics Plus 4.0 program (Manugistics, Inc., Rockville, MD, USA). A two-way analysis of variance was carried out to determine the significance of cultivar and treatment (control, calcium, Signum, and inoculation treatment) on strawberry fruit phenolic profile. The significance of the treatment on the content of individual phenolic compounds, sugars, organic acids, and total phenolic content was tested using one-way analysis of variance (ANOVA). Differences among treatments were tested with Duncan's test at a 0.05 significance level. Multivariate statistical analysis (hierarchical cluster analysis, discriminate analysis, and classification) was conducted to interpret the differences in average values of all analyzed parameters (sugars, organic acids, and phenolics) for the two strawberry cultivars among different treatments. Ward's method based on squared Euclidean distance was used to interpret the difference or similarity in determined compounds among treatments analyzed.

RESULTS AND DISCUSSION

Yield and Fruit Infection Assessment. As strawberry yield is greatly influenced by fungal attack, the infection rate of *C. nymphaeae* was assessed during strawberry fruit maturation. The numbers of infected runners and fruits among different treatments are presented in Table 1. The highest numbers of

Table 2. Content of Individual and Total Analyzed Sugars and Organic Acids, Sugars/Organic Acids Ratio of Strawberry Fruit, and Two-Way ANOVA of Cultivar (C), Treatment (T: Control, Calcium, Signum, and Infected), and Their Interaction (C × T)

	'Elsanta'				'Honeoye'						
	mean content ± SE in g/kg FW				mean content ± SE in g/kg FW				factors[a]		
	control	Signum	calcium	inoculation	control	Signum	calcium	inoculation	C	T	C × T
fructose	33.1 ± 2.2	41.0 ± 1.5	42.5 ± 2.3	50.6 ± 3.0	34.9 ± 1.7	34.7 ± 1.1	37.9 ± 2.8	55.0 ± 1.5	NS	***	NS
glucose	32.6 ± 1.9	38.5 ± 1.6	39.2 ± 2.3	45.2 ± 1.2	32.1 ± 1.8	32.1 ± 1.4	35.0 ± 2.5	47.9 ± 1.8	NS	***	NS
sucrose	10.0 ± 1.2	9.8 ± 1.3	6.7 ± 0.7	1.6 ± 0.11	9.7 ± 1.3	9.3 ± 1.1	9.4 ± 1.3	5.0 ± 0.26	NS	***	NS
total sugars	**75.7 ± 3.9**	**85.2 ± 5.1**	**88.5 ± 5.0**	**97.5 ± 4.1**	**76.8 ± 4.2**	**76.1 ± 3.5**	**82.3 ± 5.1**	**108.0 ± 2.8**	**NS**	*******	**NS**
citric acid	6.7 ± 0.3	7.0 ± 0.3	6.0 ± 0.2	4.7 ± 0.6	6.6 ± 0.2	7.4 ± 0.3	6.0 ± 0.2	5.4 ± 0.6	NS	***	NS
malic acid	2.4 ± 0.17	2.6 ± 0.2	1.7 ± 0.2	1.3 ± 0.06	2.1 ± 0.16	1.9 ± 0.2	1.9 ± 0.18	0.8 ± 0.09	*	***	NS
fumaric acid ($\times 10^{-3}$)	7.3 ± 0.6	7.6 ± 0.5	6.4 ± 0.5	11.9 ± 1.5	7.3 ± 0.4	7.0 ± 0.5	8.0 ± 0.9	12.4 ± 1.6	NS	***	NS
shikimic acid ($\times 10^{-3}$)	9.8 ± 0.5	9.0 ± 0.6	8.5 ± 0.4	8.2 ± 0.5	9.9 ± 0.4	9.0 ± 0.6	8.9 ± 0.4	10.7 ± 0.2	NS	NS	NS
total organic acids	**9.2 ± 0.4**	**9.6 ± 0.5**	**7.3 ± 0.4**	**6.1 ± 0.5**	**8.8 ± 0.3**	**9.3 ± 0.5**	**7.9 ± 0.2**	**6.2 ± 0.7**	**NS**	*******	**NS**
sugars/organic acids ratio	8.4 ± 0.6	8.9 ± 0.8	12.3 ± 1.2	16.4 ± 1.4	8.8 ± 0.5	8.2 ± 0.4	10.3 ± 0.7	18.4 ± 2.1	NS	***	NS

[a]*, statistically significant differences at P value <0.05; **, statistically significant differences at P value <0.01; ***< statistically significant differences at P value <0.001.

infected fruits and runners were recorded in the inoculation treatment. This indicates that the fungus caused a significant increase of infected fruits and a moderate increase of infected runners. However, no statistical differences in the numbers of infected fruit were recorded among control, Signum, and calcium treatments. Due to unfavorable natural conditions for anthracnose infection in 2012 the control plants did not develop visible signs of infection of strawberry fruit. Nevertheless, the infection has been detected on runners, particularly on the 'Elsanta' cultivar, which was less infected if treated with Signum and calcium. The infection rate was also significantly correlated with combined fruit yield: lowest yields were documented in the inoculation treatment for 'Elsanta' cultivar and in inoculation and control treatments for tolerant 'Honeoye' cultivar. Compared to the 'Elsanta' cultivar the latter blooms earlier, and in 2012 late frosts in April damaged many blooms. Long-term strawberry yields are approximately 60% higher compared to total yield in 2012 due to late spring frosts causing flower and fruit deformations.

Content of Sugars and Organic Acids in Strawberry Fruit. Previously identified sugars in strawberry fruit mainly belong to mono- and disaccharides (glucose, fructose, and sucrose).[23] Fructose and glucose represent the major sugars in strawberries, and sucrose accounts for only 10% of total sugars (Table 2). *C. nymphaeae* infected fruit contained up to 1.4-fold levels of total sugars compared to the control treatment (noninfected fruit). Several other studies on different plants also report overaccumulation of sugars in infected organs. Namely, when plants are exposed to stress, they commonly react with an increased accumulation of sugars in fruits[24] and leaves.[25] Presumably, carbohydrate consumption is required for production of energy to support the biosynthesis of defensive phenolic compounds induced by wounding.[26] Phytochemical analysis provides strong evidence that some sugars play a key role in the antimicrobial defense system in plants. Naqvi et al.[27] reported that the presence of a significant concentration of sugars is in correlation with antimicrobial compounds produced by plants which possess the capability to kill pathogens or inhibit their growth. It is also likely that, due to damaged cuticle of the infected fruit, the content of total sugars increases as a result of partial desiccation. Interestingly, higher total sugar content was measured in calcium-treated fruits with respect to the control. Calcium increases fruit firmness, altering the composition of cell walls and potentially causing modification in primary metabolite content levels. Lara et al.[28] also determined high sugar levels in fruits of plants subjected to calcium application. Calcium-treated fruits were able to retain more sugars in their cell walls, probably as a consequence of calcium deposition in pectin polysaccharides. This also greatly improves strawberry fruit texture.[28]

The main organic acids determined in strawberry fruit were citric and malic acids. This is in accordance with previous results.[19,29] The share of citric and malic acids in strawberries represented almost 99% total organic acids (Table 2). Although organic acids in strawberries are present in much lower concentration than sugars, their effect on fruit flavor is considerable. *C. nymphaeae* had an opposite effect on total organic acid content levels compared to sugars. Infected fruits of both analyzed cultivars contained lowest levels of organic acids compared to other treatments. This can be explained by the fact that the *Colletotrichum* fungus secretes ammonia and increases the pH level of fruits, which favors pectate lyase enzyme secretion.[30] Similarly, Kamilova et al.[31] reported that the content of organic acids was lower in *Fusarium oxysporum* f. sp. *radicis-lycopersici* infected tomato fruit. The reason for this effect is that pathogenic fungi utilize carbon from organic acids for their growth and development[31] and at the same time secrete cell wall degrading enzymes.[30] Several studies have evaluated the antimicrobial effect of organic acids on the growth of microorganisms in food. Organic acids and their salts alone or in combination have been reported to inhibit the growth of bacteria or fungi.[6,32]

The sugar to acid ratio is defined as the proportion of total sugars compared to the total organic acids in plant sample. The sugar/acid ratio is largely accountable for the taste and flavor of fruits. The calculated ratio is often utilized as an index of sweetness for specific fruits, and those with high sugar to acid ratios are considered to be sweeter than fruits with a low ratio. Fruits that taste sweet do not necessarily have a high sugar content, but they generally contain characteristically low levels of organic acids, especially malic acid.[33] Moreover, individual sugar-to-acid ratios influence the perception of sweetness;

Table 3. Identification of Phenolic Compounds in Strawberry Fruit in Positive and Negative Ions with HPLC-MS, MS^2, and MS^3

λ (nm)	$[M - H]^-$ (m/z)	MS^2 (m/z)	MS^3 (m/z)	tentative identification	content expressed as
280	783	481, 301	257	bis-HHDP[a] glucose 1	ellagic acid
	783	481, 301	257	bis-HHDP glucose 2	ellagic acid
	577	425, 407, 289		procyanidin dimer 1	procyanidin B2
	577	425, 407, 289		procyanidin dimer 2	procyanidin B2
	865	577,425	407, 289	procyanidin trimer 1	procyanidin B2
	289	245		epicatechin	epicatechin
	849	577, 425, 407	425, 407, 289	procyanidin trimer 2	procyanidin B2
	325	163		*p*-coumaroyl glucoside	*p*-coumaric acid
	633	301	257	HHDP-galloyl-glucose	ellagic acid
	849	577, 425, 407	425, 407, 289	procyanidin trimer 3	procyanidin B2
	935	633, 301	301	galloyl-bis-HHDP glucose 1	ellagic acid
	935	633, 301	301	galloyl-bis-HHDP glucose 2	ellagic acid
350	463	301	257	ellagic acid hexoside	ellagic acid
	433	301	257	ellagic acid pentoside	ellagic acid
	447	301	257	ellagic acid deoxyhexoside	ellagic acid
	463	301	179, 151	quercetin-3-*O*-glucoside	quercetin-3-*O*-glucoside
	477	301	179, 151	quercetin-3-*O*-glucuronide	quercetin-3-*O*-glucuronide
	447	285		kaempferol-3-*O*-glucoside	kaempferol-3-glucoside
	461	285		kaempferol-3-glucuronide	kaempferol-3-glucoside
	489	285		kaempferol-3-*O*-acetylglucoside	kaempferol-3-glucoside
	593	285		kaempferol-3-*O*-coumaroylglucoside	kaempferol-3-glucoside
530	449[b]	287		cyanidin-3-*O*-glucoside	cyanidin-3-*O*-glucoside
	433[b]	271		pelargonidin-3-*O*-glucoside	pelargonidin-3-glucoside
	519[b]	433/271		pelargonidin-3-*O*-malonylglucoside	pelargonidin-3-glucoside

[a]HHDP, hexahydroxydiphenic acid. [b]$[M + H]^+$ (m/z) anthocyanins were obtained in the positive ion mode.

however, in strawberry, total sugar and acid content was shown to have a greater effect on fruit flavor.[5] The sugar/acid ratio was statistically highest in infected fruit (values of >16 were recorded) (Table 2). This ratio was approximately 2-fold higher than the sugar/acid ratio of noninfected strawberries and can be correlated to sweeter-tasting fruit. Similarly, calcium-treated fruits were characterized by higher sugar/acid ratios compared to fruits of the control and Signum treatments. Other researchers reported a higher sugar to acid ratio of calcium-treated fruits.[34,35] The present study also indicates that this ratio can be altered by different factors, that is, pathogen infection.

Content of Individual and Total Phenolics in Strawberry Fruit. The study of phenolic compounds during the progression of infection is very relevant on the basis of their proposed defensive role in plants. In strawberry fruit, 24 different phenolic compounds (Table 3) have been determined and grouped into the following phenolic classes: ellagic acid derivatives, flavonols, flavan-3-ols, derivatives of hydroxycinnamic acids, and anthocyanins. The greatest share of all identified phenolic compounds (AIP) in strawberry fruit was represented by flavan-3-ols (60% AIP) and anthocyanins (33% AIP) (Table 4).

Ellagic acid conjugates have frequently been identified in *Fragaria* genus. Previous studies on strawberry have reported the presence of several derivatives of ellagic acid in different plant organs, that is, fruits and leaves.[10,11] The content of ellagic acid derivatives also differed between the inoculation treatment and other treatments (Table 4). However, no differences have been determined between calcium, Signum, and control treatments. This suggests that the infection caused the increase of ellagic acid derivatives. Specifically, 'Elsanta' and 'Honeoye' fruits of the inoculation treatment contained significantly higher levels (from 1.6- to 2-fold, respectively) of some individual and total ellagic acid conjugates. Higher content of ellagic acid conjugates can be ascribed to modifications in the metabolic processes within the plant as several studies report increased accumulation of specific compounds as a defense mechanism to stress. With up-regulation of certain metabolic processes, the plant aims to stop or reduce the growth of the pathogen, and ellagic acid and its derivatives are known to possess antimicrobial activity.[36,37]

Flavonols represented approximately 2% AIP in strawberry fruit. Several authors report health-related importance of flavonols, and particularly antimutagenic and anticarcinogenic effects have been studied.[38] The results of our study indicate that infected strawberry fruit contained significantly higher levels of quercetin-3-glucuronide and all glycosides of kaempferol (Table 4). Again, no differences have been determined among other treatments (calcium or Signum application and control). Similar to these findings, higher content levels of several flavonols have been recorded in fruit infected with various pathogens, bacteria, or viruses compared to healthy fruit tissue.[7,39] It has been shown that apple scab infection increased the synthesis of flavonols, for example, rutin and quercetin-3-rhamnoside.[8,40]

Epicatechin was the most abundant flavan-3-ol in strawberry fruit and ranged from 50 to 70% of total analyzed flavan-3-ols (Table 4). The tolerant 'Honeoye' cultivar contained 50% higher levels of epicatechin in healthy fruit compared to the more susceptible 'Elsanta' cultivar. Different authors have also reported higher levels of individual flavan-3-ols in fruits of resistant cultivars.[33,41] Moreover, infected fruits of the 'Honeoye' cultivar were characterized by 1.2−2-fold higher levels of epicatechin compared to other treatments. In contrast, the 'Elsanta' cultivar contained the lowest levels of epicatechin

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Table 4. Content of Individual and Total Analyzed Phenolic Compounds and Total Phenolic Content in Strawberry Fruit and Two-Way ANOVA of Cultivar (C), Treatment (T: Control, Calcium, Signum, and Infected), and Their Interaction (C × T)

	'Elsanta'				'Honeoye'						
	mean content[a] ± SE in mg/kg FW				mean content[a] ± SE in mg/kg FW				factors[b]		
polyphenol[c]	control	Signum	calcium	inoculation	control	Signum	calcium	inoculation	C	T	C × T
1	10.1 ± 1.1	11.7 ± 2.0	9.6 ± 0,7	13.7 ± 1.1	12.3 ± 0.9	15.9 ± 1.1	11.4 ± 2.1	15.9 ± 2.4	*	*	NS
2	20.1 ± 6.5 ab	32.1 ± 4.1 a	10.1 ± 4.7 bc	0.15 ± 0.01 c	3.4 ± 0.2 c	0.28 ± 0.05 c	2.4 ± 0.2 c	13.8 ± 1.9 bc	**	NS	***
3	4.9 ± 0.4	4.5 ± 0.6	6.1 ± 0.9	6.8 ± 0.8	4.6 ± 0.3	5.0 ± 0.09	5.3 ± 0.8	6.0 ± 0.7	NS	*	NS
4	0.7 ± 0.06	0.7 ± 0.03	0.8 ± 0.07	1.5 ± 0.2	2.1 ± 0.2	2.1 ± 0.09	2.5 ± 0.3	3.2 ± 0.4	***	***	NS
5	6.8 ± 0.4	7.1 ± 0.5	7.5 ± 0.8	7.5 ± 0.9	7.2 ± 0.5	7.3 ± 0.2	7.4 ± 0.8	5.8 ± 0.4	NS	NS	NS
6	7.3 ± 1.0	9.8 ± 1.1	6.2 ± 0.8	8.9 ± 0.9	3.9 ± 0.4	4.3 ± 0.5	5.5 ± 0.5	11.8 ± 1.1	NS	*	NS
7	2.8 ± 0.4	2.0 ± 0.2	4.8 ± 0.6	5.1 ± 0.5	2.5 ± 0.4	2.8 ± 0.4	3.2 ± 0.5	5.2 ± 0.8	NS	***	NS
8	2.3 ± 0.1 cd	1.3 ± 0.1 d	2.6 ± 0.4 cd	7.8 ± 0.9 a	3.5 ± 0.3 c	3.9 ± 0.2 c	4.2 ± 0.6 bc	6.0 ± 0.5 ab	NS	***	*
9	**34.2 ± 2.4**	**30.9 ± 2.1**	**42.1 ± 4.8**	**67.2 ± 7.5**	**39.8 ± 3.3**	**41.8 ± 1.7**	**42.1 ± 5.2**	**64.6 ± 6.5**	**NS**	***	**NS**
10	5.8 ± 1.1	4.0 ± 0.6	5.2 ± 0.9	11.3 ± 0.1	9.2 ± 1.0	7.4 ± 0.8	7.0 ± 0.9	14.5 ± 1.8	*	***	NS
11	2.5 ± 0.1	2.5 ± 0.3	2.9 ± 0.1	4.5 ± 0.5	3.7 ± 0.3	4.3 ± 0.3	4.4 ± 0.3	6.6 ± 0.8	***	***	NS
12	4.4 ± 0.4	4.2 ± 0.7	3.5 ± 0.5	7.3 ± 0.9	5.8 ± 0.7	8.8 ± 0.2	7.4 ± 0.8	9.3 ± 0.8	***	***	NS
13	0.2 ± 0.06	0.8 ± 0.07	0.14 ± 0.02	1.8 ± 0.2	0.7 ± 0.08	0.6 ± 0.09	0.9 ± 0.1	1.5 ± 0.3	NS	***	NS
14	1.3 ± 0.17	0.9 ± 0.11	1.0 ± 0.12	3.3 ± 0.3	1.0 ± 0.1	0.9 ± 0.1	1.1 ± 0.11	2.9 ± 0.3	NS	***	NS
15	1.6 ± 0.2	1.9 ± 0.25	1.2 ± 0.19	3.5 ± 0.39	1.7 ± 0.09	1.9 ± 0.08	1.6 ± 0.17	3.1 ± 0.33	NS	***	NS
16	**15.8 ± 0.1.9**	**13.5 ± 1.4**	**14.1 ± 1.9**	**31.8 ± 2.8**	**22.2 ± 0.17**	**24.0 ± 0.8**	**22.6 ± 0.87**	**38.0 ± 3.8**	***	***	**NS**
17	281.5 ± 27.8 cd	198.0 ± 20.9 d	315.2 ± 32.4 cd	554.2 ± 53.7 b	424.0 ± 45.2 bc	724.6 ± 67.3 a	450.1 ± 48.1 bc	873.0 ± 88.7 a	***	***	***
18	36.4 ± 2.4	37.9 ± 4.2	38.8 ± 4.2	57.3 ± 6.3	51.3 ± 4.7	53.6 ± 6.2	53.5 ± 7.4	79.4 ± 8.1	***	***	NS
19	28.1 ± 3.4 cde	73.4 ± 8.1 ab	31.8 ± 4.1 cd	54.3 ± 5.9 bc	21.3 ± 2.9 de	31.4 ± 3.5 cd	4.2 ± 0.5 e	95.9 ± 10.2 a	NS	***	**
20	58.2 ± 6.3	55.1 ± 4.4	42.1 ± 5.3	81.5 ± 9.1	43.1 ± 5.1	79.0 ± 8.9	64.3 ± 8.2	104.5 ± 12.0	NS	***	NS
21	36.1 ± 4.7	23.3 ± 3.4	41.4 ± 4.7	140.1 ± 18.7	48.9 ± 6.8	37.4 ± 4.8	63.5 ± 4.0	145.4 ± 17.9	NS	***	NS
22	41.7 ± 5.8 d	28.7 ± 4.2 d	46.5 ± 6.1 cd	127.8 ± 13.7 b	52.4 ± 7.8 d	113.6 ± 12.0 bc	90.2 ± 10.6 bc	160.8 ± 15.4 a	***	***	*
23	**478.6 ± 39.0 de**	**416.5 ± 33.1 e**	**515.9 ± 62.0 de**	**1015.3 ± 42.9 b**	**641.1 ± 62.6 cd**	**1039.6 ± 75.0 b**	**722.5 ± 33.1 c**	**1459.1 ± 109.0 a**	***	***	**
24	19.6 ± 2.0	16.3 ± 2.3	15.1 ± 2.1	30.4 ± 4.4	20.6 ± 1.8	27.4 ± 3.4	17.6 ± 2.6	33.0 ± 4.2	NS	***	NS
25	210.4 ± 14.0 c	347.3 ± 15.6 b	345.1 ± 40.2 b	186.7 ± 20.4 c	173.7 ± 11.7 c	416.8 ± 16.9 a	414.5 ± 30.6 a	197.8 ± 20.5 bc	NS	***	*
26	20.9 ± 8.2 c	72.2 ± 9.6 b	105.2 ± 2.8 a	70.6 ± 7.2 b	61.5 ± 8.3 b	76.3 ± 4.3 b	107.5 ± 6.5 a	66.4 ± 7.3 b	*	***	*
27	**269.7 ± 22.3 cd**	**205.3 ± 13.0 d**	**308.8 ± 41.2 cd**	**371.6 ± 47.5 bc**	**477.5 ± 32.7 ab**	**535.2 ± 26.5 a**	**517.4 ± 42.4 a**	**244.6 ± 26.4 d**	***	**NS**	***
28	5.8 ± 0.5 bc	3.5 ± 0.4 c	6.7 ± 0.9 bc	20.0 ± 3.1 a	8.9 ± 0.8 b	10.0 ± 0.5 b	10.8 ± 1.9 ab	16.7 ± 1.1 a	*	***	*
29	1299.1 ± 45.0	1585.0 ± 59.1	1634.8 ± 80.3	1822.1 ± 78.4	1267.7 ± 49.1	1550.3 ± 65.0	1543.8 ± 61.1	1904.7.0 ± 76.1	NS	***	NS

[a]Different letters in rows denote significant differences (Duncan's test, $p < 0.05$). [b]*, statistically significant differences at P value <0.05; **, statistically significant differences at P value <0.01; ***, statistically significant differences at P value <0.001. [c]Polyphenol names: 1, bis-HHDP glucose isomer 1; 2, bis-HHDP glucose isomer 2; 3, ellagic acid deoxyhexoside; 4, ellagic acid hexoside; 5, ellagic acid pentoside; 6, galloyl bis-HHDP glucose 1; 7, galloyl bis-HHDP glucose 2; 8, HHDP galloyl glucose; 9, total ellagic acid conjugates; 10, kaempferol-3-glucoside; 11, kaempferol-3-glucuronide; 12, kaempferol-3-acetylglucoside; 13, kaempferol-3-coumaroylglucoside; 14, quercetin-3-glucoside; 15, quercetin-3-glucuronide; 16, total flavonols; 17, epicatechin; 18, procyanidin dimer 1; 19, procyanidin dimer 2; 20, procyanidin trimer 1; 21, procyanidin trimer 2; 22, procyanidin trimer 3; 23, total flavan-3-ols; 24, cyanidin-3-glucoside; 25, pelargonidin-3-glucoside; 26, pelargonidin-3-malonylglucoside; 27, total anthocyanins; 28, *p*-coumaroyl glucoside; 29, total phenolic content (mg gallic acid equiv/kg FW).

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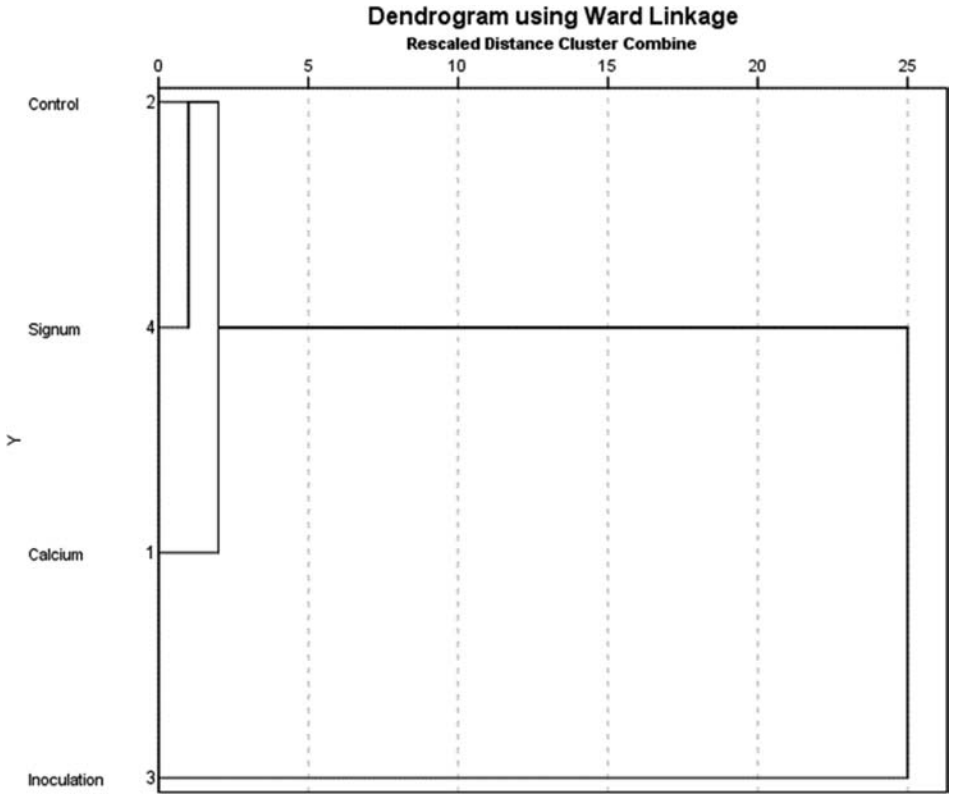

Figure 1. Dendrogram combining data on primary and secondary metabolites analyzed in strawberry fruit subjected to different treatments (control, calcium, Signum, and inoculation), using Ward's method based on squared Euclidian distance.

in fruit treated with Signum fungicide and the highest content in inoculated fruits. It seems that the fungus caused increased accumulation of epicatechin in infected tissue. Some researchers[8,40] have also observed a dramatic increase in monomeric flavanols and their polymers in the boundary zones near the infection site, potentially being the reason for its restricted spread. The role of flavan-3-ols in pathogen defense of plants might be their interaction with proteins and the inhibition of enzyme activity secreted by pathogenic fungi.[9] A high diversity of procyanidins has also been determined in strawberry fruit; di-, tri-, and tetramers have been identified. Oligomeric procyanidins were increased up to 4.5-fold as a result of infection with *C. nymphaeae*. Correspondingly, a 36% procyanidin increase has been reported in virus-infected grapevine leaves[39] and a 200% increase in fungus-infected cacao leaves.[42] Higher levels of individual procyanidin forms and epicatechin in infected strawberry fruit have also caused a 2.2−2.4-fold higher levels of total flavan-3-ols compared to other treatments. Previous studies report similar plant reactions in terms of phenolic synthesis as a response to disease or pest attack.[9] It has been suggested that a rapid accumulation of monomeric and polymeric flavanols at the infection site stops further dissemination of the pathogens.

Among hydroxycinamic acid derivatives *p*-coumaroyl glucoside has been identified in the range of 3.51−20.08 mg/kg FW in strawberry fruit (Table 4). The inoculation treatment caused a significant increase of *p*-coumaroyl glucoside, and 1.8−5.7-fold higher levels were measured in infected fruit compared to other treatments. Likewise, *p*-coumaroyl glucoside was increased in strawberry fruit after powdery mildew infection.[43] Our previous investigation also reported that apple scab infection increased specific hydroxycinnamic acid content.[8,40] It has been determined that hydroxycinnamic acid derivatives play a major role in plant resistance and exhibit a fungitoxic effect against different pathogens, because they inhibit the growth and sporulation of fungi.[33,44]

The major anthocyanins identified in strawberry fruit were pelargonidin-3-glucoside and pelargonidin-3-malonylglucoside, which combined represented 85−95% total analyzed anthocyanins (TA) (Table 4). Cyanidin-3-glucoside has also been determined in strawberries and accounted for 5−15% TA. The content of anthocyanins is greatly influenced by fruit maturity level and cultivar. On the other hand, inoculation with fungi, fungicide, or calcium treatment did not cause significant changes in anthocyanin content levels. However, the bordering tissue near the infection site is darker and possibly accumulates more anthocyanins (visual observations). Similarly, Slatnar et al.[45] reported higher content levels of anthocyanins at the infected apple peel site, which also resulted in intensified red coloration.

The phenolic profile (content and abundance of different compounds) is suitable as an indicator for stress conditions that occur in plants. The first step of the defense mechanism in plant involves a rapid accumulation of phenols at the infection site, which inhibit or restrict pathogen growth and development.[46] TPC in strawberry ranged from 1267 to 1904 mg/kg gallic acid equivalents (GAE) (Table 4). TPC is closely related with the levels of analyzed phenolics, and thus higher levels of individual compounds are indicative of a higher TPC in plant tissue. Therefore, similarly to individual phenolic increase, inoculation with *C. nymphaeae* caused from 1.4- to 1.5-fold higher levels of TPC compared to other treatments (healthy fruit). Other papers indicate a significantly higher TPC as a

reaction to fungal, bacterial, and viral infection[47,48] or pest attack.[49]

Multivariate statistical analysis was employed to determine which treatments differentiated the content of primary and secondary metabolites in strawberries, and a dendrogram revealed similarities and differences among treatments in all analyzed parameters (Figure 1). Fruits of the inoculation treatment were characterized by almost 2-fold higher levels of ellagic acid conjugates, flavan-3-ols, and flavanols and a higher content of total sugars compared to other treatments. This is indicative of the involvement of these phenolic groups in plant defense mechanisms against *C. nymphaeae*. Strawberries of the control and Signum treatments were very similar in all analyzed parameters, which could be attributed to similar health status of these fruits. Also, it is an indication that fungicide spraying did not alter the polyphenolic level in plant tissue. Both treatments yielded completely healthy fruit. Likewise, fruits of the calcium treatment were similar to the control and Signum treatments in all analyzed parameters except for sugars. Again, calcium-treated fruits contained somewhat higher levels of specific compounds. Hernandez-Munoz et al.[50] reported that calcium applications reduce fungal decay incidence on strawberries. The authors explain that postharvest treatment with calcium salts is expected to reinforce the cell wall and middle lamella of fruits and vegetables, thus enhancing tissue resistance to fungal enzyme activity.

The accumulation of some phenolic compounds may explain the broad and unspecific prevention of plant diseases such as black spot disease. Our results demonstrate that *C. nymphaeae* infection caused a significant increase in sugars, conjugates of ellagic acid, epicatechin, procyanidins, a derivate of hydroxycinnamic acid, and some flavonols (mostly glycosides of kaempferol) in infected fruits. It has been demonstrated that the runners and fruits of the 'Honeoye' cultivar were less infected compared to the 'Elsanta' cultivar. The cultivars also differed in the content levels of flavonols and flavanols; both phenolic groups were more abundant in fruits of the 'Honeoye' cultivar. Results support the hypothesis that phenolic compounds play an important role in host resistance in infected tissue and that the mechanism of resistance may be influenced by responses linked to the host−pathogen interaction. However, it is obvious that the strong defense reaction is not enough to overcome the disease. In addition to polyphenols, other biochemical compounds are potentially involved in strawberry defense response. Additional studies may help clarify the precise mechanisms, processes, and important resistance indicators within the plants, which take place after the pathogen attack. Specific studies are of great importance to help minimize the negative effects of fungal diseases such as strawberry anthracnose, causing large yield losses due to its fast spread, ineffective chemical treatments, and long withdrawal periods of fungicides. Therefore, the development of strawberry cultivars resistant to *C. nymphaeae* in terms of high accumulation of specific phenolics is promising for controlling anthracnose as a method that is both economical and environmentally acceptable.

AUTHOR INFORMATION

Corresponding Author

*(M.M.-P.) E-mail: maja.mikulic-petkovsek@bf.uni-lj.si. Fax: +386 1 423 10 88. Phone: +386 1 320 31 41.

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