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Metabolic elicitors of *Pseudomonas fluorescens* N 21.4 elicit flavonoid metabolism in blackberry fruit

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Abstract

Background: The beneficial rhizobacterium, *Pseudomonas fluorescens* N 21.4, and its metabolic elicitors were inoculated in commercial cultivars of blackberry plants (*Rubus* cv. Loch Ness). Phenolic compounds present in red and black fruit and the expression of structural marker genes of the phenylpropanoid pathway during fruit ripening were studied.

Results: An inverse relationship between gene expression and accumulation of metabolites was seen, except for the *RuDFR* gene, which had a direct correlation with cyanidin 3-*O*-glucoside synthesis, increasing its content 1.3 times when *Ru*DFR was overexpressed in the red fruit of plants inoculated with the metabolic elicitors of *P. fluorescens* N 21.4, compared with red fruit of plants inoculated with N 21.4. The *RuCHS* gene also had a fundamental role in the accumulation of metabolites. Both rhizobacterium and metabolic elicitors triggered the flavonoid metabolism, enhancing the catechin and epicatechin content between 1.1 and 1.6 times in the case of red fruit and between 1.1 and 1.8 times in the case of black fruit. Both treatments also boosted the anthocyanin, quercetin, and kaempferol derivative content, highlighting the effects of metabolic elicitors in red fruit and the effects of live rhizobacterium in black fruit.

Conclusion: The metabolic elicitors' capacity to modulate gene expression and to increase secondary metabolites content was demonstrated. This work therefore suggests that they are effective, affordable, easily manageable, and ecofriendly plant inoculants that complement, or are alternatives to, beneficial rhizobacteria.

Keywords: *Pseudomonas fluorescens* N 21.4; metabolic elicitors; blackberries; flavonoid metabolism; *RuDFR* gene; *RuCHS* gene

INTRODUCTION

Blackberry consumption is becoming increasingly popular due to its human health benefits.[1] The increase in consumption, linked with the high economic price of blackberry in the market, makes this crop very attractive for the agro-food industry.[2]

Blackberries are among the fruits with more beneficial properties due to they are extremely rich in flavonoids, among which are flavonols, flavanols, and anthocyanins.[2] These compounds have demonstrated cytotoxic, anticancer, antiviral, antibacterial, anti-inflammatory, antiallergenic, antithrombotic, cardioprotective, and hepatoprotective properties.[3,4] Moreover, their content directly affects the nutritional and organoleptic quality of the fruit.[5]

On the other hand, flavonoids are secondary metabolites that have a vital function in plants, acting as protectors against biotic and abiotic stress.[6] They also have an important role as repellents, visual attractors, phytoalexins, phytoanticipins,[7,8] or auxin controlers.[9]

As flavonoids are secondary metabolites, their biosynthesis is highly inducible. An accepted and effective biotechnological practice to elicit secondary metabolism, enhancing the biosynthesis of compounds with agro-alimentary interest, is the use of beneficial rhizobacteria[10-13] and their derived elicitors (structural molecules such as flagellin,[14] or metabolic elicitors released to the medium, such as antibiotics, surfactants, or other chemicals).[15,16]

As flavonoids have numerous beneficial properties, the engineering of their biosynthetic pathways for the deliberate accumulation and isolation of active molecules has been used extensively in the biotechnological industry.[17] However, the regulation of this pathway during blackberry ripening has not been deeply studied yet. Knowledge of this pathway during blackberry ripening and upon challenge with beneficial rhizobacteria and metabolic elicitors would be useful for the development of new blackberry crop techniques by stimulating the synthesis of secondary metabolites, sustainably improving fruit nutritional qualities.[11,12]

For all the above, the beneficial rhizobacterium *Pseudomonas fluorescens* N 21.4 and its metabolic elicitors (ME) were used in

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the present work to study their capacity to elicit the flavonoid biosynthetic pathway during blackberry fruit ripening. The *P. fluorescens* N 21.4 strain has widely demonstrated its beneficial effects on the physiology of blackberry plant, and on the flavonoid metabolism in the fruit. N 21.4 was able to trigger secondary metabolism in *Rubus fruticosus*, enhancing plant defenses and fruit quality and production.[18] In the work of Ramos-Solano *et al.*,[11] it also demonstrated its capacity to increase fruit production and fruit quality, enhancing the quantity of phenolic compounds throughout the year, mainly when environmental conditions were tougher for the plant's survival. Finally, it was seen that elicitation of blackberry plants by the N 21.4 strain modulated gene expression in the fruit of *Rubus* cv. Loch Ness affected the profiles of secondary metabolites during fruit ripening, boosting the expression of some flavonoid biosynthetic genes and enhancing the concentration of certain flavonoids in the fruit.[12] This allowed the identification of regulatory genes involved in the phenylpropanoid pathway.

Since the N 21.4 strain had demonstrated an extraordinary ability to influence the physiology of blackberry plants and to elicit the flavonoid secondary metabolism, it was proposed, in the present work, to evaluate the potential of its ME to mimic the capacities of the live strain. A previous study demonstrated the capacity of ME to elicit flavonoid metabolism in blackberry leaves, enhancing the amount of epicatechin, kaempferol, and quercetin derivatives.[19] It was also seen that ME were able to reinforce the blackberry plant's immune system, activating pathogenesis-related proteins, and to improve plant fitness, reducing oxidative stress and increasing photosynthesis in the leaves.[19] In other plant species, the ME of N 21.4 also elicited secondary metabolism (e.g. isoflavone metabolism in *Glycine max*,[20] and defensive secondary metabolism in *Arabidopsis thaliana*).[13] As a result of these data, the present study hypothesized that it was feasible that the ME could modify the biosynthetic pathway of flavonoids during blackberry ripening.

The scientific contribution of the present research is therefore to advance the study of metabolic elicitors, using them as alternative plant inoculants to live rhizospheric bacteria, as they have many reported advantages related to the maintenance and management of the inoculums and because they do not cause biosecurity problems. The latter is the innovative aspect of this work, as the capacity of N 21.4 to trigger secondary metabolism in blackberry has already been demonstrated by our group.[11,12]

To pursue these goals, a thorough study of the content and variety of phenolic compounds present in blackberry fruits of plants inoculated with *Pseudomonas fluorescens* N 21.4 and with its metabolic elicitors was carried out in red and in black fruit, as well as an expression analysis of structural marker genes of the phenylpropanoids pathway in both fruit stages.

MATERIAL AND METHODS

Blackberry experimental design

The *Rubus* cv. Loch Ness plants used in this work were kindly provided by Agricola El Bosque S.L. 'La Canastita' (Lucena del Puerto, Huelva, Spain). Plants and greenhouses were managed according to regular agricultural practices.[11] Plants were grown in Huelva from November 2017 to June 2018 under a 'winter cycle'. Before being transplanted to greenhouses in November, plants underwent an artificial 5-month cold period at 4 °C to start their regular cycle. A total of 540 plants were in the trial, arranged in five greenhouses. Each greenhouse had two lines (200 m long each) with 120 plants in total, each line being one replicate with 60 plants. Three lines were inoculated with N 21.4 at root level; three lines were inoculated with metabolic elicitors (ME) of N 21.4 by aerial spraying; and three lines were left as non-inoculated controls. N 21.4 and ME were inoculated every 15 days during the whole plant cycle with 0.5 L of inoculum per plant.

Fruits for analysis were sampled in April 2018, when the two stages of the fruit (red and black) were present in the plants at the same time, and were rapidly frozen in liquid nitrogen and brought to the laboratory to be maintained at −80 °C. Three replicates were taken, each comprising 125 g of fruit, randomly sampled from 60 plants.

Bacterial experimental design

The bacterial strain used in this study was *Pseudomonas fluorescens* N 21.4 (Spanish Type Culture Collection accession number CECT 7620), a gram-negative bacillus, which was isolated from the rhizosphere of *Nicotiana glauca* Graham.[21] The strain N 21.4 is able to trigger defense and phenolic metabolism in *Solanum lycopersicum*,[22] *Arabidopsis thaliana*,[13,22] *Glycine max*,[23] *Hypericum* sp.,[10] *Papaver* sp.[24] and blackberry. In blackberry, it improves fruit yield and quality by increasing flavonoid content.[11,12]

Bacterial strain was stored at −80 °C in nutrient broth (CONDA) with 20% glycerol. Inoculum was prepared by streaking strains from −80 °C onto Plate Count Agar (CONDA) plates, incubating them at 28 °C for 24 h. Then, bacterial cells were scraped off of the plates into sterile nutrient broth and were incubated for 24 h on an orbital shaker at 28 °C, with strong agitation, to keep the medium oxygenated and to obtain a 10^9 cfu mL^{-1} inoculum. Inoculum of N 21.4 was diluted from 10^9 to 10^7 cfu mL^{-1} and it was delivered at root level to the 60 plants (0.5 L per plant) every 15 days during the whole plant cycle.

The inoculum of ME was prepared by centrifuging N 21.4 (grown for 24 h at 28 °C on an orbital shaker) at 2890×*g* for 20 min at 4 °C. Cells were discarded and the remaining supernatant was diluted following the same proportion as the live strain (from 10^9 to 10^7 cfu mL^{-1}) and it was sprayed onto the 60 plants (0.5 L per plant) every 15 days during the whole plant cycle.

Fruit production

Fruit was collected from mid-March to early June. They were collected every 3 days from all the plants in each line and treatment. They were weighed and this weight was divided by the number of plants, obtaining the weight (in g) per plant on each day of harvest. Fruit production corresponds to the accumulated production of all the harvesting moments.

Extract preparation for measuring bioactives by colorimetry

The fruit extracts (red and black fruit) used to measure the total flavonols and phenols were prepared by adding 9 mL of 80% cold methanol to 1 g of fruit powder, mixing by vortex (protected from light), sonicating for 10 min and centrifuging at 2890×*g* for 5 min at 4 °C. The remaining supernatant was collected and stored at 4 °C.

The fruit extracts (red and black fruit) to measure total of anthocyanins were prepared by adding 9 mL of 80% methanol and 0.1% of cold HCl to 1 g of fruit powder, mixing by vortex (protected from light), sonicating for 10 min and centrifuging at 2890×*g* for 5 min at 4 °C. The remaining supernatant was collected and stored at 4 °C.

Measurement of bioactives

Total flavonols

Total flavonols were quantitatively determined through the test described by Jia *et al.*,[25] using catechin as standard (Sigma-Aldrich, St Louis, MO, USA). One milliliter of the methanolic extract was added to a flask with 4 mL of distilled water and 0.3 mL of 5% $NaNO_2$ (w/v). After 5 min, 0.3 mL of 10% $AlCl_3$ were added. Five minutes later, 2 mL of NaOH 1 M was finally added. The solution was mixed and measured at 510 nm with an UV–visible spectrophotometer (Biomate 5). A catechin calibration curve was made ($r = 0.99$). The results were expressed as g of catechin equivalents per kg of fresh weight (FW). All samples of red and black fruit were measured in triplicate.

Total phenols

Total phenols were determined quantitatively with Folin–Ciocalteu agent (Sigma-Aldrich) by a colorimetric method described by Singleton and Rossi[26] with some modifications.[27] Gallic acid was used as standard (Sigma-Aldrich). Twenty µL of the methanolic extract were mixed with 250 µL of Folin–Ciocalteu agent 2 N (Sigma-Aldrich) and 3 mL of distilled water. After 5 min at room temperature, 0.75 mL of 20% Na_2CO_3 solution was added. After 8 min at room temperature, 950 µL distilled water was added and after 2 h in obscurity, absorbance was measured at 760 nm with an UV-visible spectrophotometer (Biomate 5). A gallic acid calibration curve was made ($r = 0.99$). Results were expressed in g of gallic acid equivalents per kg of fresh weigh (FW). All samples of red and black fruit were measured in triplicate.

Total anthocyanins

Total anthocyanins were determined quantitatively through the pH differential method described by Giusti and Wrolstad.[28] Methanolic extracts were diluted in pH 1 buffer (0.2 M KCl) and pH 4.5 (1 M CH_3CO_2Na) in 1:15 proportion. After that, absorbance was measured at 510 and 700 nm respectively, in a UV–visible spectrophotometer (Biomate 5). A cyanidin-3-glucoside calibration curve was made ($r = 0.99$). Results were expressed in g of cyanidin-3-glucoside equivalents per kg of fresh weigh (FW). All samples of red and black fruits were measured in triplicate.

Characterization of phenolics and flavonoids by ultra-high performance liquid chromatography with electrospray ionization source, coupled to quadrupole time-of-flight mass spectrometry analyzer (UHPLC/ESI-qTOF-MS)

Phenolic acids, including, citric acid, gallic acid, genistic acid, salicylic acid, vanillic acid, ferulic acid, ellagic acid, and chlorogenic acid, were purchased from Sigma-Aldrich (St Louis, MO, USA); flavonoids including kaempferol, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, quercetin, quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-rhamnoside, luteolin, naringenin (aglycone), hesperetin, (+)-catechin, (−)-epicatechin, aesculetin, phloridzin, delphinidin, anthocyanin, delphinidin-3-*O*-rutinoside, and malvidin and other polyphenols like 6,7-dihydroxicoumarin were purchased from Sigma-Aldrich and from Extrasynthese Co.™ (Geney, France).

The standard solutions (10 ppm) were dissolved in methanol. All the solvents used, as methanol and acetonitrile (Honeywell Riedel-de Haen (Seelze, Germany)) were liquid chromatography–mass spectrometry (LC/MS) grade. Purified water was obtained from the Milli-Q Plus™ system from Millipore (Milford, MA, USA). Formic acid was purchased from Sigma-Aldrich (St Louis, MO, USA).

Sample preparation

The extraction of phenolics was conducted as follows: 10 mg of powder of lyophilized fruit were added to 1000 µL of methanol. The mixture was vortexed for 1 min, sonicated for 5 min, and centrifuged at 2890×*g* for 5 min at 4 °C. Supernatants were collected and stored at −20 °C until their use for LC/MS analysis. During the process, extracts were protected from light.

UHPLC/ESI-qTOF-MS analysis

Samples were injected on a 1290 Infinity series UHPLC system associated with an electrospray ionization source (ESI) with Jet Stream technology to a 6550 iFunnel QTOF/MS system (Agilent Technologies, Waldbronn, Germany).

For the dissociation, a volume of 2 µL was injected into a reversed-phase column (Zorbax Eclipse XDB-C18 4.6 × 50 mm, 1.8 µm, Agilent Technologies) at 40 °C. The flow rate was 0.5 mL min^{-1} with a mobile phase consisted of solvent A: 0.1% formic acid, and solvent B: methanol. Gradient elution consisted of 2% B (0–6 min), 2–50% B (6–10 min), 50–95% B (11–18 min), 95% B for 2 min (18–20 min), and returned to starting conditions 2% B in 1 min (20–21 min) to finally keep the re-equilibration with a total analysis time of 25 min.

The detector was functioning in full scan mode (m/z 50 to 2000) at 1 scan/s. Accurate mass measurement was confirmed through an automated calibrator delivery system that constantly introduced a standard solution, containing m/z 121.0509 (purine) and m/z 922.0098 (HP-921) in positive ESI mode; on the other hand m/z 112.9856 (TFA) and m/z 922.009798 (HP-921) were introduced in negative ESI mode. The capillary voltage was ±4000 V for positive and negative ionization mode. The temperature was set at 225 °C. The nebulizer and gas flow rate were 35 psig and 11 L min^{-1} respectively, fragmentor voltage was 75 V, and radiofrequency voltage in the octopole (OCT RF Vpp) was 750 V.

For the investigation, MassHunter Workstation Software LC/MS Data Acquisition version B.07.00 (Agilent Technologies) was used for control and acquisition of all data obtained with UHPLC/ESI-qTOF-MS.

For measurement, each sample was injected twice in six different concentrations to create calibration curves in which sample peak areas were extrapolated. The UHPLC-MS data analysis was performed by MassHunter Qualitative Analysis (Agilent Technologies) Software version B.08.00 using molecular feature extraction (MFE).

All measurements were carried out using red and black fruit.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

Prior to RNA extraction, the fruits were removed from the −80 °C freezer and ground to a fine powder with liquid nitrogen using an RNAase free mortar and pestle. Total RNA was isolated from each replicate with a Plant / Fungi Total RNA Purification kit (50) (Norgen™ (Thorold, ON, Canada)) (DNAase treatment included) and RNA integrity was confirmed by using Nanodrop™ (ThermoScientific).

The retrotranscription was carried out using iScript tm cDNA Synthesis Kit (Bio-Rad). All retrotranscriptions were executed using a GeneAmp PCR System 2700 (Applied Biosystems): 5 min 25 °C, 30 min 42 °C, 5 min 85 °C, holding at 4 °C. The

Table 1 Forward and reverse primers used in qPCR analysis

Gene	Gene code	Forward primer	Tm (°C)	Reverse primer	Tm (°C)
RuCHS	Chalcone synthase [EC 2.3.1.74]	5′ATGGTGGTTGTTGAAATTCC	61.1	5′CTGGATTGCACACCCAGGTGGCCC	79.4
RuFLS	Flavonol synthase [EC 1.14.20.6]	5′CCTACAGGGAAGTCAATGAGAAA	63.1	5′CACATGGGATTTCAGTACCTTCT	62.9
RuF3′H	Flavonid-3′-hydroxylase [EC 1.14.14.82]	5′CCTATCTCCAAGCTGTCATCAAG	63.8	5′GTGGTATCCGTTGATTTCACAAC	64.1
RuDFR	Dihydroflavonol reductase [EC 1.1.1.219]	5′AATCAGAAGAAGGTGAAGC	55.9	5′CATTAKSACAAGTTTGGTG	50.2
RuLAR	Leucocyanidin reductase [EC 1.17.1.3]	5′GTGGAGTCCCATACACGTACATT	63.6	5′CTGAAACTGATCTAACGGTGGAA	64
RuANS	Anthocyanidin synthase [EC 1.14.20.4]	5′TTGGTCTGGGATTAGAAGAAAGG	64.2	5′CTGAGGGCATTTTGGGTAGTAAT	63.9
RuANR	Anthocyanidin reductase [EC 1.3.1.77]	5′ TCGCAATGTACTTCCAAGAAAC	62.9	5′CTTCATCAGCTTACGGAAATCAC	63.6
RuMYB5	MYB family transcription factor	5′ACTCAATCCAGACTCCTCATCTG	63.5	5′AGGAAGTGATTGGACTTTTAGGG	63.2
RuACT	Actin	5′ATGTTCCCTGGTATTGCAGAC	62.7	5′CCACAACCTTGATCTTCATGC	64.4

Garcia-Seco *et al.*[12] The primers were designed with the Primer3Plus program. Under the design conditions, a PCR fragment size of 100–120 bp was specified.

amplification was performed with a MiniOpticon Real Time PCR System (Bio-Rad): 3 min at 95 °C and then 39 cycles consisting of 15 s at 95 °C, 30 s at 50 °C, and 30 s at 72 °C, followed by a melting curve to verify the results. To explain the expression obtained in the analysis, a cycle threshold (Ct) was used. Standard curves were calculated for each gene, and the efficiency values ranged between 90 and 110%. The regulatory genes of the phenylpropanoids biosynthetic pathway analyzed in blackberry were: *RuCHS*, *RuFLS*, *RuF3′H*, *RuDFR*, *RuLAR*, *RuANS* and *RuANR*. The transcription factor Ru*MYB5* was also analyzed. The reference gene was *Actin*. The primers used are given in Table 1. The primers were designed with the Primer3Plus program. Under design conditions, a PCR fragment size of 100–120 bp was specified. Results for gene expression were expressed as differential expression by the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

To check the statistical differences in the results obtained, an analysis of variance (ANOVA) was used. For the quantitative polymerase chain reaction (qPCR) experiment and the bioactive measurements by colorimetric methods (with only one independent variable), a one-way ANOVA was used. In the bioactive analysis by UHPLC/ESI-qTOF-MS, with two independent variables, a two-way ANOVA (a factorial ANOVA) was used. In both cases, prior to ANOVA analysis, homoscedasticity and normality of the variance were checked with Statgraphics plus 5.1 for Windows, meeting the requirements for analysis. When significant differences appeared ($P < 0.05$) a Fisher test was used.[29]

RESULTS

Fruit production

In control plants, 6167 ± 75 g per plant was recorded; in plants inoculated with N.21.4, 6277 ± 50 g per plant, and in plants inoculated with ME, 5680 ± 45 g per plant was recorded. Plants inoculated with ME showed statistically significant differences when compared with those inoculated with live strain (N 21.4) and control plants, which registered a higher fruit yield.

Bioactive content of fruit from non-inoculated plants

The differences between the red and black fruit of control plants (not inoculated with any bacterial treatment) were analyzed.

The amount of flavonols, measured by colorimetry, was very similar in red control fruit (0.46 ± 0 g of catechin equivalents kg^{-1} of FW) and in black control fruit (0.47 ± 0 g of catechin equivalents kg^{-1} of FW). However, in the case of phenols, measured by colorimetry, black control fruit showed significant higher content (3.02 ± 0.03 g of gallic acid equivalents kg^{-1} of FW) than red control fruit (2.83 ± 0.02 g of gallic acid equivalents kg^{-1} of FW). In the case of anthocyanins, measured by colorimetry, black control fruit also showed significant higher content (0.63 ± 0 g of cyaniding 3-*O*-glucoside equivalents kg^{-1} of FW) than red control fruit (0.12 ± 0 g of cyaniding 3-*O*-glucoside equivalents kg^{-1} of FW).

Regarding the specific bioactives measured by UHPLC/ESI-qTOF-MS, it was seen that black control fruit had a significantly lower concentration of all the measured bioactives, except for quercetin, aglycone, and phloridzin (1.7 times higher in black control fruit) and cyanidin-3-*O*-glucoside (3.7 times higher in black control fruit than red control fruit).

Measurement of bioactives

The total amount of bioactives in red and in black fruit was measured by colorimetry. Figure 1 shows the fold increase and fold-decrease in bioactives in red fruit and in black fruit of each treatment compared to the bioactives present in the fruit of control plants: Fig. 1 (i) fold-increased in flavonols compared to control; (ii) fold-increased and fold-decreased in phenols compared to control and (iii) fold-increased and fold-decreased in anthocyanins compared to control.

In flavonols (Fig. 1(A)), significant differences between both treatments (N 21.4 and ME) were seen in red and in black fruit. With both treatments and in both fruit stages there was an increase in flavonol content compared with controls, the greatest increase occurring in black fruit of plants inoculated with the

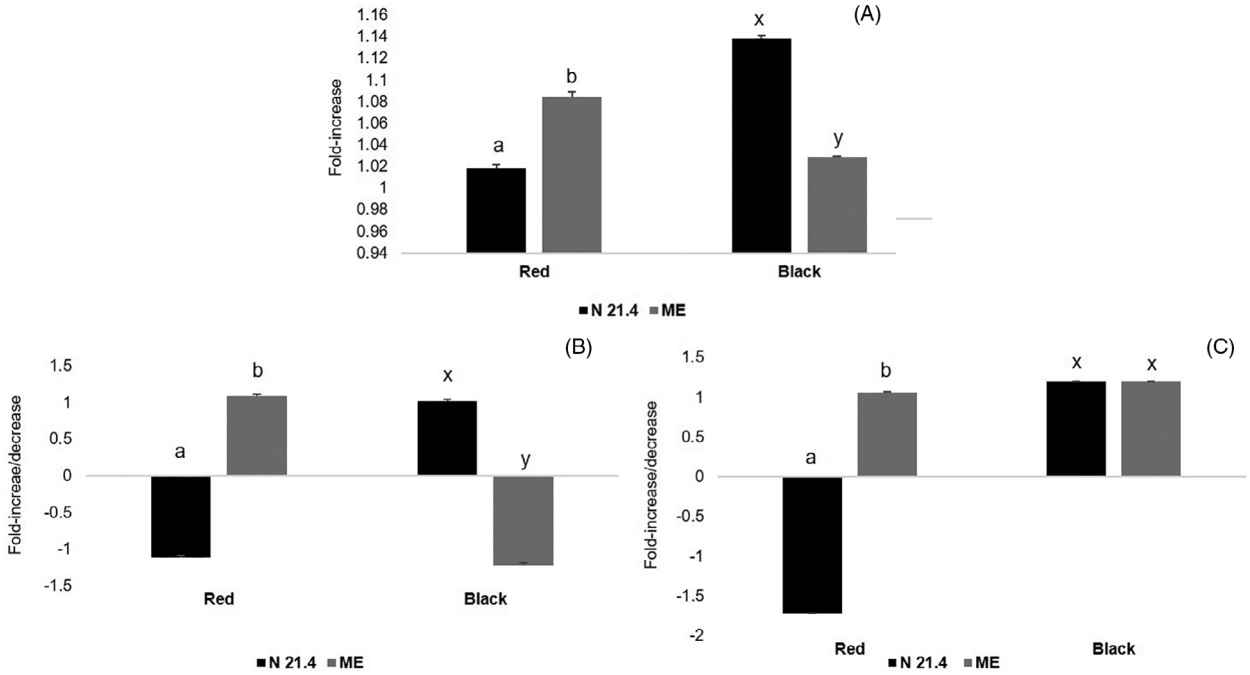

Figure 1 Fold-increase and fold decreased in the content of bioactives, measured by colorimetry, with respect to control: (A) flavonols; (B) phenols and (C) anthocyanins in red and black fruit of blackberry plants treated with *P. fluorescens* N 21.4 and with its metabolic elicitors (ME). Different letters indicate significant differences ($P < 0.05$): a and b indicate differences between treatments in red fruit; and x and y between treatments in black fruit.

strain N 21.4 (1.13 fold) and in red fruit of plants inoculated with ME (1.08 fold).

In phenols (Fig. 1(B)), significant differences between both treatments (N 21.4 and ME) were seen in red and in black fruit. A slight increase was recorded in red fruit of plants inoculated with ME and in black fruit of plants inoculated with N 21.4. However, a decrease in the amount of phenols was seen in red fruit of plants inoculated with N 21.4, and in black fruit of plants inoculated with ME.

In anthocyanins (Fig. 1(C)), significant differences between both treatments (N 21.4 and ME) were only seen in red fruit. A deep decreased in the quantity of anthocyanins was seen in the red fruit of plants inoculated with N 21.4. However, a 1.2 fold-increased was seen in the black fruit of plants inoculated with both treatments.

Bioactives analysis by UHPLC/ESI-qTOF-MS

Methanolic extracts were analyzed by liquid chromatography with electrospray ionization, coupled to quadrupole-time-of-flight mass spectrometry (LC/ESI-qTOF-MS). All the compounds were identified by comparing the retention times and spectra with reference standards. The main compounds found (11) and their concentration ($\mu g\ g^{-1}$) are shown in Table 2. They are grouped as flavonols (5), flavanols (2), dihydrochalcone (1), anthocyanine (1), hydroxycinnamic acid (1), and flavone (1). Red and black fruit of control and inoculated plants (N 21.4 and ME) were compared.

In red fruit, all compounds had significant higher values (between 1.2 and 1.6 times higher) in ME-treated plants than in N 21.4-treated plants. Values of N 21.4-treated-plants were similar to those of control plants, except for (−)-epicatechin and (+)-catechin values, which were 1.2 times higher in treated plants (this being significant), and for cyaniding-3-*O*-glucoside that were significantly lower in treated plants.

In black fruit, the opposite trend was observed: all compounds (except cyaniding 3-*O*-G) had significantly higher values in N 21.4-treated plants (between 1.1 and 1.5 times higher) than in ME-treated plants. The values for ME-treated-plants were similar to those of control plants, except for (−)-epicatechin and (+)-catechin values, which were 1.1 times significantly higher in treated plants, and for quercetin aglycone and phloridzin, which were significantly lower in treated plants.

The variation in the content of (+)-catechin, (−)-epicatechin and cyaniding 3-*O*-G, the phenolic compounds most affected by our treatments, appears in Fig. 2, in a chromatogram.

RT-qPCR analysis

The differential expression of flavonoids pathway genes in the fruit of treated plants appears in Fig. 3, showing red fruit (3 A) and black fruits (3 B).

In the red fruit, (Fig. 3(A)) of plants treated with N 21.4, *RuF3′H*, *RuFLS*, *RuDFR*, *RuANS* and *RuANR* genes appeared upregulated. *RuCHS*, *RuLAR* and *RuMYB5* were not significantly affected by this treatment. In the case of ME-treated-plants, *RuCHS* and *RuDFR* genes appeared upregulated and *RuF3′H*, *RuFLS*, *RuANS*, *RuLAR* and *RuANR* were downregulated. Differential expression of all genes, except the one for the transcriptional factor *RuMYB5*, had significant differences ($P<0.05$) between treatments (N 21.4 versus ME).

In the black fruit (Fig. 3(B)) of plants treated with N 21.4, *RuFLS*, *RuANS*, and *RuANR* genes were upregulated. The rest of genes were not significantly affected by this treatment. In ME-treated plants, *RuF3′H*, *RuFLS*, *RuANS*, and *RuANR* genes were upregulated and *RuLAR* was downregulated. Differential expression of all genes, except the one of the transcriptional factors, *RuMYB5*, gave significant differences ($P<0.05$) between treatments.

Table 2 Main flavonoid and phenolic compounds found in blackberry fruit and their concentration in μg g-1. Control, N 21.4 and ME treatments were compared in red and black fruit

Polyphenol class	Compound	Control		N 21.4		ME	
		Red	Black	Red	Black	Red	Black
Flavonols	Quercetin aglycone	<LOQ	0.621 ± 0 (α)	<LOQ	0.172 ± 0.08 (β)	0.235 ± 0.11 a	0.185 ± 0.01 b (β)
	Quercetin 3-O-glucoside	13.8 ± 0.3 a (x)	13.2 ± 0.1 a ($\alpha\beta$)	13.1 ± 0.55 a (x)	14.1 ± 0.3a (α)	17.7 ± 0.3 a (y)	12.9 ± 0.4 b (β)
	Quercetin 3-O-rutinoside	7.5 ± 0.25 a (x)	6.2 ± 0.1 b (α)	7.4 ± 0.35 a (x)	7.6 ± 0.15 a (β)	9.7 ± 0.25 a (y)	6.4 ± 0.15 b (α)
	Kaempferol 3-O-rutinoside	0.661 ± 0 a (x)	0.44 ± 0.01 b (α)	0.631 ± 0.01 a (x)	0.501 ± 0 b (β)	0.814 ± 0.03 a (y)	0.426 ± 0.2 b (α)
	Kaempferol 3-O-glucoside	1.1 ± 0.05 a (x)	0.8 ± 0.05 b (α)	0.9 ± 0 a (y)	0.8 ± 0.05 b (α)	1.3 ± 0 a (z)	0.7 ± 0.05 b (α)
Flavanols	(−)-Epicatechin	302.4 ± 8.3 a (x)	226.7 ± 3.4 b (α)	356 ± 0.45 a (y)	304.4 ± 0.45 b (β)	386.8 ± 8.05 a (z)	247.8 ± 1.8 b (γ)
	(+)-Catechin	20.8 ± 0 a (x)	9.5 ± 0.35 b (α)	25.4 ± 0.65 a (y)	17.2 ± 0.55 b (β)	33.7 ± 0.75 a (z)	11.1 ± 0.25 b (γ)
Dihydrochalcone	Phloridzin	0.263 ± 0.01 a (x)	0.442 ± 0.02 b (α)	0.216 ± 0 a (y)	0.397 ± 0 b (β)	0.329 ± 0 a (z)	0.392 ± 0 b (β)
Anthocyanine	Cyanidin 3-O-glucoside	838.3 ± 21.16 a (x)	3162.26 ± 42.808 b (α)	560.74 ± 18.18 a (y)	2968.72 ± 46.28 b (β)	727.23 ± 17.99 a (x)	3117.82 ± 38.40 b (α)
Hydroxycinnamic acid	Chlorogenic acid	0.603 ± 0.04 a (x)	0.333 ± 0.01 a (α)	0.597 ± 0.01 a (x)	0.256 ± 0.21 b (α)	0.779 ± 0.05 a (x)	0.335 ± 0.03 b (α)
Flavone	Luteolin	0.05 ± 0.01 a (x)	0.029 ± 0 a (α)	0 (y)	0 (α)	0.091 ± 0.02 a (x)	0 b (α)

The letters a and b indicate significant differences between red and black fruit within the same treatment; x, y and z indicate significant differences between treatments in red fruit; and letters α, β, and γ indicate significant differences between treatments in black fruit. < LOQ means sample under the limit of quantification.

DISCUSSION

Food security, as well as the improvement and protection of crops in a more ecofriendly environment, is nowadays a topic of great importance. This calls for more efficient and non-polluting agricultural methods and one of the most challenging tools to achieve this goal is the use of biological agents,[30] such as beneficial rhizobacteria or their derived elicitors. In the present work, the beneficial rhizobacterium *Pseudomonas fluorescens* N 21.4 and its metabolic elicitors have been used as plant inoculants in commercial cultivars of blackberry (*Rubus* cv. Loch Ness) to elicit flavonoid metabolism in the fruit.

Blackberries are strongly rich in flavonoids, which have essential functions in plant defense against biotic (fungus, bacteria, herbivores)[4,31,32] and abiotic stress (light, temperature, water supply, minerals, CO_2, etc.)[33] and they also have many benefits for human health[34,35] when they are included in the diet. For this reason, plant flavonoid metabolism has been studied in depth with the aim of modifying and obtaining better performance (higher accumulation of beneficial secondary metabolites, higher antipathogenic capacity, better plant fitness, etc.).

The biosynthesis of flavonoid compounds starts from the amino acid phenylalanine and produces phenylpropanoids, which are channeled into the flavonol-anthocyanin pathway by chalcone synthase (CHS). Further reactions involve chalcone isomerase (CHI), which generates naringenin, flavanone-3-hydroxylase (F3H), which hydroxylates naringenin until dihydrokaempferol, which is then hydroxylated by flavonoid-3-hydroxylase (F3′H) and transformed into dihydroquercetin. Flavonols are synthesized at this point by the flavonol synthase (FLS), forming kaempferol or quercetin (depending on where FLS introduces a double bond). Dihydroquercetin is then reduced by dihydroflavonol reductase (DFR) to obtain leucocyanidin. Anthocyanins are synthetized at this point by the anthocyanidin synthase (ANS) obtaining cyanidin. (+)-Catechin is obtained when leucocyanidin reductase (LAR) reduces leucocyanidin, and (−)-epicatechin is obtained when anthocyanidin reductase (ANR) reduces cyanidin.[4,36]

The study of regulatory genes encoding those enzymes of the phenylpropanoid and flavonol-anthocyanin pathways is crucial for modifying the accumulation of secondary metabolites of interest at the end of the route. In our work, an inverse relationship (in red and black fruit) between gene expression and accumulation of secondary metabolites has been seen, except for the *RuDFR* gene, the first gene of the anthocyanins route, which had a direct effect in the increase of cyanidin 3-O-glucoside (Fig. 3 and Table 2). However, in the study by Chen *et al.*,[37] some genes involved in anthocyanin and proanthocyanidin biosynthesis were investigated and the expression levels of genes agreed with the final products accumulated. Furthermore, they saw that enzymes encoded by the structural genes of the pathway had two peaks of maximum activity: at the beginning of the fruit ripening and at the end. The same pattern of enzyme activity was observed in strawberry by Halbwirth *et al.*,[38] except for DFR and FLS enzymes, which only had one peak of activity at the red-black stage (also seen by Almeida *et al.*).[39] This last was consistent with our results in which *RuDFR* gene was upregulated in red stages of the fruit, but downregulated in black, suggesting that dihydroflavonol reductase (DFR) only had one peak of activity at this stage of ripening (in red fruits).

Regarding the effects of our treatments in red blackberry fruit, ME treatment had effects in all the studied genes, downregulating *RuF3′H*, *RuFLS*, *RuANS*, *RuLAR* and *RuANR* and upregulating *RuDFR*.

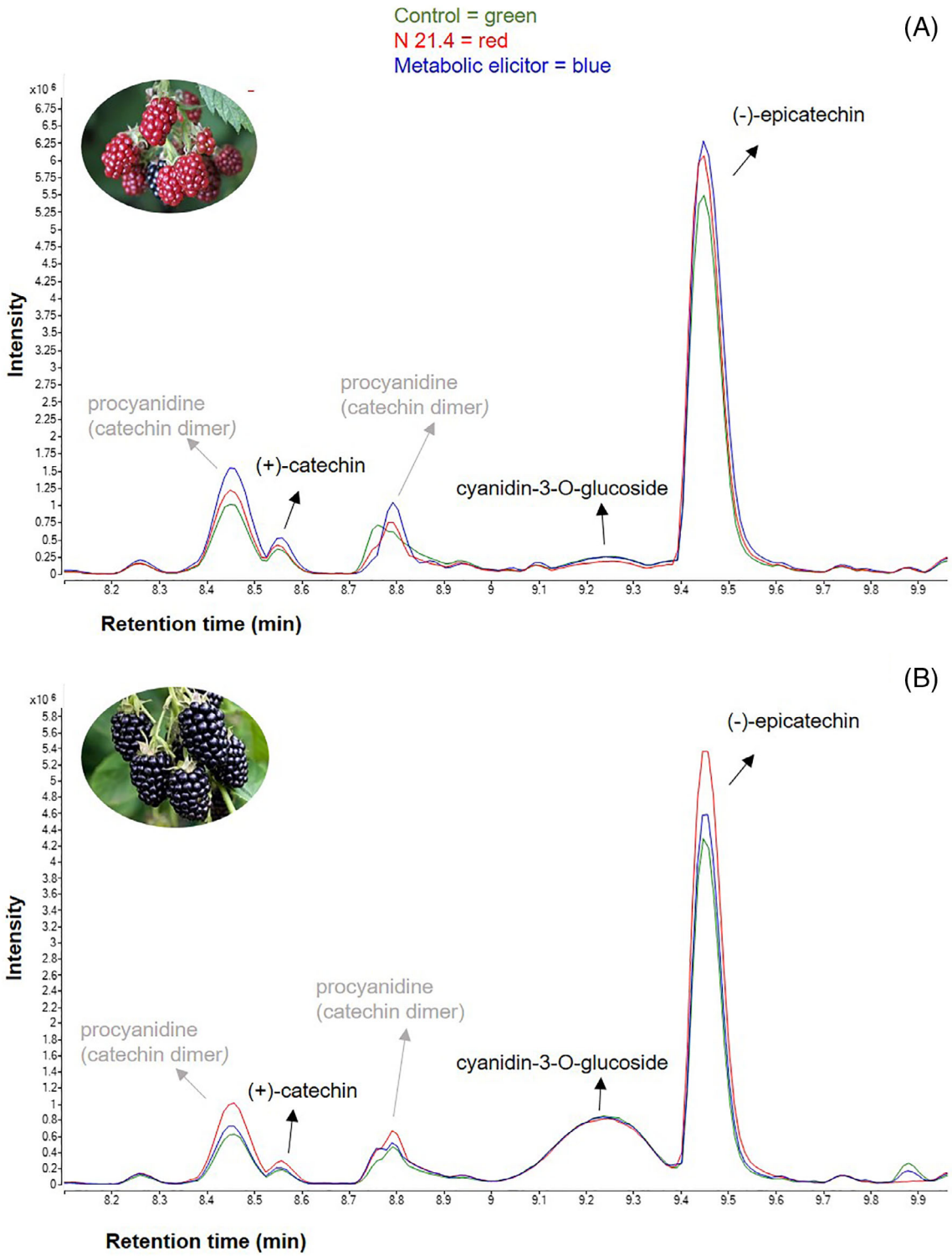

Figure 2 Enlarged chromatogram (from 8.0 to 9.9 min) visually comparing the content variation of three main phenolic compounds in red (A) and black (B) fruit and between treatments. Green line represents control, red line *P. fluorescens* N 21.4 and blue line ME treatments.

This was directly linked to an increase in all the compounds derived from the activity of the enzymes encoded by the above downregulated cited genes. The plants treated with ME had red fruit with a higher concentration of flavonoids, highlighting quercetin and kaempferol derivatives, catechin, epicatechin and anthocyanins. However, red fruits of N 21.4-treated-plants showed higher differential expression and lower concentration of flavonoid compounds, which reinforces our idea of a supposed inverse relationship between gene expression and final compound accumulation. A hypothesis that could explain this phenomenon is that, since there were more transcripts of these genes, all the biosynthetic machinery of phenolic compounds

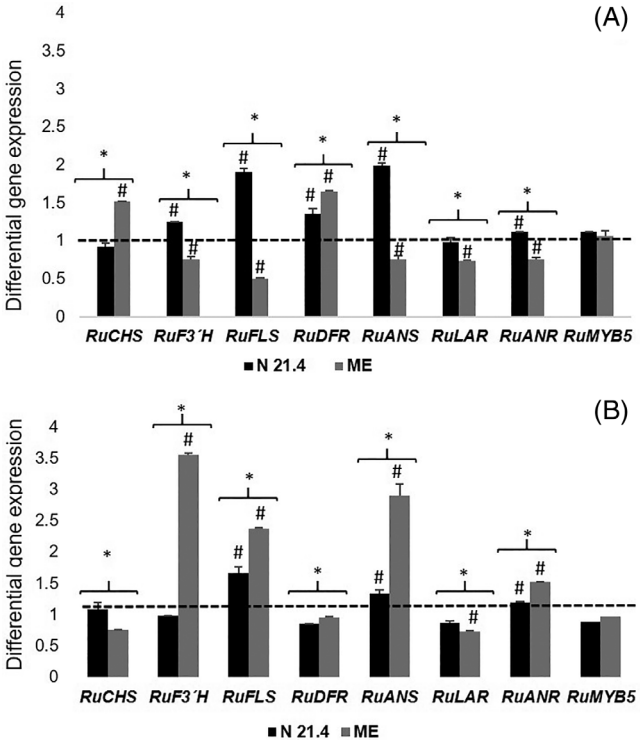

Figure 3 Differential expression of phenylpropanoids pathway genes in red fruit (A) and in black fruit (B) of blackberry plants inoculated with *P. fluorescens* N 21.4 and with its metabolic elicitors (ME). Asterisks represent statistically significant differences ($P < 0.05$) between treatments and hashtags represent statistically significant differences ($P < 0.05$) with the control. Expression of 1 is that of the control (horizontal black dashed line).

worked faster and bioactives were accumulated in later stages of the route as derivatives of the compounds that we had measured. Probably, if compounds had been measured in subsequent steps of the route, an accumulation of them would have been found (https://www.genome.jp/kegg-bin/show_pathway?map00944; https://www.genome.jp/kegg-bin/show_pathway?map00942).

In black fruit, the same inverse relationship was observed, but in this case it was the plants treated with N 21.4 that showed less differential expression and greater accumulation of all the secondary compounds, except anthocyanins, which accumulated more in fruits of ME-treated plants. This is also consistent with a greater expression of *RuDFR* observed with the ME treatment. *RuDFR* is the only gene in which a direct relationship between differential expression and anthocyanin accumulation was observed in red and in black fruit. *RuDFR* had the same behavior as that of *DFR* in the work by Almeida *et al.*[39] and Garcia-Seco *et al.*[12]

At the same time, *RuCHS* seem to be a fundamental regulatory gene related to the accumulation of secondary metabolites. When *RuCHS* is upregulated, compound accumulation occurs along the pathway. This had been previously seen in the work of Garcia-Seco *et al.*[12]

MYB transcription factors are among the most important regulators involving flavonoid biosynthesis.[12,40] In the present work, the gene expression of *Ru*MYB5 was analyzed, since in previous works and with other beneficial rhizobacteria from our collection, *Ru*MYB5 was found to be a positive regulator of the RuDFR, RuANR and RuLAR genes.[41] In that work, the positive regulation supposed an increase in catechin synthesis and their accumulation in the fruits. However, in our study, *RuMYB5* did not exceed the control differential expression. In the work of Thole *et al.*,[42] it is said that *RuMYB5* from cultivated blackberry has a peak of expression at the early intermediate ripening fruit stage, which could be related to a higher concentration of catechin and epicatechin in green-red fruit stages. In that study it is also said that Ru*MYB5* interacts with other transcription factors (*RuTTG1* and *RubHLH1*) related to proanthocyanidins synthesis, showing decreasing transcript levels during ripening. Despite not having seen differential gene expression of *RuMYB5* in our work, the tendency discussed in Thole *et al.*[42] work has been seen, with a higher concentration of catechin and epicatechin in red fruit and a lower concentration in black fruit. However, in strawberry, *FaMYB5* transcripts have been seen to accumulate steadily during fruit development. Moreover, Chang *et al.*[43] saw that not *RuMYB5*, but *RuMYB10* was the transcription factor that better controlled proanthocyanidin biosynthesis. Hence, it can be concluded that these transcription factors might be affected differently by diverse bioeffectors or by different crop conditions.

On the other hand, it has been seen that in early maturation stages (red fruit), (−)-epicatechin and (+)-catechin were the predominant phenolic compounds and their concentration decreased during ripening, when anthocyanins increased. This was consistent with the gene expression of *RuANR*, *RuLAR*, and *RuANS*, respectively. Chang *et al.*[43] and Garcia-Seco *et al.*[12] observed the same in blackberry and Almeida *et al.*[39] in strawberry. Hence, there is an obvious redirection of flavonoid biosynthesis from flavanol to anthocyanin formation during the complex developmental process of fruit ripening.[38,43] The different groups of polyphenols formed during fruit ripening fulfill different important functions, like herbivore deterrence with the presence of astringent flavanols in early stages[12] or visual attraction of ripen fruits. Anthocyanin, epicatechin, and catechin have been seen as key factors affecting fruit flavor and nutrition properties in persimmon, grape, berries, and many other fruits.[44-46] However, in grapes, catechin and epicatechin accumulation occurs immediately after fruit-set and maximum levels of accumulation are reached around véraison.[47] Consequently, these species might be under control of different regulatory mechanisms.

In our *Rubus* cv. Loch Ness plants, treatments inoculated (rhizobacterium N 21.4 and ME) affected not only the total phenolic and flavonoids during ripening but also the profile of different flavonols, flavanols, and anthocyanins (also seen by Garcia Seco *et al.*[12] with N 21.4). In general, the fruits of inoculated plants, and especially the fruits of ME-inoculated plants, accumulated secondary metabolites in greater concentrations than non-inoculated control plants. A remarkable fact was a major increase of flavanols ((−)-epicatechin and (+)-catechin) at both stages of fruit ripening with both treatments. These compounds have strong antioxidant capacity and they are able to reduce oxidative stress in plants.[48,49] They have also important cardiovascular benefits for humans.[50,51] Epicatechin also acts as phytoanticipins in some fruits[52] giving fungal and bacterial resistance to infection.

The elicitation of the secondary metabolism of *P. fluorescens* N 21.4-treated plants was again demonstrated and the elicitation of secondary metabolism of ME-treated plants was also evidenced. This secondary metabolism activation not only suggested an improvement in the nutritional quality of the fruit by increasing their metabolite content but it also suggested that plants trigger their defenses as a result of the activation of this secondary metabolism.[53] In general, ME had more marked effects on plant elicitation, enhancing the amount of many of the

metabolites accumulated in fruits. Hence, it is understood that ME-treated plants had a more active secondary defensive metabolism and therefore they were more protected against biotic and abiotic stress; they had better fitness. The fruit production in ME-inoculated plants was slightly lower, which is a clear symptom of the ME's elicitation capacity, which improved the quality of fruit slightly compromising fruit yield. Thus, ME-treated plants were more primed, were more fit, and had slightly fewer fruits, but fruits with better nutritional quality. Previous results from our group, with the same plant and with another rhizobacterium,[54] verified that elicitation supposes a redirection of resources towards defensive metabolism, slightly compromising fruit yield. They also support the view that metabolic changes inherent in the primed status[55] resulted in the allocation of carbon sources to the defense metabolism.[56]

In summary, the effectiveness of ME as plant inoculants for the elicitation of blackberry secondary metabolism was shown, as well as their capacity to modify the flavonoid biosynthetic pathway. The ME of *P. fluorescens* N 21.4 were able to modulate gene expression in the fruit of *Rubus* cv. Loch Ness, and to affect the profiles of secondary metabolites, increasing the synthesis and accumulation of them mainly during the red stage and potentially increasing nutritional properties of subsequent black fruit. The ME used as plant inoculants also have advantages related to the management and maintenance of inoculums. They are effective, cheap to produce, easy to manage, environmentally friendly, and they do not cause the biosecurity problems that live rhizobacteria could cause.[57] The application of ME of beneficial rhizobacteria as plants inoculants therefore opens a feasible new window towards the improvement of the nutritional qualities of crops using innovative and more ecofriendly agro-food techniques.

From all the above, we conclude that the efficacy of ME of *P. fluorescens* N 21.4 in the elicitation of blackberry secondary metabolism has been demonstrated. Metabolic elicitors are efficient, profitable and ecological plant inoculants that could be alternatives to agrochemicals, or could be either alternatives or complementary to rhizobacteria-based products. We can also conclude that, through the study of the phenylpropanoid pathway in blackberry fruit, the regulatory role of *RuCHS* in the accumulation of secondary metabolites at the final stages of the pathway has been shown, as well as the role of *RuDFR* in the increase of synthesis and accumulation of cynidine-3-*O*-glucoside.

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