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Bacterial bioeffectors delay postharvest fungal growth and modify total phenolics, flavonoids and anthocyanins in blackberries

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ABSTRACT

Blackberries are naturally rich in functional components beneficial for human health. The postharvest period of these fruits is very short due to fungal development, therefore, it is of great economic interest. Flavonoids and anthocyanins are secondary metabolites, and thus, strongly inducible. The aim of this study was to evaluate the ability of 6 bacteria with biocontrol traits and demonstrated Induced Systemic Resistance capacity, to prevent fungal growth during the postharvest period; the secondary aim was to identify whether the bacterial determinant was structural or metabolic, and if the treatment would affect flavonoid and anthocyanin levels. To achieve this goal, bacterial strains were sprayed dead or alive; fungal growth and phytochemicals were recorded.

Only one strain delayed fungal growth by 50%, being structural and metabolic elicitors independently as efficient as the strain itself (dead or alive). This protection was associated to a decrease in the evaluated metabolites (28% total phenolics, 33% total flavonoids, 24% anthocyanins), suggesting transformation of flavonoids and anthocyanins (phytoanticipins) onto other molecules (phytoalexins) involved in defense and confirming induction of natural immunity.

This study shows the potential of beneficial bacteria to develop a biological product to extend fruit-shelf life of blackberries, increasing benefits for health and economic profit.

1. Introduction

Blackberry (*Rubus* spp.) is an aggregate fruit, composed of small drupelets, belonging to the Rosaceae family. Nowadays, blackberries are gaining importance due to its richness in functional components, beneficial for human health in prevention of chronic diseases (Kaume, Howard, & Devareddy, 2012; Martin, Butelli, Petroni, & Tonelli, 2011). These compounds are mainly represented by polyphenols such as anthocyanins and flavonoids, which are strong natural antioxidants. Due to the high polyphenol concentration and diversity, berry fruits including blackberries, are increasingly often referred to as natural functional foods (Paredes-Lopez, Cervantes-Ceja, Vigna-Perez, & Hernandez-Perez, 2010), and the beneficial components are referred to as phytonutrients (Butelli et al., 2008) or bioactives (Szajdek & Borowska, 2008).

Plant secondary metabolites significantly contribute to adaptations to changing environmental conditions and plant communication (Dörnenburg, 2004). Therefore, these pathways are strongly inducible and are especially sensitive to abiotic (Ayala-Zavala, Wang, Wang, & González-Aguilar, 2004; Wu, Frei, Kennedy, & Zhao, 2010) and biotic stimuli (Capanoglu, 2010). Systemic induction during plant growth has been widely demonstrated in many plant species as a tool to increase plant fitness (Kumar, Shivay, Dhar, Kumar, & Prasad, 2012), either stimulating plant growth or preventing disease progression (Ramos-Solano, Barriuso, & Gutiérrez Mañero, 2008). On the other hand, during development of commercially valuable plant organs and after their harvest, natural disease resistance generally declines leading to the inevitable infection, disease and ultimately death (Terry & Joyce, 2004). For this reason, there is a great interest to identify the conditions leading to disease and the elicitors able to trigger plant metabolism to prevent postharvest decay.

Among the great efforts done to increase natural disease resistance in harvested horticultural crops, physical, biological and/or chemical elicitors have received increasing attention over the recent years (Terry & Joyce, 2004). Most studies carried out with

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biological elicitors involve pathogenic microorganisms or derived elicitors, but the use of free-living non-pathogenic rhizobacteria known as plant growth promoting rhizobacteria (PGPR) are currently overtaking a relevant position (Algar et al., 2013).

PGPR are able to trigger secondary metabolism by the means of different chemical molecules. These molecules are frequently highly conserved through evolution in such a way that plants have been able to develop receptors for these structures to prevent invasion (Lakshmanan et al., 2012); these bacterial determinants are called MAMPs (Microbe-Associated Molecular Patterns). Recognition of MAMPs represents the major trait of innate immunity common to both plants and animals. In plant immunity, MAMPs, more commonly known as general elicitors are able to elicit a defense response in hosts by binding to specific PRRs (Zamioudis & Pieterse, 2012). In addition, there are other bacterial metabolites not as well conserved but still effective, as they may overcome plant defensive metabolism; these are grouped as metabolic determinants or effectors (Dodds & Rathjen, 2010).

On the other hand, the beneficial effects of PGPR bacteria also have a well-documented background for biocontrol during plant growth (Bevivino et al., 1998; Lorito, Di Pietro, Hayes, Woo, & Harman, 1993). This statement supports the notion for preventing fungal growth on fruits during the postharvest period.

The aim of this study was to evaluate the ability of six bacterial strains to prevent fungal growth during the postharvest period in blackberries. Consequently, the final objective is to develop a safe biotechnological procedure to extend fruit shelf-life with improved quality. To achieve these goals, bacterial strains, dead or alive, will be delivered to blackberries with a double aim, first to identify if the putative elicitor is able to prevent fungal growth, and second, if the process involves a biocontrol or a systemic induction mechanism, affecting flavonoids and anthocyanin contents in the edible fruit.

2. Materials and methods

2.1. Plant material

Blackberries harvested from hybrid plants of *Rubus* spp. var. Loch Ness were kindly provided by the company Agricola El Bosque, S.L.L. (Lucena del Puerto, Huelva, Spain) in March 2012, July 2012, March 2013 and July 2013. The variety *Rubus* spp. var. Loch Ness is a high yield tetraploid (4n = 28), and one of the most cultivated worldwide. .

2.2. Bacterial strains

The 6 bacterial strains used in this study were isolated from the rhizosphere of wild populations of *Nicotiana glauca* and identified by 16s rDNA sequencing: N6.8 as *Stenotrophomonas maltophilia*, N17.35 as *Pseudomonas aeruginosa*, N19.27 as *Pseudomonas corrugata*, N5.18 as *S. maltophilia*, N21.4 as *Pseudomonas fluorescens* and N11.37, as *Bacillus subtilis* (Ramos-Solano, Algar, et al., 2010; Ramos-Solano, Lucas-García et al., 2010). These strains have been selected for their ability to trigger systemic metabolism and because all of them produce siderophores and chitinases, that can reduce the fungal growth (García-Seco et al., 2013; Ramos-Solano, Algar, et al., 2010, Ramos-Solano, Lucas-García et al., 2010; Ramos-Solano et al., 2014).

2.3. Inoculum preparation

Bacterial strains were maintained at −80 °C in nutrient broth (Pronadisa, Spain) with 20 ml/L glycerol. Inocula were prepared by streaking strains onto plate count agar (PCA) plates, incubating plates at 28 °C 24 h, and scraping bacterial cells off the plates into sterile 10 mmol/L $MgSO_4$ buffer. The inoculum was delivered to fruits at 10^7 colony forming units (c.f.u.)/mL.

Inoculum of each strain with the desired concentration density were irradiated with ultraviolet light (UV) for 1 h under sterile conditions to obtain non-viable inoculum; the inoculum was confirmed to be non-viable by plating the UV-treated bacterial solution.

2.4. Bacterial elicitors from N17.35

Strain N17.35 survived extremely long UV irradiation periods so bacterial elicitors were obtained with a different approach. Fresh cultures (10^7 ufc/mL) were split in two fractions, one was kept viable (alive inoculum) and the other part was autoclaved for 20 min, at 121 °C and 98.07 kPa. The autoclaved inoculum was divided in two fractions: one was centrifuged to separate the supernatant and the pellet; the supernatant (S) would contain putative soluble elicitors and the pellet (P) would contain putative structural elicitors; and the other was not separated and contained both fractions (dead inoculum).

2.5. Experimental set up

Two types of experiments were set up. First, a single screening experiment to identify effective strains. Once the effective strain was identified, the second type of experiment was carried out, and repeated 3 times, to confirm effectiveness. The first experiment was set to identify the potential of the 6 strains to prevent or delay fungal growth during the postharvest period. Since control of fungal growth could be achieved either by induction of secondary metabolism on fruits or by a biocontrol mechanism, blackberries were divided in two groups, one to detect potential systemic induction (Group A), and one to screen for biocontrol ability (Group B). The first group (Group A) consisted on three 125 g-trays, with 25–30 fruits in each tray, (n = 3); 3 days after treatment (see below), bioactive contents and nutritional characterisation were determined. The second group (Group B) was used to screen for biocontrol capacity of the 6 strains, it consisted of one 125 g tray for each treatment, with 25–30 fruits in each tray; effective treatments were assayed with a larger number of replicates in the second experiment. Therefore, a total of 11 treatments (5 strains UV-treated and alive + N17.35 alive) and a non-inoculated control were assayed in the first experiment. Inocula (viable and non-viable) were prepared as described above for each strain.

Blackberries were removed from 125 g plastic trays and gently placed on a flat surface in a flow cabinet and sprayed with a 10^7 ufc/mL bacterial solution (10 mL); controls were mock inoculated with 10 mmol/L $MgSO_4$ phosphate buffer to reproduce the same moisture conditions as in treated fruits. Fruits were allowed to dry, placed back on plastic trays and incubated at 20 °C in a SANYO MLR-350H growth cabinet with a 12 h light/dark. The first group (Group A) was incubated for 3 days, and determinations were carried out. The second group (Group B) was incubated in the growth chamber for 8 days, when the number of fruits showing fungal growth over total number of blackberries were recorded. Results are expressed as a relative fungal growth in order to compare the different experiments.

The second experiment was carried out with the most efficient strain controlling fungal growth, N17.35. The experimental set up and conditions were as described above, but using a higher number of replicates (n = 8). Treatments were N17.35 (alive), N17.35 dead supernatant (S) and pellet (P), and the non-inoculated control. Blackberries were treated as described above, and fungal growth was recorded after 8, 10 and 12 days. Three independent experiments were carried out. In these, an additional treatment with the

complete autoclaved strain was evaluated. Fungal growth was recorded 4, 6 and 10 days after the onset of the experiment, since after 12 days fungal development was excessive.

2.6. Determination of chemical composition

For determinations, all berries in each 125 g plastic tray were pooled and crushed to obtain the raw juice, which was stored at −80 °C for further analyses.

2.6.1. Nutritional characterisation

For nutritional characterization, 50 g of each fresh raw juice was centrifuged for 10 min at 3000 *g*. Determinations were done on supernatants.

Soluble solids were determined with a Portable refractometer at 20 °C. Results are expressed in °Brix.

Citric acid content was measured following the AOAC (2000) official method 932.05 (2000), by acid-base evaluation with NaOH 0.1 mol/L.

pH was measured with MicropH2001 (CRISON) pHmeter at room temperature.

2.6.2. Bioactive content characterisation

For bioactive content characterisation, 1 g of each raw juice was extracted with 80 g/L methanol (1/10 w/v), sonicated with an ultrasonic cleaner (Selecta 50 Hz) for 5 min, and centrifuged at 2300 *g* for 5 min at 4 °C.

2.6.2.1. Total phenolic content. The phenolic content was determined quantitatively with Folin-Ciocalteau reagent (Sigma-Aldrich, St Louis, MO) by colorimetry (Xu & Chang, 2007), with modifications (García-Seco et al., 2013), using gallic acid as a standard (Sigma-Aldrich, St Louis, MO). Results were expressed as mg gallic acid equivalent (GAE) per 100 g of fresh weight (FW). All samples were analysed in triplicate.

2.6.2.2. Total flavonoids content. Total flavonoids content was determined quantitatively by an aluminium chloride colorimetric assay (Zhishen, Mengcheng, & Jianming, 1999) with modifications, using catechin as a standard (Sigma-Aldrich, St Louis, MO). Total flavonoid content of fruit extracts was expressed as mg catechin equivalents (CE) per 100 g FW. All samples were analysed in triplicate.

2.6.2.3. Anthocyanins content. Methanolic extracts were filtered through 0.45 μm nylon filters and injected in HPLC. Determinations were performed according to Burdulis et al. with modifications (2008), on a Beckman HPLC provided with a two-pump 125 solvent module and a 168 diode array detector. Separation of anthocyanins was carried out using a Thermo Hypersil ODS (C18) column (100 × 4 mm; particle size: 3 μm), provided with a Hypersil ODS (10 × 4 mm i.d.; particle size: 5 μm) guard column (Thermo). The column was kept at room temperature. The flow rate was kept constant at 1.0 ml/min for a total run time of 45 min. Mobile phases were A: acetonitrile and B: 4 ml/L aqueous ortophosphoric acid in water (V/V). The system was run with the following gradient 0–31 min, 8–22% A, 31–36 min, 22–8% A and 36–46 min 8% A. The injection volume was 10 μL. The UV/Vis detector was set at 520 nm (Burdulis, Janulis, Milašius, Jakštas, & Ivanauskas, 2008). The calibration curve of cyanidine-3-glucoside (Polyphenols, Norway) was used to quantify other anthocyanins since the sugar moiety does not contain an ultraviolet chromophore, it was hypothesized that the absorption properties of the glycoside structures at 520 nm should not be modified by a conjugation, and that their response factor only depended on their molecular weight (Berger, Rasolohery, Cazalis, & Daydé, 2008).

To identify the peaks, the samples were ran on a Surveyor LC system attached to a DecaXPplus ion trap MS (both from Thermo). Anthocyanins were separated on a 100 × 2.1 mm 2.6 μ Kinetex XB-C18 column (Phenomenex) using the following gradient of acetonitrile versus 0.1 ml/L formic acid in water, run at 300 μL.min-1 and 30 °C, (0–1 min, 2% A, 1–6 min, 2–10% A, 6–26 min, 10–30%, 26–36.5, 30–90% A, 36.5–37.5, 90% A, 37.5–38 min, 90-2% A, 38–45 min, 2%). Anthocyanins were detected by absorbance, collecting spectra from 200 to 600 nm, and by positive mode electrospray MS.

All solvents were HPLC grade (VWR, Spain).

2.7. Statistical analyses

To evaluate bacterial effects on all parameters measured, one-way analysis of variance was performed. When differences were significant, the least significant differences (LSD) posthoc test was also performed (Sokal & Rohlf, 1979) with the software Statgraphics plus 5.1 for Windows.

Pooled variance is a method for estimating variance given several different samples taken in different circumstances where the mean may vary between samples but the true variance (equivalently, precision) is assumed to remain the same. Pooled variance is calculated for a series of replicated measurements as the sum of individual variances pondered by degrees of freedom (sum of VarixDFi/sumDF). The square-root of a pooled variance estimator is known as a pooled standard deviation (Gurevitch & Hedges, 1993; Wolf, 1986).

3. Results

Delivering bacterial bioeffectors (dead or alive) to blackberries during the postharvest period (3 days after harvest) resulted in higher presence of fungal growth 8 days after treatment compared to non-inoculated controls (Table 1) in five out of the 6 strains under trial. Only strain N17.35 decreased fungal presence by approximately 50%.

Interestingly, N17.35 survived long UV-exposure times (over 2 h) so a different approach to inactivate the bacterium was applied and evaluated in a second experiment (Table 2). The bacterial strain N17.35 was tested alive, and inactivated by autoclaving the culture;

Table 1
Relative Fungal Infection on postharvest period on blackberries treated with different bacterial bioeffectors (alive strains (A) or UV-inactivated (D)) 8 days after challenge. The control is expressed as 100% of fungal infection (fungal infection index for control = 51.61).PS: *Pseudomonas fluorescens*, PC: *P. corrugata*, PA: *P. aeruginosa*, SM: *Stenotrophomonas maltophilia*, BS: *Bacillus subtilis*. Asterisks indicate significant differences with non -inoculated controls after bacterial challenge within each experiment according to LSD test ($p < 0.05$).

Treatments	Number of fruits with fungi	Total number of fruits	% Blackberries affected
C	16	31	52
PS N21.4 A	23	24	96*
PS N21.4 D	23	24	96*
PC N19.27 A	29	29	100*
PC N19.27 D	30	30	100*
PS 17.35 A	7	24	29*
SM N5.18 A	23	27	85*
SM N5.18 D	25	29	86*
SM N6.8 A	26	27	96*
SM N6.8 D	20	21	95*
BS 11.37 A	27	31	87*
BS 11.37 D	24	28	86*

Table 2
Relative fungal infection (%) at three time points along the postharvest period on blackberries treated with N17.35 and derived bioeffectors. Assay A) 8, 10 and 12 days after bacterial challenge; B) 4, 6 and 10 days after bacterial challenge; C) 4, 6 and 10 days after challenge ($n = 3$). The control is expressed as 100% of fungal infection (fungal infection index for control = 32.7%; 43.75%; 81.25% (A); 0%; 25.71%; 93.39% (B); 30.22%; 58.06%; 70.51% (C)). Asterisks indicate significant differences with non -inoculated controls after bacterial challenge within each experiment according to LSD test ($p < 0.05$).

	Treatment	TIME 1	TIME 2	TIME 3
Assay A	N17.35 Alive A	39 ± 7*	82 ± 24	104 ± 15
	N17.35 Dead S	67 ± 13*	79 ± 21	79 ± 12*
	N17.35 Dead P	84 ± 26	107 ± 17	95 ± 16
Assay B	N17.35 Alive A	0 ± 0	26 ± 14*	79 ± 5
	N17.35 Dead S	0 ± 0	71 ± 15	101 ± 6
	N17.35 Dead P	0 ± 0	17 ± 8*	88 ± 4
Assay C	N17.35 Alive A	0 ± 0*	9 ± 4*	53 ± 10
	N17.35 Dead S	25 ± 3*	44 ± 18*	61 ± 10
	N17.35 Dead P	15 ± 7*	57 ± 29*	64 ± 31
	N17.35 Dead S + P	0 ± 0*	41 ± 21*	74 ± 16

two fractions were assayed, supernatants (S) and precipitate (P). The two assayed fractions aimed to unravel if the elicitor was structural (precipitate) or metabolic (supernatant). Eight days after the elicitor challenge, fungal presence was significantly decreased by the bacterial strain, and by the metabolic elicitor (S), showing approximately 60% and 30% protection relative to controls, respectively (Table 2).

The experiment was repeated two additional times with similar results (Table 2). A supplementary treatment was included in the last experiment with the complete autoclaved N17.35 (P + S), in addition to the two separated fractions (S and P). Similar results were obtained; the fraction including the complete bacteria (alive or dead) 4 days after treatments was especially effective (100% protection).

Citric acid content in blackberries averaged 1.4 g/kg citric acid, pH averaged 3.2 and total soluble solids averaged 10.3 °Brix (data not shown). None of these parameters were affected by any treatment.

Total phenolic contents in control fruits averaged 580 mg gallic acid equivalents/100 g FW, total flavonoids 98 mg catechin equivalents/100 g FW and total anthocyanin content averaged 254 mg cyanidin equivalents/100 g FW (Table 3).

A total of 3 anthocyanins were identified by HPLC-MS (Fig 1, Table 4). Cyanidin-3-Glucoside was the predominant anthocyanin in blackberries (90%), being its contents around 250 mg/100 g FW; Cyanidin-3-Galactoside 19 mg/100 g FW and Cyanidin-3-Glucoside-Malonate 15 mg/100 g FW (Table 5).

Total phenolic content was the less affected parameter by inoculation, while total flavonoids and anthocyanins were the most affected; no significant increases were observed after any treatment (Table 3). The pooled variance is a method for estimating variance given several different samples taken in different circumstances where the mean may vary between samples but the true variance is assumed to remain the same. According to this pooled variance, total phenolics and total flavonoids were not significantly affected by treatments, while anthocyanins were. However, unidirectional ANOVAS highlighted a significant decrease in total phenolics for N17.35, and in total flavonoids on N17.35 and N11.37 treated fruits while total anthocyanins were significantly decreased in all fruits treated with the living strains, and only under the dead fractions of N6.8 and N11.37. Decreases in anthocyanins were detected only on cyanidin-3-glucoside under all treatments (Table 5).

Organisation of these results according to the bacterial genera indicates that the two *Stenotrophomonas* strains (N5.18 and N6.8) did not affect total phenolics or flavonoids, but anthocyanins were significantly decreased by N6.8 (dead or alive) and by N5.18 (only alive) (Table 3). Among the three *Pseudomonas* (N21.4, N19.27 and N17.35), *P. aeruginosa* N17.35 is the most effective strain modifying bioactives, decreasing significantly all the evaluated parametres. Similarly to the two *Stenotrophomonas*, N19.27 significantly decreased flavonoids and anthocyanins alive, while N21.4 is only effective on anthocyanins when delivered alive.

4. Discussion

Induction of natural disease resistance (NDR) in harvested horticultural crops using physical, biological and/or chemical elicitors, has received increasing attention over recent years (Capanoglu, 2010; Terry & Joyce, 2004). Biological induced resistance has been successfully approached to identify microbial antagonists for postharvest biocontrol (Ippolito & Nigro, 2000). Consistent with this rationale, the six bacterial strains tested in this experiment had demonstrated their ability to release siderophores and chitinases to specific culture media (Ramos-Solano, Lucas-García et al., 2010) and therefore, they had a good potential to prevent fungal growth. This potential is based on the two mechanisms described for biocontrol, iron scavenging by siderophores (Lorito et al., 1993) and hydrolysis of fungal cell walls with chitinases (Bevivino et al., 1998). Among the 6 strains tested, only N17.35 decreased fungal presence supporting the notion of effective biocontrol activity (Table 1). However, a dose factor may also be

Table 3
Bioactives contents of blackberries treated with different bacterial bioeffectors (alive strains (A) or UV-inactivated (D)) 24 h after challenge. Column 1, Total phenolics (mg gallic acid equivalents/100 g FW), B) total flavonoids (mg catechin equivalents/100 g FW), C) total anthocyanins (mg cyanidin-3-glucose equivalents/100 g FW). Asteriks indicate significant differences between treatments and control according to LSD test ($p < 0.05$) ($n = 3$). PS: *Pseudomonas fluorescens*, PC *P. corrugata*, PA: *P. aeruginosa*, SM: *Stenotrophomonas maltophilia*, BS: *Bacillus subtilis*. SD_{pooled} = pooled standard deviation ($n = 3$).

Treatment	Phenolics content (meq gallic acid/100 g FW)	Flavonoids content (meq catechin/100 g FW)	Anthocyanin content (meq cyanidin/100 g FW)	Antioxidant potential (EC50) (μl FW/ml DPPH)
C	573	96	254	0.04
PS N21.4 A	573	85	187*	0.05
PS N21.4 D	558	99	237	0.04
PC N19.27 A	555	73*	196*	0.05
PC N19.27 D	491	76	201*	0.05
PS N17.35 A	409*	64*	193*	0.04
SM N5.18 A	559	83	182*	0.04
SM N5.18 D	549	89	247	0.04
SM N6.8 A	600	82	188*	0.04
SM N6.8 D	604	99	188*	0.04
BS N11.37 A	633	81	225*	0.04
BS N11.37 D	535	72*	211*	0.04
Pooled Standard Deviation	38	7	8	0.003

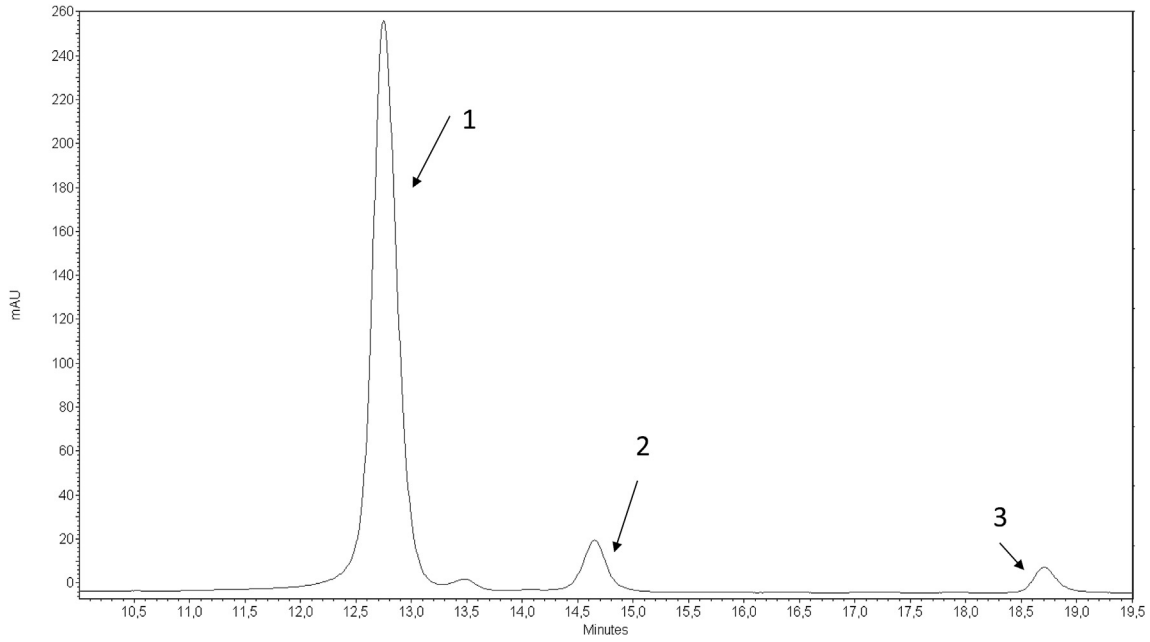

Fig. 1. HPLC chromatogram of anthocyanins (Cyanidin-3-Glucoside (1), Cyanidin-3-Arabinoside (2), Cyanidin-3-Glucoside-Malonate (3)) in an extract of *Rubus* sp. Var. Loch Ness detected at 520 nm (20 nm).

responsible for failure of the other strains showing the same potential traits for biocontrol (Raaijmakers et al., 1995). Since nutritional requirements of pathogenic fungal load and activation of fungal pathogenicity factors upon environmental conditions have also been reported as factors affecting the decline of natural disease resistance (Prusky, 1996), prevention of fungal growth by delivering these strains may change if harvest is done on other environmental conditions. Supporting this concept, the four experiments carried out in this study with the effective strain N17.35 show a variable inhibition of fungal growth, depending on the harvest time of fruits (Table 2).

Since the postharvest treatment is done on the edible fruit, health concerns may arise due to the bacterial load. Therefore, there is an interest to identify the bacterial elicitors responsible for the effect. In order to identify these bacterial determinants, a parallel approach was carried out with UV-inactivated strains, since a postharvest application of the dead microorganisms would prevent bacterial growth and therefore, it would be safer for consumer's health. Effectiveness of dead bacteria supports systemic induction of plant metabolism by ruling out bacterial biocontrol activity that calls for the living microorganism. As in the case of living bacteria, no strain was effective preventing fungal growth (Table 1), suggesting that structural bacterial determinants did not trigger plant defensive metabolism to enhance natural disease resistance (Terry & Joyce, 2004). However, bioactive levels were modified (Table 3)

Table 4
Retention Times (HPLC-diode array and HPLC/MS) and High-Resolution Electrospray MS for Anthocyanins Identified in *Rubus* sp. Var. Loch Ness.

Peak number	Compound	Retention time (min)		Mass (MS^2)
		HPLC-diode array	HPLC/MS	
1	Cyanidin-3-Glucoside	12.5–13.00	8.33	449 (287)
1	Cyanidin-3-Glucoside		11	449 (287)
2	Cyanidin-3-Arabinoside	14.5	12.57	419 (287)
3	Cyanidin-3-Glucoside-malonate	18.5	13.58	535 (491,449,287)

indicating changes in metabolism, although it did not result in fruit protection.

Effects of N17.35, the effective strain, were approached in a different way. The strain was inactivated by autoclaving the culture and two fractions were tested, aiming to unravel if the elicitor was structural (P), like flagellin (Lakshmanan et al., 2012) and oligogalacturonide-like (OGs) (Ferrari et al., 2007), or metabolic (S) (Sánchez-Sampedro, Fernández-Tárrago, & Corchete, 2005). Since the most effective elicitor varied depending on the experiment, an additional treatment was included to evaluate the potential of the metabolic (S) and structural (P) elicitors together. Interestingly, the combination was more effective than the individual fraction, and equivalent to the living bacteria. This speaks about several determinants involved in the effect and ensures a successful result in the market. Since these determinants are able to trigger plant immune system, prior to commercialisation, they should be evaluated for potential effects for human health in order to ensure safety for consumers.

Measuring fruit response upon exposure to different bacterial fractions (dead or alive) can be useful to know the nature of the immune mechanism involved in *Rubus-P. fluorescens* N21.4 interaction (García-Seco et al., 2013). Based on our data, N17.35 dead and alive is able to trigger a response in the plant, speaking of a plant-based defensive mechanism able to detect potential bacterial threats. This mechanism is based on known determinants (Van Wees, Van der Ent, & Pieterse, 2008) termed MAMPs (Erbs & Newman, 2012).

In addition to surface biocontrol, a number of antagonistic microorganisms have been shown to induce defense reactions in host tissues (Adikaram, Joyce, & Terryc, 2002), increasing natural disease resistance by enhancing contents in defensive compounds, which may further report health benefits (Cantos, Espín, & Tomás-Barberán, 2001; García-Seco et al., 2013). Therefore, the experimental set up addressed evaluation of secondary metabolites (bioactives) in blackberries, irrespective of the lack of effect of these bioeffectors to prevent fungal growth.

Citric acid content, pH and total soluble solids were not affected by any treatment probably because changes in these parameters

Table 5
Anthocyanins content (Cyanidin-3-Glucoside, Cyanidin-3-Arabinoside, Cyanidin-3-Glucoside-Malonate) (mg/100 g FW) in an extract of *Rubus* sp. Var. Loch Ness of blackberries treated with different bacterial bioeffectors (alive strains (A) or UV-inactivated (D)) 24 h after challenge. Asterisks indicate significant differences with non-inoculated controls after bacterial challenge within each experiment according to LSD test ($p < 0.05$).

Treatment	Cyanidin-3-glucoside (mg/100 g FW)	Cyanidin-3-Arabinoside (mg/100 g FW)	Cyanidin-3-glucoside-Malonate (mg/100 g FW)
C	254 ± 6	18.8 ± 1.7	15.1 ± 0.9
PS N21.4 A	187 ± 14*	15.8 ± 0.8	13.9 ± 0.9
PS N21.4 D	237 ± 2	16.6 ± 0.1	14 ± 0.7
PC N19.27 A	196 ± 1*	17.7 ± 0.5	13.8 ± 0.4
PC N19.27 D	201 ± 5*	16.5 ± 0.5	13.4 ± 0.1
PS N17.35 A	193 ± 13*	17 ± 0.6	13.4 ± 0.4
SM N5.18 A	182 ± 6*	15.4 ± 0.8	12.8 ± 0.1
SM N5.18 D	247 ± 1	18.4 ± 0.4	15.1 ± 0.3
SM N6.8 A	188 ± 10*	16.4 ± 0.6	12.5 ± 0.2
SM N6.8 D	188 ± 11*	16.6 ± 1.1	12.9 ± 0.4
BS N11.37 A	225 ± 5*	16.8 ± 0.7	13.8 ± 0.8
BS N11.37 D	211 ± 0*	15.8 ± 0.5	13.4 ± 0.6

mostly occur during maturation, as they are related to photosynthesis and respiration (Zhang et al., 2008). In non-climacteric fruits, such as blackberries, most organoleptic changes occur during maturation but once harvested, the post-harvest organoleptic changes are caused by fruit senescence.

As contents of anthocyanins, flavonoids and other phenolics have been reported to increase after microbial attack, chemicals, wounding or other factors (Capanoglu, 2010), an increase in these compounds would be expected after biological elicitation (Ballester, Izquierdo, Lafuente, & González-Candelas, 2010). However, results did not confirm this hypothesis since the evaluated secondary metabolites were lower in elicited fruits than in non-inoculated controls (Table 3), being total phenolic content the less affected parameter, while total flavonoids and anthocyanins were most affected. Interestingly, N17.35 which was the only effective delaying fungal growth, decreased all bioactives, supporting the notion of transformation of these molecules into other defensive compounds (Boue et al., 2008). Hence, the observed decreases in flavonoids and anthocyanins suggest transformation of these compounds into phytoalexins upon challenge, indicating that flavonoids and anthocyanins accumulate as phytoanticipins (Algar et al., 2014; Capanoglu, 2010) that are able to respond to challenges even after detaching fruits from the plant. The versatility of secondary metabolism to overcome any challenge is shown in the different effects achieved by strains (Weston et al., 2012).

5. Conclusions

Due to the particularly practical purpose of this research, the results are really encouraging as treatment delays fungal growth, ensuring a longer shelf-life of the product. In addition, the effect is achieved with the non-viable strain that holds a number of effective determinants against natural fungal populations. Further studies need to be carried out to unravel the nature of these elicitors for commercial uses.

On the other hand, characterisation of compound formed from flavonoids and anthocyanins upon bacterial challenge is to be studied. Moreover, consistent effects will allow defining bioactive contents in elicited fruits within certain limits, contributing to provide a complete nutritional labelling in blackberries. This will hopefully result in an added value for the fruits and more information to the consumer, contributing to the Great Challenge on health for the next 50 years (Daar et al., 2007).

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