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RESEARCH ARTICLE

Enhanced blackberry production using *Pseudomonas fluorescens* as elicitor

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Abstract This report shows for the first time the ability of *Pseudomonas fluorescens* N21.4 to trigger the secondary metabolism of blackberries, *Rubus fruticosus*, in the field. Blackberries are an excellent source of bioactive compounds as compared to other marketable berries. Biotic elicitation with plant growth-promoting rhizobacteria has been proposed to improve biomass production and to trigger secondary metabolism. However, most reports that support this statement involve controlled, in-door experiments, not real field conditions under continuous environmental changes. Furthermore, most investigations are carried out using model plants. Strain *P. fluorescens* N21.4 has been able to trigger secondary metabolism of plant species. Therefore we studied *P. fluorescens* ability to increase blackberry fitness, fruit quality, and protection against natural pests under field conditions. *P. fluorescens* N21.4 was delivered in the root or shoot system of blackberry plants along its entire production period, evaluating fruit quality and yield. Our results show an average increase up to 800 g per plant in total production, directly related to the increase in flowering buds. Protection against *Spodoptera littoralis* in inoculated plants was similar to control plants, hence contributing to increase in yield. Fruits from inoculated plants showed significant increases of up to 3 °Brix, total phenolics of up to 18 %, and flavonoids of up to 22 %. We conclude that *P. fluorescens* N21.4 enhances plant defense and fruit quality together with an increased productivity as compared to current management practices, already obtaining high yields with economic profit.

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1 Introduction

Blackberry (*Rubus fruticosus*) is an aggregate fruit, composed of small drupelets, belonging to the *Rosaceae* family (Fig. 1). Nowadays, blackberries are gaining importance due to its richness in functional components, beneficial for human health in prevention of chronic diseases. Those compounds are mainly represented by polyphenols such as anthocyanins, phenolic acids, and flavonoids, 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-López et al. 2010).

However, because of the inducible nature of secondary metabolism, polyphenols levels change according to environmental conditions (Poulev et al. 2003; Boué et al. 2008); therefore, effects on health through the diet are not consistent, since a relation between the dose and the response may not be established. This lack of reproducibility may be overcome by means of elicitation that is, triggering plant's metabolism with elicitors (Capanoglu 2010). So far, elicitors have been grouped into two distinct blocks abiotic factors (light intensity, temperature, and chemicals) and biotic factors (pathogenic bacteria, beneficial bacteria, fungi, and insects) (Radman et al. 2003). Biotic elicitation with plant growth-promoting rhizobacteria (PGPR) is proposed as a useful strategy to improve biomass production and to trigger secondary metabolism at the same time (Gutierrez Mañero et al. 2003; Zhang et al. 2004). Upon recognition of the non-pathogenic biotic agent, a series of metabolic changes are systemically initiated throughout the plant to activate defensive metabolism (van Wees et al. 2008). Using PGPR as elicitors to trigger secondary metabolism has a double

Fig. 1 *Rubus fruticosus* var. Lochness

advantage. First, defensive metabolites are bioactive compounds and therefore, elicited edible plant species constitute food products with an added value for human health; second, from a physiological point of view, these metabolic changes indicate that the biotic agent would be priming the plant. Primed plants show a specific metabolic state that allows a better performance upon stress challenge although plant growth may be compromised in the first stages (van Hulten et al. 2006). Despite simultaneous induction of growth and accumulation of secondary metabolites is rare in nature, the use of selected PGPR bacteria or certain cellular components to increase levels of secondary metabolites has been demonstrated in various studies as in *Digitalis lanata* for cardenolides (Gutierrez Mañero et al. 2003), in *Glycine max* for isoflavones (Ramos Solano et al. 2010a), or in *Arabidopsis thaliana* to enhance defense against pathogens (van Wees et al. 2008), among others.

In view of the above, there is a great interest in determining effectiveness of PGPRs as biological agents to obtain consistent and reproducible improvement on secondary metabolism. These improvements will result in an increased food quality, with consistent high bioactive content, together with high production rates, while enhancing plant protection. On the basis of the foregoing, the objective of this study was to evaluate the ability of the PGPR strain *Pseudomonas fluorescens* N21.4 (CECT 7620) to cause a systemic stimulation of blackberry metabolism throughout the growth period, evaluating plant growth and fitness as markers of the induction, and effects on fruit quality parameters and yield, in field conditions.

2 Materials and methods

2.1 Plant material

R. fruticosus var. Lochness was used. This is a high-yield thornless variety. All assays were carried out at production fields of the company Agricola El Bosque (Lucena del Puerto, Huelva, Spain). Plants and greenhouses were kindly provided by the company and all were handled according to regular agricultural practices. Plants were grown under "winter cycle" that is after an artificial cold period, from July to November 2010 under natural light conditions.

2.2 Bacterial strain

The bacterial strain used was *P. fluorescens* N21.4 (Spanish-Type Culture Collection accession number CECT 7620), a gram-negative bacilli isolated from the rhizosphere of *Nicotiana glauca*. It is able to release siderophores and chitinases and triggers defensive metabolism in *Solanum lycopersicum* (Ramos Solano et al. 2010b), *A. thaliana* (Domenech et al. 2007) and *G. max* (unpublished results). It also increases isoflavone contents in *G. max* (Ramos Solano et al. 2010a).

2.3 Inoculum preparation

Bacterial strain was maintained at −80 °C in nutrient broth with 20 % glycerol. Inoculum was prepared by streaking strains from −80 °C onto plate count agar (PCA) plates, incubating plates at 28 °C for 24 h, and scraping bacterial cells off the plates into sterile 10 mM $MgSO_4$ buffer. Inoculum was delivered to plants at 10^7 c.f.u./mL.

2.4 Experimental design

The experimental area was defined within a blackberry production greenhouse, arranged in 200-m long tunnels, and each one covers two lines. Within one tunnel, two lines were marked, one for root treatments and another for leaf inoculations. In each line, a total of four blocks, with six plants each were separated: two blocks were devoted to bacterial treatments, a third block to bacteria plus regular chemical treatments and a fourth block was left as a non-inoculated control, with regular chemical treatments; therefore, a total of 48 plants were in the trial. The chemical treatments used were devoted to plague control and are under a non-release policy agreement. Root inoculations were carried out by soil drench at 10^7 c.f.u./mL in water; leaf inoculations were delivered by spraying leaves with a 10^7 c.f.u./mL bacterial suspension with wetting agents; the combined treatments with chemicals involved the usual chemical treatments of commercial exploitation, only when it was necessary. In summary, the following treatments were

delivered: root inoculation (R) (n=12); root inoculation plus chemicals (RQ) (n=6); leaf inoculation (F) (n=12); leaf inoculation plus chemicals (FQ) (n=6); controls: the usual chemical treatments of commercial exploitation were applied systematically (products for control of *Spodoptera littoralis*, *Botrytis cinerea* and acaricides; n=12).

Plants were transplanted to production greenhouses in July 2010. One week after, plants were inoculated throughout the growth period every 2 weeks until production finished, constituting a total of nine inoculations.

Plant fitness was evaluated in the middle of the flowering period, recording effects on growth. Systemic plant protection against *S. littoralis* in flowers as macroscopic indicator related to induction of systemic responses was evaluated.

Fruits were handpicked and weighed throughout the production period (8 weeks) to evaluate effects in yield. Fruit quality was assessed at three time points of the production period (beginning, middle, and end; T1, T2, and T3, respectively) by the following parameters: pH, degrees Brix, antioxidant potential, quantitative determination of phenols, flavonoids, and anthocyanins. Yield was recorded weekly.

2.5 Evaluation of plant fitness

Effects on growth were evaluated as number of flowering stems per square meter and number of flowers per square meter. Plant protection against *S. littoralis* was evaluated in flowers. For this purpose, a 30×50-cm wooden framework was placed at the middle level of plants in each treatment (between 1.3 and 1.6 m). The number of affected flowering buds over total number of flowers was recorded.

2.6 Production

Quality of fruit Blackberries were handpicked twice a week and sent at 4 °C to the lab. Upon arrival, fruits from each treatment were weighed and split in three replicates (n=3) for nutritional characterization (pH and degrees Brix), bioactive characterization (total anthocyanins, total phenolics, and total flavonoids) and antioxidant potential.

Nutritional characterization For pH and degrees Brix, four blackberries in each replicate were weighed individually, pulled and crushed and centrifuged together for 10 min at 4,000 rpm. Determinations were done on supernatants from fresh fruits.

pH: pH was measured with MicropH2001 (CRISON) pH meter.

Degrees Brix: Brix grades were measured with Portable refractometer.

Determination of total phenolic content The phenolic content was determined quantitatively with Folin-Ciocalteau reagent (Sigma-Aldrich, St Louis, MO) by colorimetry (Xu and Chang 2007) with modifications, using gallic acid as a standard (Sigma-Aldrich, St. Louis, MO). One milliliter aliquot of the extract solution was mixed with 0.250 mL of a 2 N Folin-Ciocalteu reagent (Sigma-Aldrich, St Louis, MO) and 0.75 mL of 20 % Na_2CO_3 solution. The mixture was allowed to stand at room temperature for 30 min and then, absorbance was measured at 760 nm with a UV–visible spectrophotometer (Biomate 5). A calibration curve was constructed with gallic acid (r=0.99). Results were expressed as milligrams of gallic acid equivalent per 100 g of fresh weight (FW).

Determination of total flavonoids content Total flavonoid content was determined quantitatively by the aluminum chloride colorimetric assay (Zhishen et al. 1999) with modifications, using catechin as a standard (Sigma-Aldrich, St. Louis, MO). One milliliter aliquot of the extract solution was added to 10-mL volumetric flask containing 4 mL of distilled water. Then, 300 μL of 5 % $NaNO_2$ was added. After 5 min, 300 μL of 10 % $AlCl_3$ was added. Then, after 1 min, 2 mL of 1 M NaOH was added and the total volume was brought to 10 mL with distilled water. The solution was mixed thoroughly and absorbance was measured against prepared reagent blank at 510 nm. A calibration curve was constructed with catechin (r=0.99). Total flavonoid content of fruit extracts was expressed as mg catechin equivalents (CE) per 100 g FW. All samples were analyzed in triplicate.

Determination of total anthocyanins content Total anthocyanin content was determined quantitatively by the pH differential method of Giusti and Wrolstad (2001). Extracts were diluted 1:15 with pH 1.0 buffer (0.2 M KCl pH 1) and pH 4.5 buffer (1 M CH_3COONa pH 4.5). Then, absorbance was measured at 520 and 700 nm in a UV-visible spectrophotometer (Biomate 5). Concentrations were calculated applying Lambert–Beer equation to samples absorbance. Results were expressed as mg of cyanidin-3-glucoside/100 g FW.

Determination of antioxidant activity: 1,1-diphenil-2-pricrilhidrazil The radical scavenging activity of *R. fruticosus* extract against 1,1-diphenil-2-pricrilhidrazil (DPPH) free radical (antioxidant potential) was measured using the method of Brand-Williams et al. (1995). The antioxidant potential EC50 (amount of blackberry extract needed to reduce to 50 % the amount of free radicals of DPPH solution) was determined by a curve dilution made with different blackberry extract concentrations mixed with 0.1 mM DPPH in methanol 80 %. The absorbance at 517 nm was measured after standing in the dark for 30 min. The control and blank were made with methanol. Since EC50 is the

amount of blackberry extract needed to reduce to 50 %, the amount of free radicals of DPPH solution, lower values indicate higher antioxidant potential.

Yield measurement Total yield over the production period was determined by weighting fruits, handpicked twice a week. Weight was recorded weekly and is presented as total production.

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) post hoc test was also performed (Sokal and Rohlf 1979) with the software Statgraphics plus 5.1 for Windows.

3 Results and discussion

The great interest to improve fruit quality especially in functional foods calls for efficient methods to obtain them and one of the most challenging tools to achieve this goal is the use of biological agents (Capanoglu 2010). Plant growth-promoting rhizobacteria have been used before to improve growth and production in different plant species or to increase plant protection (Conrath 2009), but to our knowledge, this is the first study that reports the use of a PGPR on *R. fruticosus* to improve quality of blackberries, triggering secondary metabolism to improve fruit bioactive content and yield, simultaneously.

The experimental design included root and leaf inoculation to provide the best way of treatment for further technology transfer, addressing the underlying mechanism of action of N21.4 either biocontrol or systemic resistance. Inoculations delivered throughout the plant life cycle ensured a constant elicitation of secondary metabolism as shown in plant fitness and fruit production.

Average fruit yield in *R. fruticosus* ranges between 2 and 2.5 kg/plant. Production of inoculated plants increased on almost 1 kg per plant over controls, while their combinations with chemicals caused non-significant changes (Fig. 2a). Inoculation with N21.4 increased the average number of flowering stems as compared with non-inoculated *R. fruticosus* plants from 80 per square meter to almost 100 flowering stems per square meter (data not shown) and the total number of flowering buds per square meter from 450 to over 600 (Fig. 3) on both root treatments and leaf inoculation alone (data not shown). Despite being non-significant, they resulted in a significant increase in fruit yield (Fig. 2a). Since chances to improve plant nutrition on such a limited environment with an optimum nutrient supply are scant (Senthil et al. 2003), these effects are probably achieved through plant growth regulators released by bacteria. Although N21.4 is not able to release auxin-like compounds or degrade 1-aminocyclopropane-1-carboxylic acid in vitro (Ramos Solano et al. 2010b), its ability to synthesize gibberellins has not been evaluated and effects on flowering suggest a certain interaction with this plant growth regulator (Lucas García et al. 2004) as responsible for the increase in production. However, increasing photosynthetic efficiency has also been reported as a mechanism by which PGPR increase plant growth, and this is consistent with increases in degrees Brix detected in this study (Fig. 4b; Zhang et al. 2008).

Supporting this hypothesis is the fact that production of non-inoculated controls (C) peaks appear only once, 2 weeks after the beginning of the harvesting period, while treatments peak appear more than once. Moreover, while maximal production

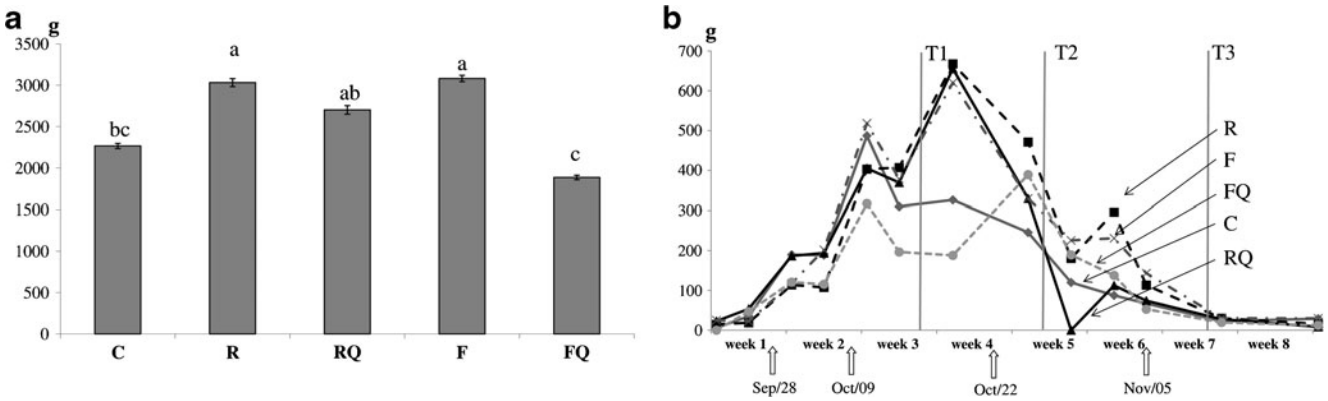

Fig. 2 Fruit yield. **a** Total fruit yield (gram) per plant and **b** fruit production progress throughout the 8-weeks harvesting period (27 September 2010 through to 22 October 2010), under the different treatments with N21.4. Treatments: *C*: non-inoculated controls with chemicals; *R*: root inoculation; *RQ*: root inoculation with chemicals; *F*: leaf spray inoculations; *FQ*: leaf spray inoculation with chemicals. *Different letters* (*a*, *b*, *c*) indicate significant differences between treatments according to LSD test ($p<0.05$). *Vertical lines* in the graph show dates on which fruit quality was determined (*T1*: beginning, *T2*: middle, and *T3*: end of the fruiting period). *Block arrows* indicate inoculation dates

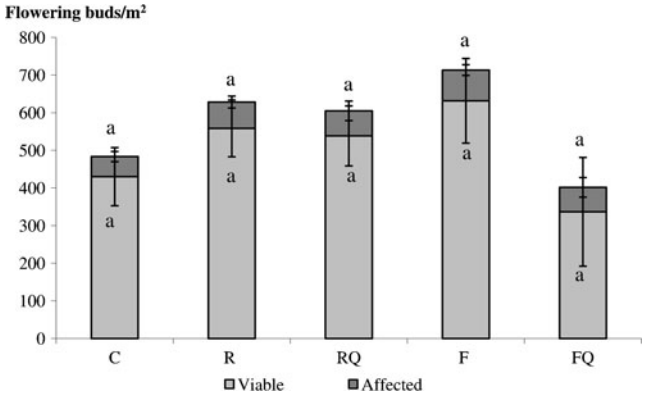

Fig. 3 Plant protection in flowers in *R. fruticosus* plants 3 days after the fifth inoculation with N21.4 under the different treatments. Protection in flowers is expressed as flower damage by *S. littoralis* in flowering buds per square meter. Treatments: *C*: non-inoculated controls with chemicals; *R*: root inoculation; *RQ*: root inoculation with chemicals; *F*: leaf spray inoculation; *FQ*: leaf spray inoculation with chemicals. *Different letters* indicate significant differences between treatments according to LSD test ($p<0.05$)

in controls at this peak reached almost 500 g/plant, it was lower than that of inoculated treatments (650 g/plant; Fig. 2b). After this first peak, fruit production decreases continuously. However, inoculated plants produce more fruits than controls. Moreover, inoculated plants show a second peak 5 weeks after beginning of production, although not as high as the first one. In view of these results, continuous delivery of the PGPR throughout the fruiting period increases productivity, and supports a plant growth regulator-mediated effect.

Supporting this systemic induction that affects flowering and fruit yield, protection against *S. littoralis*, the natural plague on greenhouse production in the evaluated climatological condition, was observed. *S. littoralis* chews on leaves and flowering buds, compromising plant fruiting potential. Damage assessment in flowers was evaluated as the number of affected buds over total number of flowering buds. The total number of flowering buds per square meter in non-inoculated controls was around 500, with 10 % damaged flowering buds (Fig. 3). Despite the non-significant increase in the number of flowering buds detected under the root treatments and the leaf treatment, the number of damaged flowers per square meter averaged 10 % as in controls (Fig. 3). This suggests that there is a systemic induction involved in plant protection due to the strain, that is altered in the leaf inoculation with chemicals.

Since leaf treatments were more effective, a biocontrol activity rather than the systemic alteration of secondary metabolism was suggested. However, an increase in glutathione reductase activity registered in all treatments confirms that there is a systemic induction and enzymatic activities involved in free radical scavenging are activated (data not shown). This suggests that N21.4 behaves as an avirulent pathogen triggering defensive metabolism associated to a hypersensitive response with an increase of free radicals and confirms the increase in this enzymatic activity as a good marker of systemic induction (Alves et al. 2004).

The systemic induction detected on vegetative growth (Fig. 3) and fruit yield (Fig. 2) extends to fruit quality (Figs. 4 and 5), confirming our working hypothesis and supporting that elicitation with beneficial strains improves fruit quality and production (Capanoglu 2010; Ramos Solano et al. 2010a). Fruit pH on blackberries from *R. fruticosus* ranged from 2.9 to 3.3, peaking at T2 and decreasing towards the end of the productive period (Fig. 4a). The most outstanding improvement for the market in nutritional quality is the increase in degrees Brix over non-inoculated controls. Blackberries from *R. fruticosus* averaged 8 °Brix, and all treatments

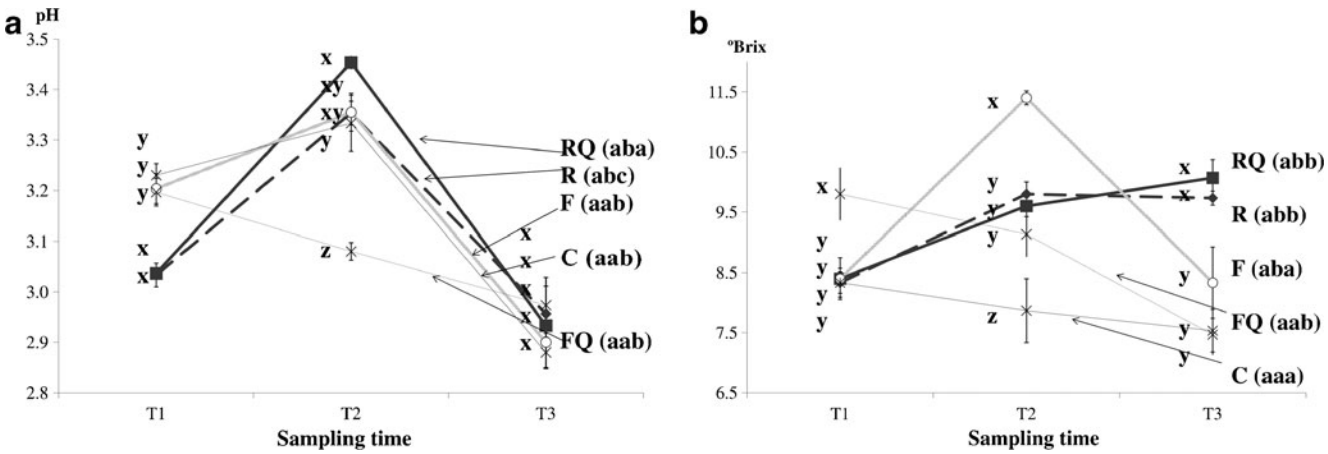

Fig. 4 Nutritional characterization of *R. fruticosus* fruits at the beginning (*T1*), middle (*T2*), and end (*T3*) of the fruiting period under the different treatments with N21.4: **a** pH, **b** degrees Brix. Treatments: *C*: non-inoculated controls with chemicals; *R*: root inoculation; *RQ*: root inoculation with chemicals; *F*: leaf spray inoculation; *FQ*: leaf spray inoculation with chemicals. *Different letters* indicate significant differences between sampling times (*a*, *b*, *c*), and among treatments within a sampling time (*x*, *y*, *z*) according to LSD test ($p<0.05$)

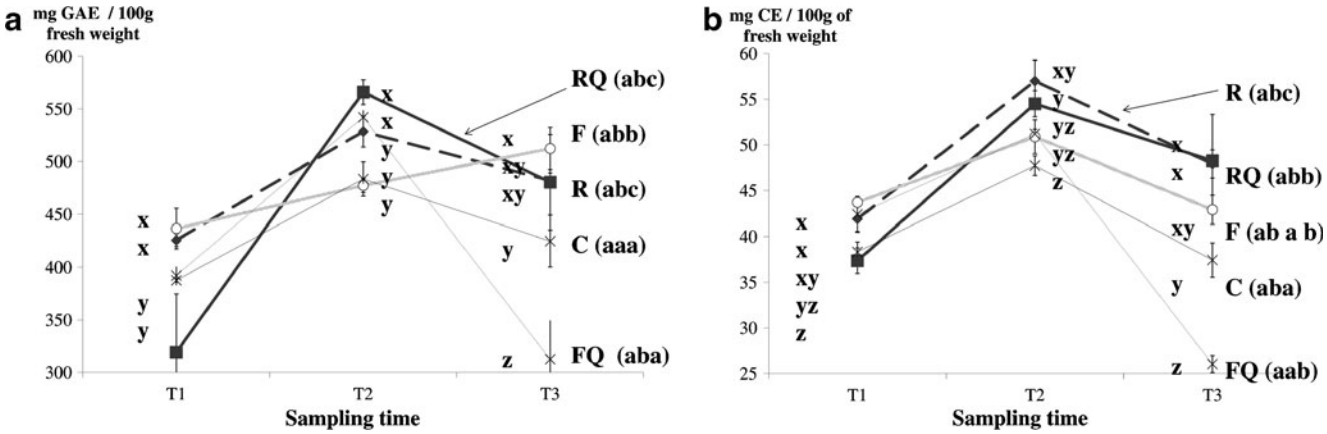

Fig. 5 Bioactive characterization of *R. fruticosus* fruits at the beginning (*T1*), middle (*T2*), and end (*T3*) of the fruiting period under the different treatments with N21.4. **a** total phenolic contents expressed as mg of gallic acid equivalents (*GAE*) per 100 g FW; **b** total flavonoid contents expressed as mg catechin equivalents (*CE*) per 100 g FW. Treatments: *C*: non-inoculated controls with chemicals; *R*: root inoculation; *RQ*: root inoculation with chemicals; *F*: leaf spray inoculation; *FQ*: leaf spray inoculation with chemicals. *Different letters* indicate significant differences between sampling times (*a*, *b*, *c*), and among treatments within a sampling time (*x*, *y*, *z*) according to LSD test ($p<0.05$)

significantly increased degrees Brix at T2, being the effect of the leaf spray striking with 3 °Brix over controls (Fig. 4b). This increase supports the effects of PGPR to enhance sucrose contents by increases in photosynthetic efficiency as suggested by the increase in flowering buds reported above. Other studies with PGPR reveal an increase of sugar accumulation as well as the suppression of classic glucose signaling (Zhang et al. 2008).

As regards to bioactive characterization, N21.4 affects total phenolics, total flavonoids, and anthocyanin profile, and therefore, antioxidant potential. Except for anthocyanins, all other parameters follow a similar trend, peaking at T2 in all treatments. Total phenolic content on *R. fruticosus* fruits ranged between 380 and 490 mg gallic acid equivalents/100 g FW (Fig. 5a). Total flavonoid content on fruits from control plants range between 37 and 47 mg catechin equivalents/100 g FW; root inoculated fruits reached 55 mg catechin equivalents/100 g FW (Fig. 5b). Root treatments significantly increased total phenolics by 18 % approximately and total flavonoids by 22 % at T2.

The antioxidant potential EC50 (Fig. 6b) of *R. fruticosus* extract ranges between 4.8 and 6.0, with significant changes along the time. Since EC50 values are inversely related to antioxidant potential, lower values indicate better antioxidant potential. Hence, changes in antioxidant potential (Fig. 6b) are related to maximal levels of total phenolics and total flavonoids (Fig. 5a, b).

Total anthocyanin content on *R. fruticosus* fruits ranged between 130 and 170 mg cyanidin-3-glucoside equivalents/100 g fresh weight. The maximal values were obtained in T1

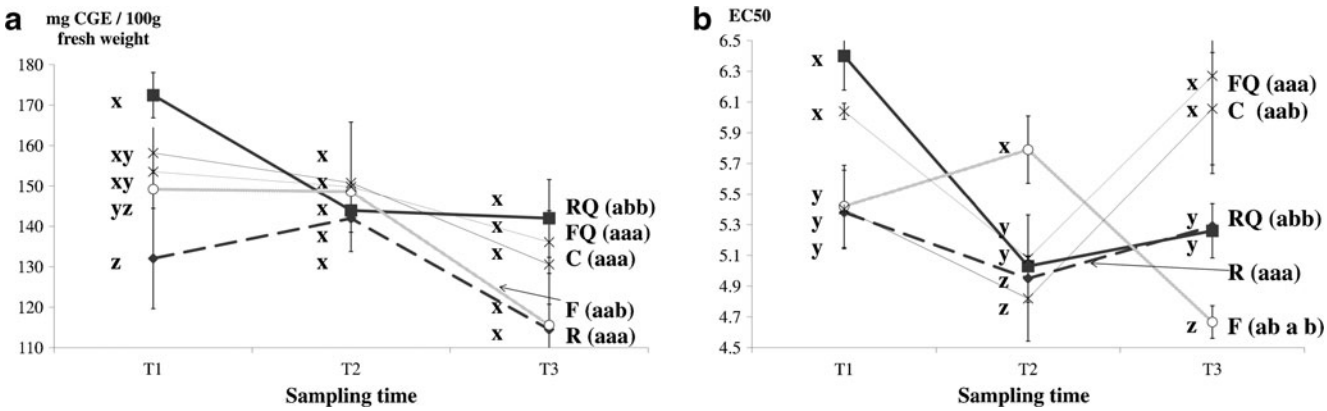

Fig. 6 Bioactive characterization of *R. fruticosus* fruits at the beginning (*T1*), middle (*T2*), and end (*T3*) of the fruiting period under the different treatments with N21.4. **a** Total anthocyanin contents expressed as mg of cyanidin-3- glucoside equivalents (*CGE*) per 100 g FW; **b** scavenging activity (EC50) of DPPH radical. The EC50 values represent the volume of extract required to reduce the absorbance of the DPPH radical by half. Treatments: *C*: non-inoculated controls with chemicals; *R*: root inoculation; *RQ*: root inoculation with chemicals; *F*: leaf spray inoculation; *FQ*: leaf spray inoculation with chemicals. *Different letters* indicate significant differences between sampling times (*a*, *b*, *c*), and among treatments within a sampling time (*x*, *y*, *z*) according to LSD test ($p<0.05$)

and the minimum at T3 in all treatments (Fig. 6a). Significant differences due to treatments appear only in T1. It should be highlighted that the variety under study (*R. fruticosus* var. Lochness) at this geographical location and seasonal conditions shows higher total phenolic contents (450 mg/100 g FW) and anthocyanins (130–170 mg/100 g FW) than other *R. fruticosus* varieties grown under natural cycle (289.3 mg/100 g FW and 90 mg/100 g FW, respectively) (Benvenuti et al. 2004), or the same variety grown at higher latitudes (Jordheim et al. 2011), probably due to the strong light dependence of anthocyanins (Rabino and Mancinelli 1986) which is evidenced also in this study by the decrease shown from T1 (October) to T3 (mid-November) (Fig. 5c).

In view of these results, it is evident that blackberries are an excellent source of bioactive compounds, overcoming contents of *V. corymbosum* (blueberries) (Giovanelli and Buratti 2009), one of the most studied berries up to date. This high bioactive content is further improved upon elicitation specially effective on root inoculated (R and RQ) and leaf inoculated (F) plants and is consistent with the systemic protection against *S. littoralis*. However, targeted enzymes must be located in the early steps of this pathway and on the flavonoid branch while anthocyanin biosynthetic enzymes do not respond to the biotic elicitation (Fig. 5) in this condition. Given the primed stated of the plant, a strong increase of anthocyanins upon a subsequent stress challenge may not be discarded (Capanoglu 2010). Furthermore, since the increase in total phenolics is not transformed only in flavonoids, it is evident that other phenolic compounds not determined in the present study are synthesized and may contribute to improve effects of blackberries in health.

4 Conclusion

Based on the evidence presented, N21.4 is able to trigger secondary metabolism in *R. fruticosus* in field conditions, being effective in plant protection and increasing fruit quality and yield. Both inoculation systems are effective and can be easily used under current cropping practices; however, despite the better performance of leaf spray for leaf protection against *S. littoralis* and fruit yield, its combination with chemicals has a general negative effect, and makes root inoculation the best alternative. Finally, based on bioactive characterization, *R. fruticosus* var. Lochness appears as an excellent source of bioactives, providing higher levels than other berries all year long.

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