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RESEARCH ARTICLE



Priming fingerprint induced by *Bacillus amyloliquefaciens* QV15, a common pattern in *Arabidopsis thaliana* and in field-grown blackberry

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ABSTRACT

The aim of this study is focused on determining the *Bacillus amyloliquefaciens* QV15 priming fingerprint in two different plant species, *Arabidopsis* and blackberry as a crop of agronomic interest, associated with protection upon pathogen challenge. To achieve this goal, *Arabidopsis thaliana* plants were challenged with *Pseudomonas syringae* DC3000 under controlled conditions, and field-grown blackberries were challenged by a powdery Mildew outbreak, finding plant protection in plants treated with QV15, in both conditions. Changes in ROS scavenging enzymes' activity, defense-related enzymes' activity and gene expression were evaluated in both plant species, before and after pathogen challenge, revealing the ability of this strain to prime both. As a result of this analysis, the priming fingerprint induced by QV15 was defined by a decrease in ROS scavenging enzymes' activity in pre- and post-challenged plants, an increase in glucanase and chitinase activity after pathogen challenge, significantly increasing the expression of *PR1*, indicating a salicylic acid (SA)-mediated pathway activation. These results suggest an excellent potential of *B. amyloliquefaciens* QV15 to protect different plant species against different pathogens in field conditions.

ARTICLE HISTORY

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Keywords

Priming fingerprint; systemic induction; *Rubus*; *Arabidopsis thaliana*; *Bacillus amyloliquefaciens*; SA

1. Introduction

Pests in agricultural systems are an important threat because of their impact on plant yield, with the consequent economic losses, which is different depending on the area. In underdeveloped areas, feeding the local population with low chemical inputs is compulsory due to limited access to chemicals while in developed countries they are strongly regulated by the regulatory authorities, so it is necessary to find new alternative tools for sustainable agriculture. Furthermore, there are some crops with limited cropping surface and a high economic value that lack specific products due to the low surface and the low number of potential consumers for those specific phytochemicals, as is the case of *Papaver somniferum* to obtain morphinanes (Bonilla et al. 2014), or blackberries in intensive production (Strik et al. 2007).

Blackberries (*Rubus* cv. Loch Ness) belong to the Rosaceae family as strawberry, raspberry, peach or apple; among berries, blackberries are one of the most important plants based on the beneficial effects on human health due to its active secondary metabolism, rich in polyphenols (Cassidy, 2017; Davicco et al. 2016; Kowalska and Olejnik 2016; Mortas and Sanlier 2017). The beneficial effects of blackberries on health have been widely reported and they constitute an excellent source of dietary polyphenols (Kaume et al. 2012).

A new approach to substitute chemical compounds to fight the impact of different pests includes the beneficial microbes known as plant growth-promoting rhizobacteria (PGPR). These bacterial strains are isolated from the rhizosphere where plants release carbon sources through the exudates, selecting those individuals that provide benefits to the plant

upon colonization (Pongsilp et al. 2016). They are able to improve nutrient availability and uptake, inhibit pathogenic microorganisms' growth among other mechanisms or to directly alter plant metabolism, either releasing plant growth regulators that affect plant metabolic balance or triggering secondary defense metabolism (Ramos Solano et al. 2008; Defago et al. 1995). The process triggered by beneficial microorganisms resulting in an increased defensive capacity of the entire plant after local contact with the beneficial microbe is called Induced Systemic Resistance (ISR) (Pieterse et al. 2014). Upon root colonization, the plant acquires a potentiated defensive capacity without a concomitant induction of specific defense genes, a phenomenon termed priming, which is accepted today as an intrinsic part of induced resistance (Mauch-Mani et al. 2017); furthermore, these specific metabolic changes taking place upon root colonization have been termed as priming fingerprint (Mauch-Mani et al. 2017) (Figure 1). Strains able to trigger ISR belong to different bacterial genera such as *Arthrobacter*, *Chryseobacterium*, *Curto-bacterium*, *Azospirillum*, *Azotobacter*, *Burkholderia*, *Pseudomonas* and *Bacillus* (Bowen and Rovira 1999; Fatima and Anjum 2017; Gutierrez Mañero et al. 2003).

The plant recognizes microbes through pattern-recognition receptors (PRRs), which can identify different molecules from beneficial or pathogenic microbes, like bacterial flagellin or fungal chitin (Boller and Felix 2009); these molecules are called pathogen or microbe-associated molecular patterns (PAMPs or MAMPs) (Henry et al. 2012). There are two different branches that can be activated for the second defense line, the salicylic acid (SA) branch and the jasmonic/

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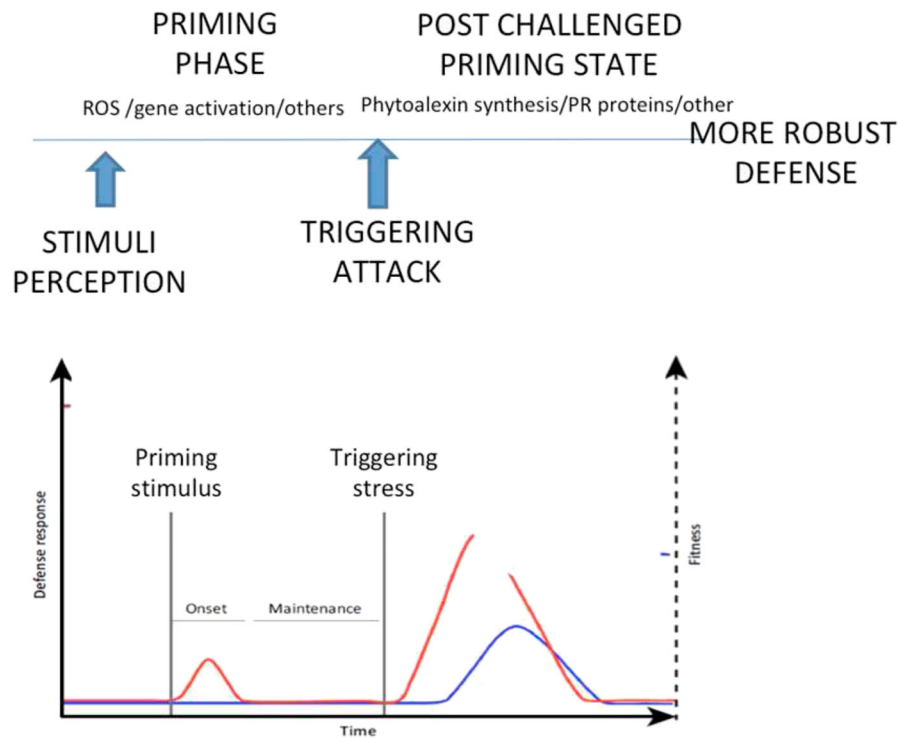


Figure 1. Relation between defense responses in primed (red) versus unprimed (blue) plants in the two stages of priming, priming phase and post-challenged priming state. Modified from Martínez-Medina et al. (2016) and Mauch-Mani et al. (2017).

ethylene acid (JA/ET) branch (Van Loon et al. 1998). The SA pathway activates the *NPR1* transcription factor that will increase the expression of the *resistance proteins* (PRs) defense genes, specifically *PR1* (Seyfferth and Tsuda 2014).

When ISR was first described, it was believed that pathogens used the SA-mediated pathway triggering Systemic Acquired Resistance, while the JA/ET branch was limited to beneficial microbes or PGPR (Pieterse et al. 1998). Furthermore, it was believed that beneficial microbes would only use the JA/ET pathway, although there is evidence that beneficial bacteria can trigger either pathway or both (Domenech et al. 2007; Barriuso et al. 2008a), depending on the determinants (MAMPs) able to trigger a response on the plant species under study. There is increasing evidence of the crosstalk between these pathways and involvement of other plant growth regulators in defense (Vos et al. 2015).

Despite the general description of ISR, the specificity of the interaction between the plant and the beneficial microbe is a fact (Walker et al. 2011), and it may become a limitation when developing field products for a general use. Therefore, effective strains to develop biological products should be able to trigger a wide range of plant species with agronomic interest.

The ability of QV15 to trigger ISR in *Arabidopsis thaliana* has been described elsewhere (Barriuso et al. 2008a). However, its metabolic and gene expression changes, the so-called *priming fingerprint*, has not been described yet. Moreover, given the specificity of the plant-microbe interaction, we wondered if the priming fingerprint would be specific to the strain or if it would depend on the plant host. Based on this background, the aim of this study was to determine metabolic and gene expression profiles on QV15-treated plants before and after pathogen challenge, in two plant species, to establish its priming fingerprint. First, the priming fingerprint was defined in *A. thaliana* under controlled conditions and, secondly, a field experiment was conducted on blackberry (*Rubus* cv. Loch Ness) as a target crop, to explore another

plant's response to QV15. To achieve this goal, the following metabolic markers were evaluated before and after pathogen challenge: reactive oxygen species (ROS) scavenging enzyme activities, superoxide dismutase (SOD) and ascorbate peroxidase (APX); defense-related enzyme activities, glucanases (PR2) and chitinases (PR3); gene expression of *PR1*, *PR2* (glucanases), *PR3* (chitinases), as marker genes of the SA-mediated induced resistance pathway; disease incidence was recorded only after pathogen challenge.

2. Materials and methods

2.1 Bacterial strains

Bacillus amyloliquefaciens (QV15) is a gram-positive sporulated bacillus; it was isolated from the rhizosphere of *Pinus pinea* (Barriuso et al. 2005). It produces siderophores, stimulates pine growth (Barriuso et al. 2008b), enhances defense against *Pseudomonas syringae* (DC3000) and protects against abiotic stress (NaCl 60 mM) (Barriuso et al. 2008a).

P. syringae (DC3000) was used as the pathogen for the ISR experiments for challenge inoculation. This strain causes bacterial speck on the model plant *A. thaliana* and is used to study the model system for plant-pathogen interactions (Van Loon et al. 1998).

Bacterial strains were maintained at -80°C in nutrient broth with 20% glycerol. An inoculum was prepared by streaking strains from -80°C onto plate count agar (PCA) plates, incubating plates at 28°C for 24 h (QV15) or 48 h (DC3000). After that, QV15 was transferred to Luria broth liquid media (LB) that was grown under shaking (1000 rpm) for 24 h to obtain a 2×10^9 cfu/mL inoculum. DC3000 was transferred to a nutrient broth liquid medium that was grown under shaking (1000 rpm) for 24 h to obtain a 2×10^8 cfu/mL inoculum.

2.2 Plant materials

A. thaliana col 0 was used as a model plant.

Rubus cv. Loch Ness is a high-yielding tetraploid ($4n = 28$) blackberry, and one of the most widely cultivated varieties. Its origin is Scottish, with parents an SCRI 74126RA8 \times SCRI 75131D2 hybrid complex, obtained in 1998 in Invergowrie, Scotland, by the Scottish Crop Research Institute. It is a mixture of races, among which the most prominent are 'Comanche' and 'Merton Thornless'. The parents came from Scotland and North America, and include European species *Rubus ulmifolius*, *R. trivialis*, *R. strigosus* and *R. armeniacus* (Brooks and Olmo 1997).

In southwest Spain, blackberries are produced under 'winter cycle' involving an artificial cold period in order to induce flowering upon transplant to greenhouses. The blackberry cycle has three stages: vegetative, flowering and flowering-fruiting; the duration of these stages is variable depending on the transplant moment, and each stage accounts for one-third of the plant's life.

2.3 *A. thaliana* experimental setup

A. thaliana seeds were sown in quartz sand and two-week-old seedlings were transplanted individually to 100 mL pots. The substrate consisted in a 12:5 (vol:vol) potting soil/sand mixture (60 g per pot). Forty-eight plants per treatment were used; plants were arranged on 3 replicates, with 16 repetitions each. Plants were cultivated in the lab under controlled conditions in a growth chamber (Sanyo MLR-350H) with a 9 h light ($350 \mu\text{E/s m}^2$ at 24°C) and 15 h dark period (20°C) at 70% relative humidity, watered with 25 mL of water twice a week and with 25 mL of $\frac{1}{2}$ Hoagland solution per plant once a week. QV15 was inoculated twice by soil drench with 3 mL of 2×10^9 cfu/mL to achieve 10^8 cfu/g soil, one week and three weeks after transplant. Two days after the last inoculation, plants were introduced into the humidity chamber for 24 h to ensure stomata opening to allow disease progress. Challenge inoculation with DC3000 was performed after 24 h of being in the humidity chamber by spraying plants with 250 mL of a suspension of 10^8 cfu/mL in 10 mM MgSO_4 ; controls were mock inoculated with a 10 mM MgSO_4 solution (Figure 2(a)). Samples were picked up 24 and 48 h after pathogen challenge, powdered in liquid nitrogen and stored at -80°C ; 24 h samples were used for gene expression analysis by qPCR and 48 h samples for ROS and defense enzyme activity determinations. Plants were harvested one week later; photosynthesis, fresh weight and disease incidence were recorded (percentage of leaves with disease symptoms (chlorotic dots) per plant). Data are shown as relative disease incidence that is relative to controls, where control plants represent the maximum disease.

2.4 Blackberry experimental setup

The *Rubus cv. Loch Ness* plants used in this study were kindly provided by Agricola El Bosque S.L. 'La Canastita' (Lucena del Puerto, Huelva, Spain). Plants and greenhouses were handled according to regular agricultural practices (Ramos-Solano et al. 2014). Plants were grown in Huelva (South Western Spain) from September 2014 to February 2015 under 'winter cycle'. A total of 360 plants were in the trial, arranged in six greenhouses; each greenhouse had two lines with 60 plants each, each line being one replicate with 60 repetitions; 3 lines were inoculated and 3 lines were left as non-inoculated controls.

QV15 was root inoculated every 15 days during the whole plant cycle with 0.5 L of inoculum at 10^7 cfu/mL per plant.

In this experiment (Figure 2(b)), plants were transplanted at the end of September, flowering (pre-pathogen challenge) took place in November and maximum fruiting (post-pathogen challenge) in January when flowers, green, red and black fruits were present in the plants at the same time. The number of flowers per square meter at flowering and accumulated fruit production were recorded. A natural mildew outbreak took place from November till harvest, and disease incidence was recorded by visual evaluation of the surface affected carried out by three independent observers. Leaves were sampled at flowering (pre-pathogen challenge) and at fruiting (post-pathogen challenge), and were immediately frozen in liquid nitrogen and brought to the lab. Three replicates were taken, each one constituted from plant material of 60 plants; a total of 25 leaves were randomly sampled and pooled constituting each replicate. Leaves were powdered with liquid nitrogen for further enzyme activity analysis and RNA extraction for qPCR analysis.

2.5 Enzyme activity analysis

Enzyme activity was determined on plant extracts. Samples were ground to a fine powder with liquid nitrogen, 1 g of the powder was taken and 9 mL of phosphate buffer 0.1M (pH 7) containing 2 mM of PMSF (extraction buffer) was added. The sample with the extraction buffer was sonicated (10 min) and centrifuged ($14,000 g$ for 20 min at 4°C) to obtain the supernatants where the proteins are; all this was carried out at $0-4^\circ\text{C}$. The following enzyme activities related to oxidative stress in plant extracts were measured spectrophotometrically: ascorbate peroxidase (APX, EC 1.11.1.11) and superoxide dismutase (SOD, EC 1.15.1.1). In addition, β -1,3-glucanase (PR2, EC3.2.1.6) and chitinase (PR3, EC 3.2.1.14) activities were also determined.

Total protein was measured with a 96-well plate, mixing the extract (5 μL) with the Bradford reagent (250 μL) and waiting for 30 min so the reaction takes place; after that the absorbance was read with an Expert 96 Bioreader. The quantity of proteins is calculated from a calibrated curve of albumin constructed in the same manner with the concentrations of 0.05, 0.1, 0.5, 1, 1.5 and 2 mg/mL in the same buffer that proteins are extracted from.

SOD analysis was performed using the 19160 SOD determination kit (SIGMA-ALDRICH). SOD activity was determined from the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) in the presence of riboflavin following the manufacturer's specifications. Results are expressed as % inhibition of formazan.

The ascorbate peroxidase activity (APX) was measured following the method described by Garcia-Limones et al. (2002). 100 μL of extract was mixed with 860 μL phosphate buffer 50 mM pH 7, 120 μL of sodium ascorbate 2.5 mM and measured (A_0) at 290 nm using a UV-visible spectrophotometer (Biomate 5); after that, 120 μL of H_2O_2 was added, and absorbance was measured again after 30 s (A). Ascorbate oxidation was determined by the decrease in A_{290} (ϵ (290 nm) = 2.8 mM cm). One unit of APX activity is defined as the amount of enzyme that oxidizes 1 mmol min^{-1} ascorbate under the above assay conditions. Enzyme activity is expressed as $\mu\text{mol/mg protein min}$.

Glucanase (PR2) activity was measured following the method described by Lee et al. (2008). 100 μL of extract,

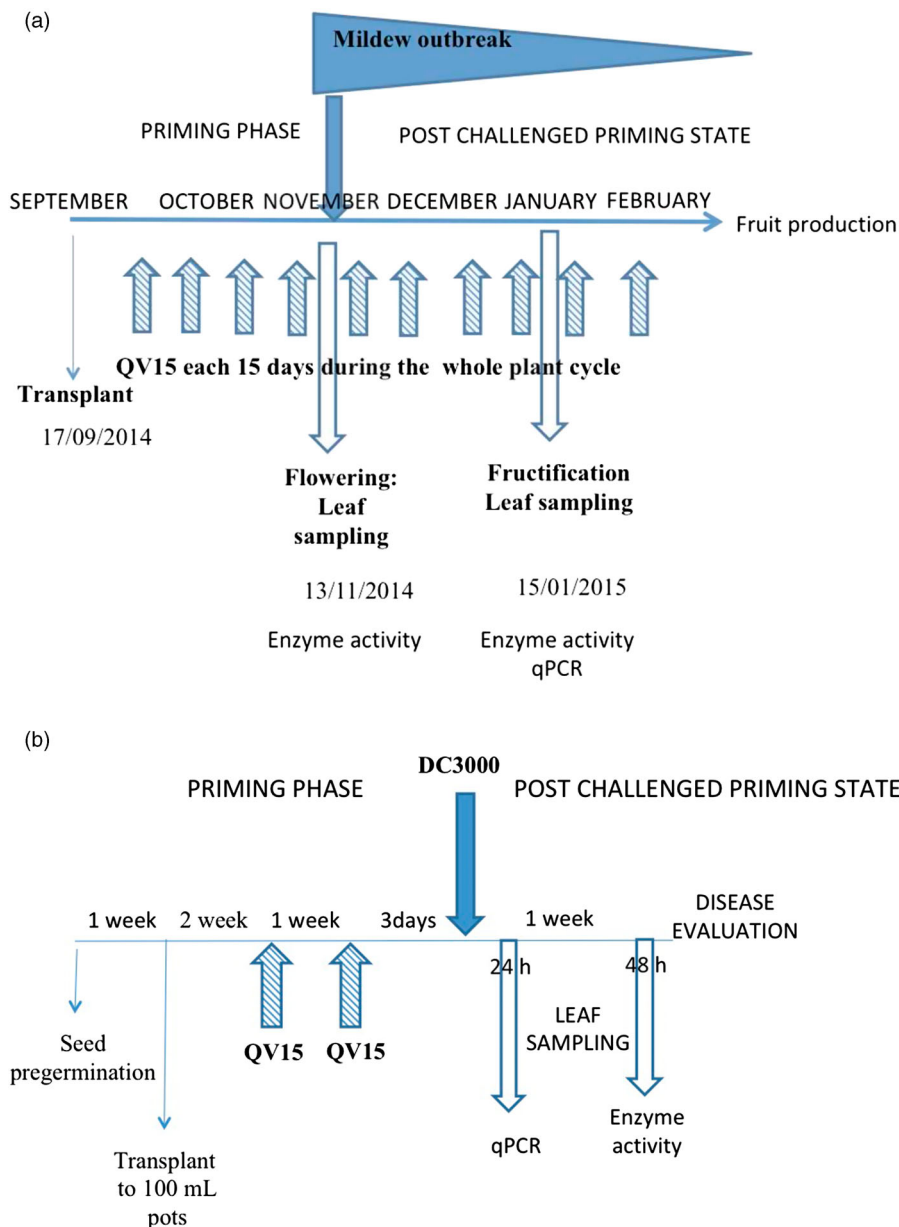


Figure 2. Experimental design for (a) *Arabidopsis thaliana* and (b) blackberry.

375 μL of sodium acetate 50 mM pH 5 buffer, and 25 μL of laminarin (Laminarin from *Laminaria digitata* (L9634, Sigma Aldrich)) were mixed and incubated for 1 h at 37°C. Then, 1.5 mL of DNS (10 g of dinitrosalicylic acid, 182 g of potassium sodium tartrate reactive, 0.5 g of sodium sulphate, 10 g of NaOH, diluted in 200 mL of distilled water and made up to 1 L) was added; it was heated at 100°C for 5 min and absorbance measured at 550 nm using a UV-visible spectrophotometer (Biomate 5). The standard curve was performed with increasing amounts of glucose following the same procedure. One unit of β -1,3-glucanase activity is defined as the amount of enzyme which produces 1 $\mu\text{mol min}^{-1}$ of reducing sugar under the assay conditions described above. Enzyme activity is expressed as $\mu\text{mol/mg protein min}$.

Chitinase (PR3) activity was measured following the method described by Lee et al. (2008). 500 μL of enzyme extract was mixed with 500 μL of sodium acetate 0.1M with colloidal chitin 1% pH 5.5 buffer and left at 37°C for 2 h. After that, 200 μL of NaOH 1 N was added and centrifuged at 10,000 g for 10 min. The supernatant was mixed with 1 mL of Schales reagent (5.29 g of sodium carbonate and

0.05 g of potassium ferrocyanate in 100 mL of water), heated at 100°C for 15 min and absorbance was measured at 420 nm using a UV-visible spectrophotometer (Biomate 5). The standard curve was performed with increasing amounts of N-acetyl Glucosamine following the same procedure described above. A unit of chitinase activity is defined as the amount of enzyme, which produces reducing sugars of 1 $\mu\text{mol min}^{-1}$ under the assay conditions described above. Enzyme activity is expressed as $\mu\text{mol/mg protein min}$.

2.6 RNA extraction and RT-qPCR analysis

Prior to RNA extraction, samples were removed from the -80°C freezer and ground to a fine powder with liquid nitrogen using a sterilized mortar and pestle. Total RNA was isolated from each replicate with Plant/Fungi Total RNA Purification kit (50) (NORGENTM) (DNase treatment included) and after confirmation of RNA integrity using NanodropTM, a retrotranscription followed by a RT-qPCR was performed.

The retrotranscription was performed using iScript tm cDNA Synthesis Kit (Bio-Rad). All retrotranscriptions were

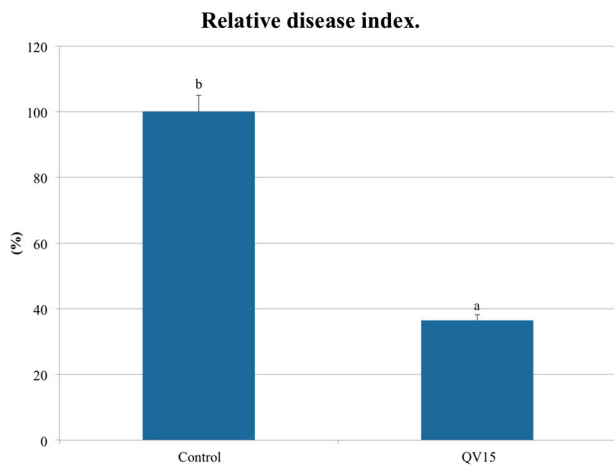


Figure 3. Relative disease index in 8-week-old *A. thaliana* plants one week after pathogen challenge. Different letters denote statistically significant differences according to the LSD test ($p < .05$).

performed using a GeneAmp PCR System 2700 (Applied Biosystems): 5 min 25°C, 30 min 42°C, 5 min 85°C and hold at 4°C. 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 55°C and 30 s at 72°C, followed by melting curve to check the results. To describe the expression obtained in the analysis, cycle threshold (Ct) was used. Standard curves were calculated for each gene, and the efficiency values

ranged between 90% and 110%. A different reference gene was used for each plant, *SAND* (AT2G28390) for *A. thaliana* and *HISTONE H3* (*HIS*) for *Rubus* cv. Loch Ness. Defense-related genes were studied for both plants and the primers used for each appear in Table 1 of supplementary material. Results for gene expression were expressed as a differential expression by the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). Control expression is set at 1; therefore, only increases above one are considered.

2.7 Statistical analysis

To evaluate treatment effects on all enzymes, SOD, APX, PR2 and PR3 values and gene expression of PRs, one-way ANOVA analysis was performed. When significant differences appeared ($p < .05$), LSD test (Least Significant Difference) from Fisher was used. Statgraphics plus 5.1 for Windows was the program used.

3. Results

3.1 *A. thaliana*

In *A. thaliana*, QV15 induced a high protection (60%) against the leaf pathogen DC3000 (Figure 3).

In control plants, ROS scavenging enzymes activities were lower in the pre-pathogen-challenged plants than in post-

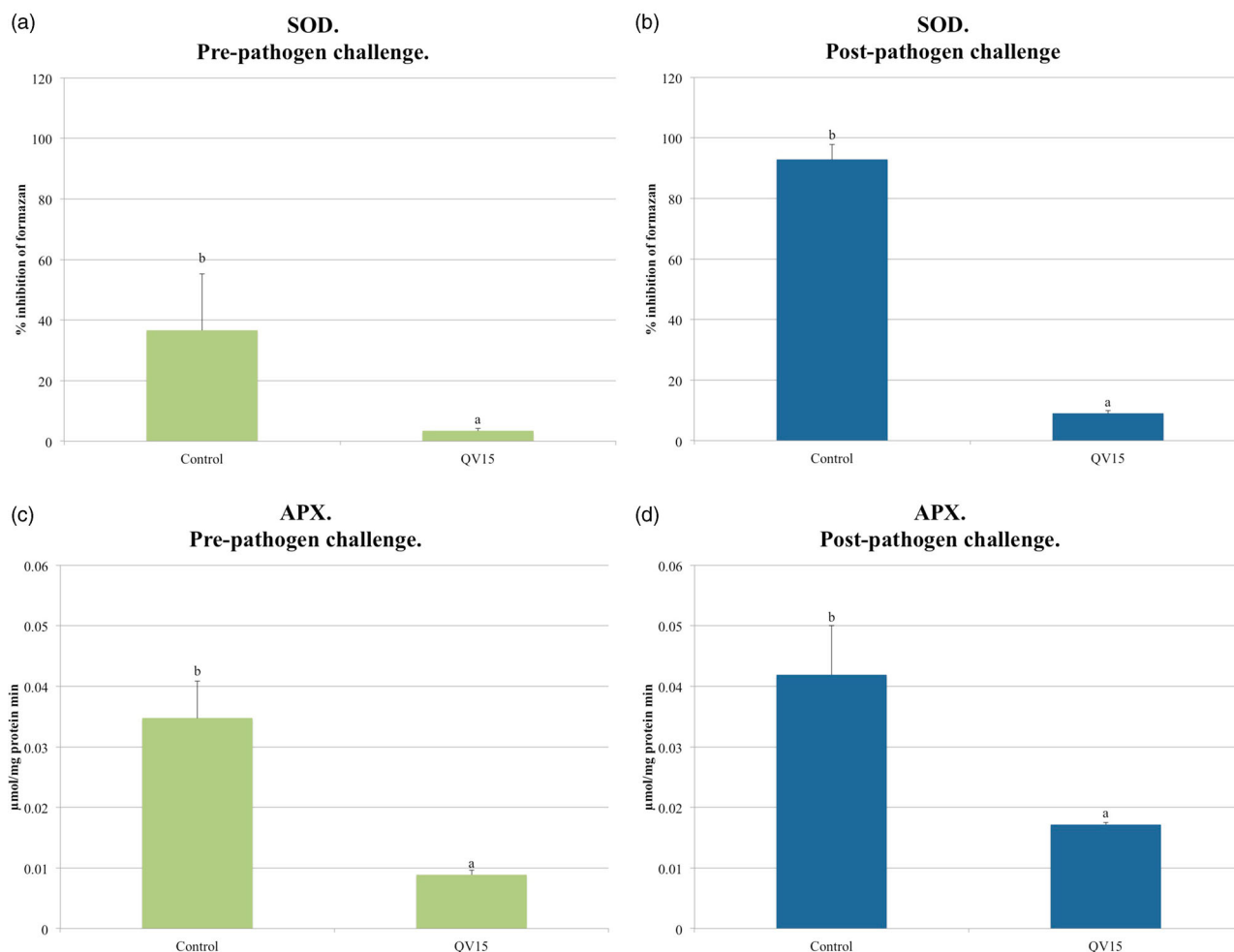


Figure 4. Enzyme activities in 7-week-old *A. thaliana* leaf extracts. Superoxide dismutase in (a) pre-pathogen challenge and (b) in post-pathogen challenge; SOD activity is expressed as % inhibition of formazan. Ascorbate peroxidase in (c) pre-pathogen challenge and (d) in post-pathogen challenge; APX activity is expressed as $\mu\text{mol/mg protein min}$. Different letters denote statistically significant differences between the controls and inoculated plants at each time point according to the LSD test ($p < .05$).

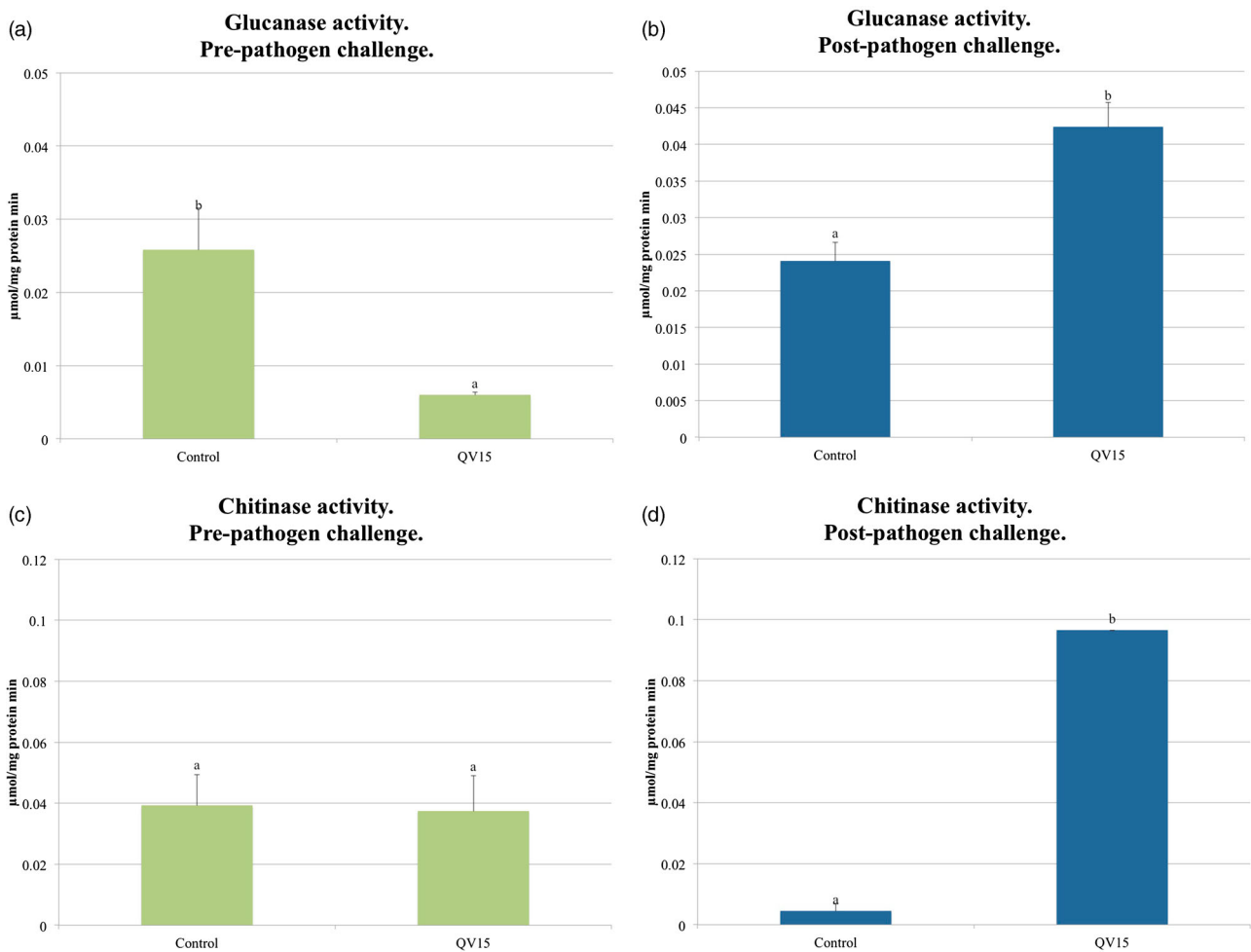


Figure 5. Seven-week-old *A. thaliana* leaf extract enzyme activities. Glucanase activity in (a) pre-pathogen-challenged and (b) post-pathogen-challenged plants. Chitinase activity in (c) pre-pathogen-challenged and (d) post-pathogen-challenged plants. Enzyme activity is expressed as $\mu\text{mol}/\text{mg}$ protein min. Different letters denote statistically significant differences according to the LSD test ($p < .05$).

challenged plants with a two-fold significant increase in SOD (Figure 4(a,b)), and not as marked in APX. In bacterial-treated plants, SOD and APX values were lower than in controls in the pre-pathogen challenge state (Figure 4(a,c)), respectively, and showed a two-fold non-significant increase in the post-pathogen challenge (Figure 4(b,d)), being significantly lower than controls.

As regards glucanase (PR2) and chitinase (PR3) activity, a different trend was detected between pre- and post-pathogen challenge (Figure 5). On QV15-treated plants, pre-pathogen challenge glucanase activity was significantly lower than in controls (Figure 5(a)) but it increased significantly (10-fold) in post-pathogen-challenged plants (Figure 5(b)), doubling control values. Chitinase activity did not change in QV15-treated plants pre-pathogen challenge as compared to the control (Figure 5(c)), but it showed a significant two-fold increase in the post-pathogen challenge (Figure 5(d)).

Gene expression of the three selected genes (*PR1*, *PR2* and *PR3*) was analyzed (Figure 6). The differential expression showed that all three were highly overexpressed in QV15-treated plants upon pathogen challenge (Figure 6), *PR1* being the most increased, overexpressed 12.21 times in inoculated plants, while *PR2* was 10.8 times overexpressed and *PR3* 7.5 times.

3.2 *Rubus cv. Loch Ness*

The significant increase of flowers per square meter in QV15-inoculated plants (Figure 7(a)) is not traduced in a significant increase of production (Figure 7(b)). The mildew outbreak

started in November and was maintained throughout the plant cycle with an average disease incidence of 15% of the affected surface in controls, while QV15-treated plants showed an average 5% disease incidence (Figure 7(c)). The relative disease index indicates an 85% protection against natural fungal disease (Figure 7(d)).

Enzyme activities were determined at two time points: flowering (pre-pathogen) and fruiting (post-pathogen challenge) (Figure 8). SOD values in control plants were lower in

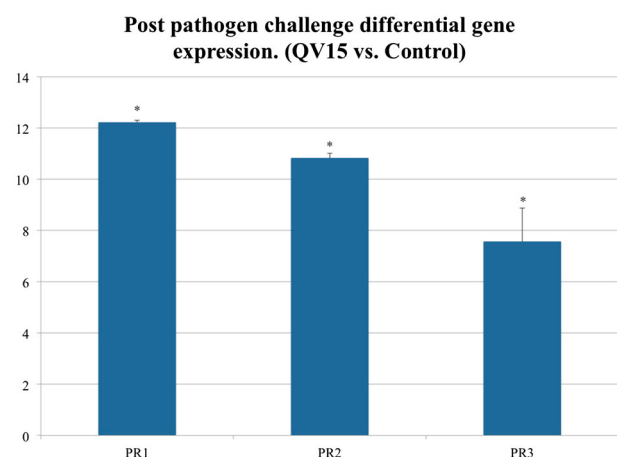


Figure 6. Differential gene expression of defense enzymes *PR1*, *PR2* and *PR3* in 7-week-old *A. thaliana* leaves, 24 h after pathogen challenge. Control vs. QV15-treated plants. Asterisks indicate significant differences, according to the LSD test ($p < .05$).

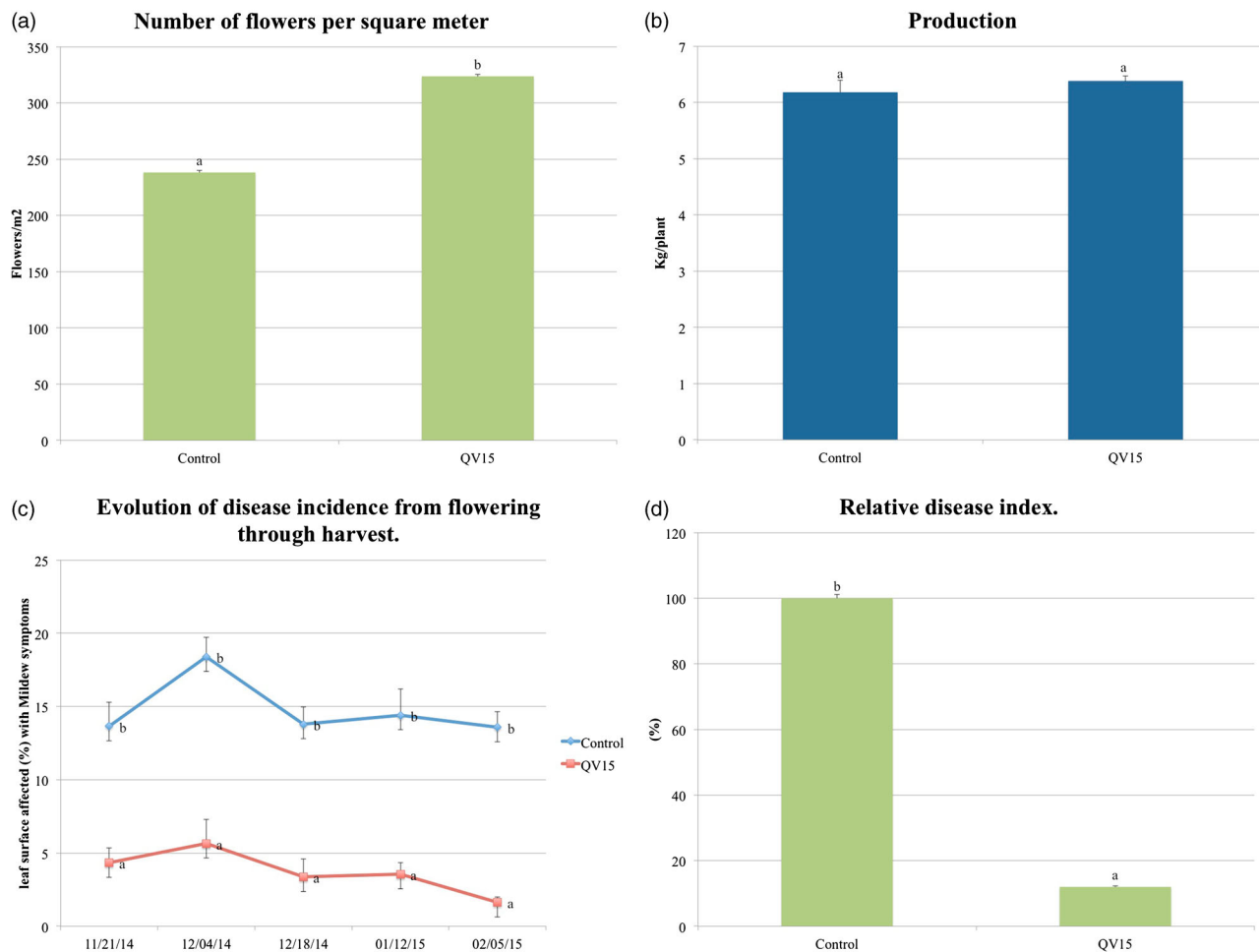


Figure 7. (a) Number of flowers per square meter of blackberry plants at flowering (pre-pathogen challenge). (b) Production (kilograms per plant) (post-pathogen challenge). (c) Disease incidence measured as leaf surface affected (%) with mildew symptoms in blackberry plants from November to harvest. (d) Relative disease incidence, expressed as accumulated leaf surface affected (%) with mildew symptoms relative to disease in controls. Different letters denote statistically significant differences according to the LSD test ($p < .05$).

pre-pathogen challenge (32.0%) (Figure 8(a)) than in post-pathogen challenge (46.9%) (Figure 8(b)) while APX behaved differently, APX values in control plants being higher in pre-pathogen challenge ($0.08 \mu\text{mol}/\text{mg protein min}$) (Figure 8(c)) than in post-pathogen challenge ($0.06 \mu\text{mol}/\text{mg protein min}$) (Figure 8(d)). However, bacterial treatment significantly decreased the activity of both enzymes at the two sampling times, this effect being especially marked in SOD at the pre-pathogen challenge when the decrease is over 10 times (Figure 8(a)).

Glucanase and chitinase activities in controls were really low at the pre-pathogen challenge (Figure 9(a,c), respectively), increasing at the post-pathogen challenge by five-fold on average (Figure 9(b,d)). On the other hand, bacterial treatment caused a significant increase of both activities, reaching $0.5 \mu/\mu\text{g min}^{-1}$ at pre-pathogen challenge (Figure 9(a,c)) and $1 \mu/\mu\text{g min}^{-1}$ at post-pathogen challenge (Figure 9(b,d)); values are two-fold in inoculated plants than in controls at the two sampling times.

Expression of genes corresponding to defense enzymes PR2 and PR3, as well as PR1, was evaluated in controls and in bacterial-treated plants (Figure 10) at the post-pathogen challenge. Differential expression is shown in Figure 10 where only a significant increase of PR1 gene expression is found.

4. Discussion

Developing effective biological products for agriculture that are applicable to different crops is a great challenge since the

specificity of the interaction between the plant and the beneficial microbe may limit effectiveness. Therefore, effective strains to develop field products should be able to trigger a wide range of plant species with agronomic interest. In this study, we have shown that *B. amyloliquefaciens* QV15 is able to trigger plant defense under controlled conditions in the model plant *A. thaliana* against *P. syringae* DC3000 and in field conditions, against a powdery mildew outbreak in blackberry grown in intensive greenhouse production, highlighting the ability of this strain to prime both plants and protect different plant species against different types of pathogens (fungi and bacteria).

This strain was isolated in a previous work (Barriuso et al. 2005) and has already proved its effect in *A. thaliana* protecting against biotic and abiotic (salt) stress (Barriuso et al. 2008a), demonstrating that protection induced by QV15 was mediated by SA, because it could not protect transgenic *Nagh Arabidopsis* while protection was maintained in *jar1* mutants. This strain also enhanced growth in *P. pinea* (Barriuso et al. 2008b), so it seemed to be a good candidate due to its broad range of activities. Blackberry was selected because it is a high added value fruit, with a low producing surface that would benefit from a specific agronomic product for its production, increasing fruit production and quality. During the experiment, a sudden and sustained Mildew outbreak took place throughout the production period, and QV15-treated plants showed less disease incidence, an 85% protection (Figure 7(d)), evidencing the ability to protect

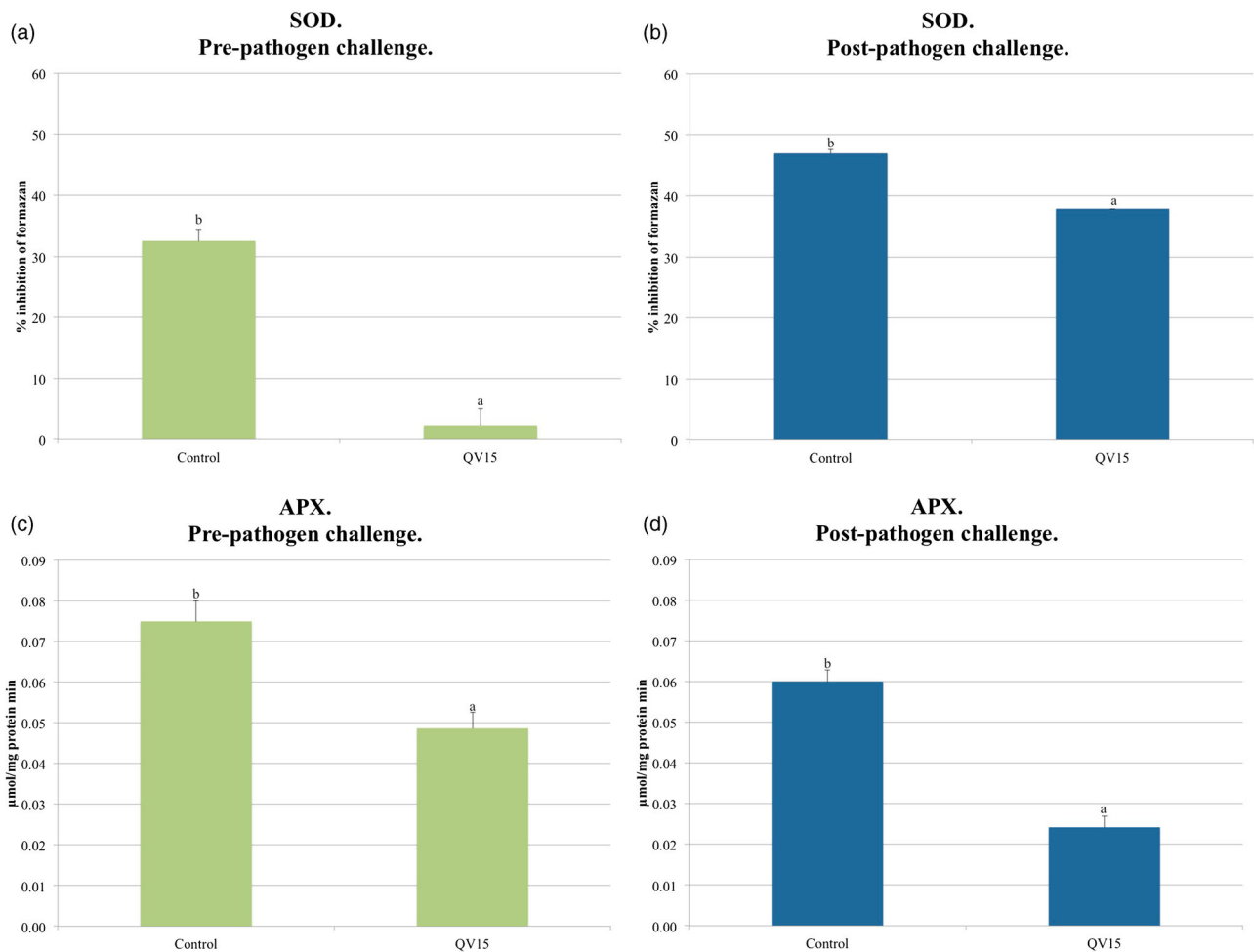


Figure 8. ROS scavenging enzyme activities of blackberry leaf extracts. Superoxide dismutase in (a) pre-pathogen-challenged and (b) in post-pathogen-challenged plants, expressed as % inhibition of formazan. Ascorbate peroxidase in (c) pre-pathogen-challenged and (d) in post-pathogen-challenged plants, expressed as $\mu\text{mol}/\text{mg}$ protein min. Different letters denote statistically significant differences between the controls and inoculated plants at each time point according to the LSD test ($p < .05$).

plants in field conditions. This decrease in pathogen incidence was also detected in *A. thaliana* (Figure 3) where protection was observed as 60% relative disease index decrease. So, these results indicate that this bacterial strain is capable of protecting both plant species, confirming the wide spectrum of actions of this strain. The significant increase in the number of flowers detected in flowering (pre-pathogen challenge) (Figure 7(a)) did not result in a significant increase in production (post-pathogen challenge) (Figure 7(b)) as expected, probably because the metabolic changes inherent to the primed status (Martinez-Medina et al. 2016) resulted in allocation of carbon sources to defense metabolism, slightly compromising fruit yield (Mauch-Mani et al. 2017). Genetic loci controlling plant immunity usually act antagonistically with regulating genes of plant growth (Ning et al. 2017).

Hence, determination of metabolic and genetic markers involved in the systemic protection was undertaken; the metabolic markers were ROS enzymes and defense proteins activity and the genetic markers were *PR* genes (*PR1*, *PR2* and *PR3*); all were evaluated before and after pathogen challenge, in order to show the more intense and quicker response of primed plants against stress challenge, the main result being the priming fingerprint of the strain.

Since a fine tuning of ROS homeostasis seems to be crucial for priming, activity of the ROS scavenging enzymes SOD and APX was determined in both plant species as metabolic markers of the priming status. The activity of both enzymes

in *Arabidopsis* (Figure 4) and blackberry (Figure 8) decreased on the pre- and post-pathogen-challenged QV15-inoculated plants, as compared to controls. Therefore, taking into account that these two enzymes are mainly responsible for ROS scavenging, a decrease in both enzymes' activities suggests a more relaxed state after PGPR inoculation (with lower free radical levels), which reflects the metabolic changes induced by the strain and appears as one feature of this strain's metabolic priming fingerprint. Interestingly, SOD activity in QV15-treated *Rubus* post-pathogen-challenged plants was markedly high, probably due to the longer exposure to powdery mildew from mid-November through January.

Conversely, the activities of glucanases (*PR2*) and chitinases (*PR3*) follow a different pattern, that is instead of being lower in pre- and post-pathogen challenge, their activity shows a two-fold increase at both sampling moments as compared to controls, with this behavior corresponding to the definition of priming (van Hulten et al. 2006). As this happens in both species (Figures 5 and 9), this feature can also be included in QV15 priming fingerprint.

PRs have been reported as markers of systemic acquired resistance mediated by SA. In view of the increase in *PR* activity, and the reported involvement of an SA-mediated protection induced by QV15 (Barriuso et al. 2008a), expression of *PR1*, *PR2* and *PR3* genes was studied in both species. This analysis confirmed that QV15 triggered systemic

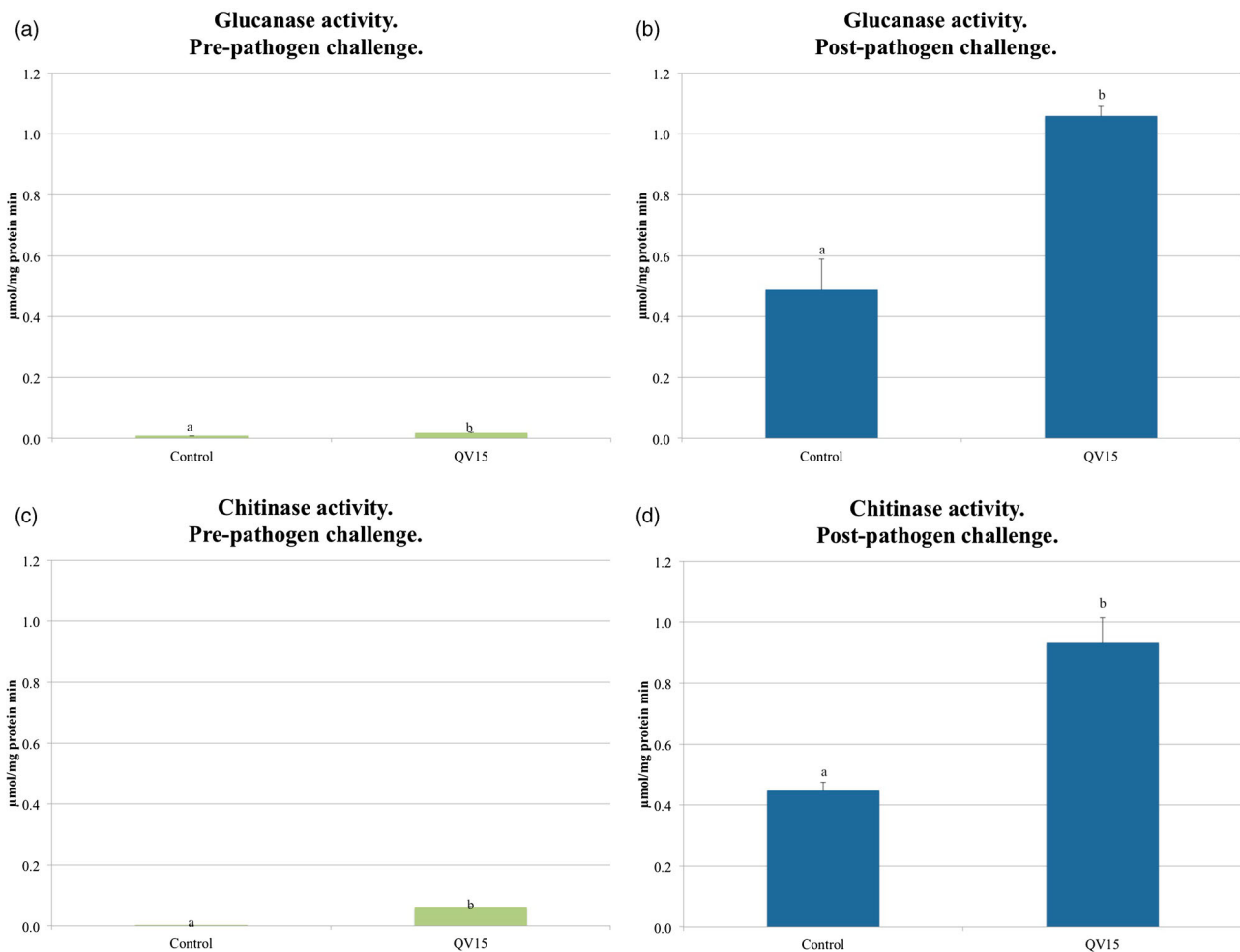


Figure 9. Blackberry leaf extract enzyme activities. Glucanase activity in (a) pre-pathogen-challenged and (b) in post-pathogen-challenged plants. Chitinase activity in (c) pre-pathogen-challenged and (d) in post-pathogen-challenged plants. All activities are expressed as $\mu\text{mol/mg protein min}$. Different letters denote statistically significant differences according to the LSD test ($p < .05$).

protection by activating the SA-mediated transduction pathway, as shown by the significant increase in the expression of *PR1*, *PR2* and *PR3* on *A. thaliana* plants inoculated with QV15 after pathogen challenge (Figure 6). The expression of *PR1* was also increased in blackberry (Figure 10) indicating that this pathway is also activated by QV15 in blackberry. Therefore, the efficient protection induced by this strain is due to activation of the SA-mediated transduction pathway, confirming that the priming fingerprint induced by QV15 depends on QV15 and not on the host plant, as we have seen it for both plant species.

Despite the increase in activity for glucanase (*PR2*) and chitinase (*PR3*), an increased expression of these genes was not detected (Figure 10), probably due to the sampling point for analyses, at fruiting (post-pathogen challenge), that is one month after the pathogen attack started. As blackberry was growing under field conditions, the pathogen attack was not controlled, so by this time, the increase in gene expression had already happened, as evidenced in the enzymes' activities determined. Supporting this hypothesis is the high increase of expression observed in *A. thaliana* 24 h after pathogen challenge under controlled conditions (Figure 6), which correlated with an increase of these enzymes' activity 48 h after pathogen challenge (Figure 5). Induction of glucanases is especially interesting on the environmental scope of this protection since oligosaccharides released from fungal cell walls upon β -glucanase activity are

among the most potent elicitors that trigger phytoalexin synthesis (Desta et al. 2016). In light of these statements, an increase in flavonols following bacterial inoculation could be expected, which could eventually contribute to enhanced plant protection (Algar et al. 2014), and therefore meet one of our goals to achieve a sustainable production with low chemical inputs, suitable for any plant species (Garcia-Seco et al. 2013; Ramos Solano et al. 2014). In view of these

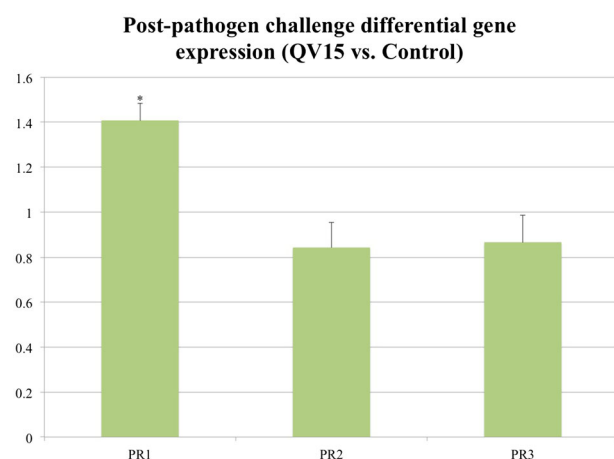


Figure 10. Differential expression of *PR1*, *PR2* and *PR3* in (a) blackberry control leaves and (b) blackberry inoculated leaves, in post-pathogen challenge. Asterisks indicate significant differences, according to the LSD test ($p < .05$).

effects in blackberry plants, the potential of this strain to be developed into a wide spectrum biofertilizer increases.

In conclusion, this PGPR has demonstrated its ability to increase plant defense in two different plant species, *Rubus* cv. Loch Ness and *A. thaliana*. The metabolic and genetic priming fingerprint induced by QV15 was defined by a decrease in ROS scavenging enzymes' activity (SOD and APX) in pre- and post-challenged plants, an increase in glucanase and chitinase activity after pathogen challenge, significantly increasing the expression of protein resistance1 (PR1), indicating a salicylic acid (SA)-mediated pathway activation. These results suggest an excellent potential of *B. amyloliquefaciens* QV15 to protect different plant species against different pathogens in field conditions. Given the phylogenetic distance of these two species and the common behavior detected in both, the potential of this strain to be effective in other plant species with economic interest is most promising and encouraging.

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Disclosure statement

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