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ORIGINAL PAPER

Characterization and potential of plant growth promoting rhizobacteria isolated from native Andean crops

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Abstract Bacteria isolated from soil and rhizosphere samples collected in Peru from Andean crops were tested in vitro and in vivo to determine their potential as plant growth promoters and their ability to induce systemic resistance to *Alternaria alternata* in tomato plants. The isolates were identified by sequencing their 16S ribosomal RNA gene. Test for phosphate solubilization, and indolacetic acid were also carried out, together with in vitro antagonism assays in dual cultures towards the plant pathogens *Fusarium solani, A. alternata* and *Curvularia lunata*. The three most promising isolates (Pa15, Ps155, Ps168) belonged to the genus *Pseudomonas*. Further assays were carried out with tomato plants to assess their plant protection effect towards *A. alternata* and as growth promoters. Inoculation of tomato seeds with all isolates significantly enhanced seed germination, plantlets emergence and plant development. Bacterial inoculation also reduce damage level caused by *A. alternata*. The expression levels of three tomato genes involved in the jasmonate (*AOS*), ethylene responsive (*ERF-2*) and pathogenesis related (*PR-P2*) pathways were determined in plants challenged with *A. alternata*, alone or with each bacterial isolate, respectively. Results showed that at 24 h after infection, in absence of the pathogen, the expression level of the tested genes was very low. The presence of *A. alternata* alone and in combination with bacteria increased the transcripts of all genes. Data showed a potential of best performing isolate Ps168 to sustain tomato plants nutrition and activate defense-related genes for protection by pathogenic fungi.

Keywords Bacteria · Gene expression · PGPR · Plant protection · Plant pathogens

Introduction

A dramatic increase in crop productivity has been achieved in the last century thanks to plant breeding, synthetic pesticides and fertilizers. Further benefits, however, are limited by progressively lower yield improvements, or threats like the heavy load of chemicals increasing pollution and depleting environment and health (Adesemoye and Kloepper 2009; Doran and Zeiss 2000). Rhizosphere is the narrow zone that surrounds the plant root and is considered the most diverse and complex ecosystem on Earth. Microorganisms from this area like bacteria and fungi are known to improve crop performance (Hinsinger et al. 2009; Jones and Hinsinger 2008). They are a key soil factor, underpinning the ecosystem functioning and determining the properties of associated biota.

Beneficial soil-borne microbes such as Plant Growth Promoter Rhizobacteria (PGPR) represent a valid tool in crop production, changing actual views in plant nutrition and protection. These bacteria portray a promising alternative to chemicals and many attempts have been carried out for their introduction in formulates alongside selected products. (Adesemoye and Egamberdieva 2013; Kloepper et al. 1989). Although the benefits conferred by PGPRs have been recognized for many years, there is still a gap in our knowledge about the biology and metabolism of these microorganisms, during the association that they establish with the host plant (Vacheron et al. 2013).

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In the last decade, several species of bacteria have been proposed as enhancers of plant growth and defense inducers, both acting against biotic and abiotic stresses or sustaining plant nutrition (Berg 2009; Compant et al. 2010; Beneduzi et al. 2012; Porcel et al. 2014). PGPRs operate at different levels by modifying the soil structure and changing the availability and distribution of nutrients in soil. Furthermore, they may act as growth stimulants through active compounds like phytohormones and siderophores production, or as defense inducers of genes involved in one or more plant defense mechanisms (Spaepen et al. 2007; Beneduzi et al. 2012). In addition to the effect on growth, fungal resistance plant induction driven by the microbial community of organic matter has been reported (Kavroulakis et al. 2005). The search for PGPRs in selected rhizosphere habitats and particular plant and microbial associations can provide more opportunities to identify the basic ingredients for novel products, useful to develop new, sustainable cropping technologies as well as to improve organic or marginal agricultural systems (Dimkpa et al. 2009).

There are many ways in which PGPRs may favour the plants, i.e. by increasing seed germination, root development and lateral root branching, shoot and root weights, or by inducing higher leaf area, stem elongation, or higher chlorophyll and protein contents (Vacheron et al. 2013). All these changes translate into higher yields. In general, the recipient plant acquires a better performance against environmental and abiotic stresses (Adesemoye and Kloepper 2009). PGPRs beneficial effects have been associated with the production of phytostimulators such as auxins, cytokinins, gibberellins, abscisic acid, and jasmonic acid, in addition or combined with other enzymatic activities modulating the plant hormonal balance (Vacheron et al. 2013; Castillo et al. 2015; Tiwari et al. 2017). These compounds play key physiological roles in plants, ranging from growth promotion to the response towards biotic or abiotic stresses (Tucci et al. 2011; Sasirekha and Srividya 2016). Plant physiology modifications exerted by beneficial microorganisms act locally at the colonizing site and distally in a systemic way. The capability of PGPRs to affect plant physiology has been the focus of many studies (Park et al. 2017; Goswami et al. 2016) that showed how PGPRs protect plants from diseases via induced systemic resistance (ISR). Bacterial activation of host defense pathways alerts plant against subsequent pathogen attacks, giving rise to a reduction in severity or disease incidence. Such ISR plant-defense effects have been observed in several species such as carnation, radish and *Arabidopsis* induced by *Pseudomonas* spp. (Verhagen et al. 2010; Goswami et al. 2016).

A survey was carried out in the Peruvian highlands to collect bacteria from soil and root samples proceeding from farms located at high altitudes (> 3300 m.a.s.l.). The sampled plants were from Andean species like maca (*Lepidium meyenii* Walpers) and aguaymanto (syn. goldenberry, *Physalis peruviana* L.). Some of the bacteria isolates showed positive effects on crops like maca (Zúñiga Dávila et al. 2011; Ortiz-Ojeda et al. 2017), potato *S. tuberosum* (Calvo et al. 2010), aguaymanto (Flores et al. 2013) and tara, *Caesalpinia spinosa* (Ogata-Gutiérrez et al. 2008). Furthermore, in vivo and in planta tests allowed the selection of the most performing cell lines (Ogata-Gutiérrez et al. 2008; Calvo et al. 2010; Zúñiga-Dávila et al. 2011; Flores et al. 2013). There is an increase awareness for the development of valid organic alternatives for crop management in view of protecting long-term soil productivity to enrich the crop system and benefit the rural farming incomes. For this purpose, we selected and further investigated the effects of three bacterial isolates proceeding from Andean crops. The aim of this study was to characterize the potential of these isolates in promoting the growth of tomato (*Solanum lycopersicum* L.) plants and in the protection against phytopathogenic fungi. Furthermore, an analysis was performed during the interaction of PGPRs and tomato plants challenged with a plant pathogenic isolate of *Alternaria alternata* to provide an insight on the molecular mechanism involved in the plant defense induction.

Materials and methods

Isolation of bacteria

The bacteria studied were isolated from Andean locations of Concepción, and San Pedro de Cajas, Tarma province (Department of Junin), located in the central-southern part of Peru, at more than 3300 m.a.s.l. Bacteria were isolated from the rhizosphere of aguaymanto and maca (Zúñiga-Dávila et al. 2011). Cryopreservation of the isolates was done with 25% glycerol (v/v) at − 80 °C (Overmann and Lepleux 2016) and deposited at the Plant Growth Promoting Bacteria Culture Collection of the Laboratorio de Ecología Microbiana y Biotecnología of the Universidad Nacional Agraria, La Molina (Lima, Peru). The isolates were reactivated on nutrient agar (NA) at 28 °C and grown in 10 ml of Yeast extract-Mannitol (YEM) broth at 28 °C on a rotary shaker (150 rev/min) for 24 h, until a concentration of 10^8 cfu/ml.

Synthesis of indolic compound

The production of indole acetic acid (IAA) was determined according the colorimetric assay by the method of Glickmann and Dessaux (1995). Bacteria were grown in YEM broth medium, supplemented with 5 mM L-tryptophan and incubated at 28 °C for 2 days. IAA was measured by mixing 250 µl of the culture supernatant with 1 ml of Salkowski's reagent (10 mM $FeCl_3$ in 36% H_2SO_4), and incubated for

30 min at room temperature in dark conditions. The mixture absorption spectra were determined at 530 nm and correlated with a standard IAA curve made from 3-indolacetic acid (Sigma–Aldrich). The experiment was repeated twice with three replicates.

Phosphate solubilization assay

For Phosphate-solubilization, the bacterial isolates were inoculated in solid media, according to the protocol by Nautiyal (1999) and supplemented with hydroxyapatite, bi- or tri-calcium phosphate. Bacterial inoculum corresponded to 10^8 cfu/ml per plate. The dishes were incubated at 28 °C and the formation of a halo was recorded for 20 days post inoculation. The Phosphate-solubilization index (SI) was determined following the formula proposed by Edi-Premono et al. (1996),

$$SI = (C\Phi + HZ\Phi)/C\Phi$$

where CΦ is the diameter of the bacterial colony and HZΦ corresponds to the diameter of the halo zone.

Molecular characterization of bacteria isolates

Genomic DNA from pure cultures was extracted with Axy-Prep Bacterial Genomic DNA Miniprep Kit (Axygen Scientific, USA) according to the manufacturer's instructions. PCR amplification of the 16S ribosomal RNA gene was performed for the identification of bacterial isolates using primers fD1 and rD1 (Table 1) as described by Weisburg et al. (1991). The amplified fragments were purified with the Axy-Prep PCR Cleanup Kit (Axygen Scientific, USA) according to the manufacturer's instructions and subsequently sequenced by a commercial service (Macrogen Inc., Seoul, S. Korea). The isolate sequences were identified through a BLASTN search on the EzTaxon database (http://www.ezbiocloud.net/eztaxon, Kim et al. 2012). Multiple alignments were compared using the Clustal X2 software (Larkin et al. 2007). Phylogenetic analyses were performed using the neighbor joining (NJ) method (Saitou and Nei 1987), with Mega6 software, applying 1000 bootstrap tests subsets with genetic distances computed using the Kimura's two-parameter model (Tamura et al. 2013).

Fungal isolates

Phytopathogenic fungi were isolated from naturally-infected plants of *P. peruviana* with disease symptoms like rotted fruit, necrotic spotting and leaf spots, according to the method by French and Hebert (1982). Fungal species isolated, were identified by the partial sequence of 18S, ITS1, 5.8S and ITS2. Approx. 500 µg of mycelium was collected by scraping the mycelial mass from the surface of the Petri dishes and grinded in liquid nitrogen with a mortar and pestle to obtain a fine powder. DNA was isolated using Axy-Prep Genomic DNA Miniprep Kit (Axygen Scientific, USA) according to the manufacturer's instructions. The purified DNA was directly used for PCR of the internal transcribed spacer (ITS1-5.8S-ITS2) ribosomal region as in White et al. 1990 (Table 1). The final PCR mixture contained 5.0 µl 10× PCR buffer, 2 U of *Taq* DNA polymerase, 200 µM each dNTPs, 1 mM of each primer and 10 µl of the template (100 ng). PCR was performed in a thermal cycler (Eppendorf) with 1 initial cycle at 94 °C for 3 min, 35 cycles with 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C, and a final extension at 72 C for 4 min. The sequence data obtained were checked on the EZfungi database algorithm for species identification (Altschul et al. 1990).

Dual culture test

Antimicrobial in vitro assays were performed using confrontation or dual culture tests (Idris et al. 2007). A mycelial plug of the isolate of the pathogenic fungi to test was placed in the centre of a PDA plate and the bacterial culture (10^8 CFU/ml) near the margins of the Petri dish. Plates inoculated with test pathogens alone served as controls. Plates were incubated in the dark at 22 °C and evaluated daily thereafter until 20 days post inoculation. Antagonism was evaluated

Table 1 Sequence of primers used for rRNA gene amplification and qRT-PCR assays

Genes	Full name	Forward (5′-3′)	Reverse (5′-3′)	Reference
16 s	16 s rRNA	AGAGTTTGATCCTGGCTCAG	AAGGAGGTGATCCAGCC	Weiburg et al. (1991)
ITS	Internal transcribed spacer	TCCGTAGGTGAACCTGCGG	TCCTCCGCTTATTGATATGC	White et al. (1990)
Ubi	Ubiquitin 3	TCGTAAGGAGTGCCCTAATGCTGA	CAATCGCCTCCAGCCTTGTTGTAA	SG[a]
AOS	Allene oxide synthase	ACGGAAGAGCCAAACAGGACCTTA	CGTTGCAAATGGTTGGTACCCGAA	SG
PR-P2	Pathogenesis related	GGAACAGGAACACAAGAAACAGTG A	CCCAATCCATTAGTGTCCAATCG	GenBank X58548
ERF2	Ethylene responsive	GTTCCTCTCAACCCCAAACG	TTCATCTGCTCACCACCTGTAGA	SG

[a]SG: https://solgenomics.net/organism/Solanum_lycopersicum/genome

by visible zones of fungal growth inhibition (FGI) using the formula

$$\text{FGI}\ (\%) = (R - r)/R \times 100$$

with r = radius of the fungal colony that grew towards the bacterial colony, R = maximum radius of the fungal colony away from the bacterial colony (Idris et al. 2007). The test was repeated three times in four replicates.

Plant material and inoculation

Seeds of *S. lycopersicum* L. var. Río Grande (kindly provided by the Erma Zaden Seed Company, NL) were surface disinfected in 3% NaOCl and then in 70% ethanol for 3 min and rinsed four times in sterile distilled water. Tomato seeds were pretreated with bacteria suspension at a concentration of 10^6 cfu/ml for 15 min and followed by an incubation in a growth chamber at 22 °C for 3 days in Petri dishes. Previous experiments showed the survival of the bacteria, in the same conditions as described before, in 10^3–10^4 cfu/ml, displaying a good adhesion of bacteria to seed surface. Germination tests were accomplished using 25 seeds for each treatment with four replications in completely randomized design, after 3 days the percentage of germination was assessed.

For plant assay, seeds of *S. lycopersicum* were inoculated respectively with each bacterial isolate and placed in sterile sand at 2 cm depth. All treatments were arranged in 40 pots, with 12 seeds per pot, control seeds were treated with sterile distilled water only. Seedlings were grown in a naturally illuminated greenhouse at 22–28 °C minimum and maximum temperature and 64–87% of relative humidity. After 192 and 240 h the percentage of the seed emergence was assessed. They were watered with hydroponic solution A and B (Centro de Investigación de Hidroponía y Nutrición Mineral UNALM, Peru) constituted of: 0.55 g/l Potassium nitrate, 0.35 g/l Ammonium nitrate, 0.18 g/l Triple superphosphate, 0.22 g/l Magnesium sulfate, 0.017 g/l Iron Chelate 6% Fe and micronutrient solution (2 mg/l manganese sulfate, 1.2 mg/l boric acid, 0.68 mg/l zinc sulfate, 0.4 mg/l copper sulfate and 0.08 mg/l ammonium molybdate). One week after emergence, tomato seedlings were thinned to two plantlets per pot for further evaluation at harvesting time. Harvest material was separate from the substrate and plant data as shoot length, root length, fresh and dry weight of aerial part, fresh and dry weight of root were recorded.

Pathogenicity assays

Three weeks old treated plants were infected with *A. alternata* by spraying a sterile distilled water suspension containing 10^4 conidia/ml in the abaxial and adaxial leaf surface. Treatments were: plants inoculated with the pathogen (with or without bacteria), non inoculated controls (with or without bacteria). Five replicated plants per treatment were used for the assay. Samples (100 mg of leaves from treated and control plants of each replicate) were collected 24 and 48 h post inoculation (hpi). The samples were immediately frozen in liquid nitrogen, pulverized, and then stored in the presence of Trizol (Thermo Scientific) at − 80 °C until use.

The damage level of the disease on the different treatments was recorded after 23 days of pathogen inoculation. Different parameters of tomato plant growth like germination and emergence percentage, aerial length, fresh weight of aerial parts, fresh weight of roots, dry weight of aerial parts, dry weight of roots, severity and pathogen disease index (foliar damage) were registered and analysed by LSD ANOVA multiple range test.

For each treatment, the severity of *A. alternata* was assessed as the average of the leaf area affected and expressed as pathogen disease index (PDI), calculated at 45 days of growth using the 0–5 scale of Durairaj et al. (2010) with the following scores: 0 = no symptom; 1 = 0.1–5.0% leaf area affected (l.a.a.); 2 = 5.1–15.0% l.a.a.; 3 = 15.1–30.0% l.a.a.; 4 = 30.1–50% l.a.a.; 5 = 50.1–100% l.a.a.

The disease incidence was calculated using the pathogen disease index according to the following formula

$$\text{PDI} = \frac{\text{Sum of all numerical grade}}{\text{Total number of leaves counted} \times \text{Maximum grade}} \times 100$$

RNA isolation, cDNA synthesis and quantitative PCR

To reveal whether bacterial inoculants activated a plant defense response towards the tested pathogens, the temporal expression of the pathogenesis-related (PR) gene *PR-P2* and of genes involved in the jasmonate (allene oxide synthase, *AOS*) and ethylene responsive (*ERF-2*) pathways were analysed by Real-time RT-PCR at 24 and 48 hpi, after challenging 21 days old tomato plants (var. Río Grande) with *A. alternata*, inoculated as previously described.

Total RNA of infected and control plants from replicates at different sampling times was extracted from frozen tomato leaves with the Purelink RNA mini kit system from AMBION (Life Technologies™, CA, USA) following manufacturer's recommendations. The extracted RNAs were subjected to DNAse digestion with the Turbo™ DNA free kit, according to the manufacturer's instructions. Total RNA integrity was analysed by native agarose gel electrophoresis, while the concentration and purity of the RNA samples using a NanoDrop spectrophotometer (Thermo Scientific). Total RNA (2 µg) was reverse transcribed in presence of random hexanucleotides using the Superscript® III reverse transcription kit (Invitrogen™, Ca, USA). Real-time PCR with SYBR Green detection was performed on the Eppendorf Mastercycler ep *realplex*™ system (Eppendorf, Germany). Prior the DNAse treatment, the primers used were tested

through an end point PCR with total RNA-DNA contaminated as template (Table 1). The following PCR program was used: 1 cycle of 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s, annealing and extension at 60 °C for 60 s. Reaction specificity was confirmed by dissociation curves after the amplification was completed. Ubiquitin was used as the housekeeping gene for the normalization of expression signals. All reactions were assayed in triplicates, with two technical replicates per each biological one, the assays were repeated twice. The relative fold change of gene expression was determined in healthy plants compared with infected leaves, and with bacteria treated plants. Student's *t* test was carried out to determine significant differences from the bacteria pre-inoculated plants alone or from those challenged with the pathogen (Table 2).

Results

Biochemical characterization

Bacterial isolates were characterized for their plant growth promoter activity by considering different parameters. Soils at the collection sites had a high content of organic matter, available phosphate and potassium and were found to be strongly acidic, with pH between 4.5 and 5.5. All isolates were capable of producing IAA at different extents with a range of 10.7–56.6 ppm in presence of precursor tryptophan, with Pa15 showing the best performance (Table 3).

The Phosphate-solubilization assays showed that the three isolates were able to mobilize at least one of the inorganic phosphate sources tested. The solubilization index varied between 1.6 and 2.1, considering one as no capability to solubilize the phosphate source. All the isolates were able to solubilize hydroxyapatite as phosphate source and two of them only bi-calcium phosphate but with lower SI (Table 3).

Molecular characterization of bacterial isolates

Based on primers homology to conserved regions of the 16S rRNA, the corresponding fragments were amplified and obtained the sequences of the expected nucleotidic dimensions, including a partial 16S region. When compared with similar fragments of other bacteria, available in GenBank as identified by BLAST, two of the selected isolates showed nucleotide sequence identities in the range of 99–100%. Phylogenetic analyses showed that Pa15 isolate has close similarities to *P. baetica* and *P. jessenii*, whereas Ps155 and Ps168 to *P. aeruginosa*. The NJ rooted tree (Fig. 1) showed that the isolate sequences clustered preferentially in two branches, as shown by the significant bootstrap values at major nodes that clearly distinguished Pa15 from the closer Ps155 and Ps168. The sequences determined in this study for isolates Pa15, Ps168 and Ps155 have been deposited in the GenBank database under Accession Numbers KU095844, KU095845 and KU095846, respectively.

Identification of fungal isolates

The sequencing data aligned with the isolates Ctom0, Ftom01 and Atom01 rDNA and compared with the nearest corresponding entries available in the Ezfungi database as identified by BLAST resulted in 100, 98.95 and 100% identity with *Cochliobolus lunatus* (JQ936202), the anamorph of *Curvularia lunata; Haematonectria haematococca* (JX868703), the teleomorph of *Fusarium solani*, and *Alternaria alternata* (JX418354) respectively.

Dual culture assays

Results showed a significant antagonistic effect of bacteria on fungal growth (Fig. 2). All isolates showed > 20% inhibition of radial growth against the three fungi tested. Isolate Ps168 performed better against *C. lunata* (> 90%) and was active almost at the same extent against *F. solani* and *A. alternata* (53 and 41.9%, respectively). Ps155 displayed a better performance against *A. alternata* and *F. solani* compared to the other treatments, with a 67.2 and 75.9% rate of growth inhibition, respectively (p value < 0.05). Compared to the other bacteria tested Pa15 showed the lowest inhibition in the case of *C. lunata* and *F. solani*, with a higher activity against *A. alternata*. The surface of the control plates not inoculated with the bacterial isolates was completely covered (> 90%) by the growing mycelium of the phytopathogens showing no inhibition.

Seed germination and emergence in tomato plantlets

The response of *S. lycopersicum* to the three selected bacteria was evaluated after seed inoculation with pure bacterial culture. All isolates showed an enhancement in seed germination at 48 and 72 h in vitro assays compared to control. At 72 h, the isolates Ps168 and Ps155 induced a significantly better plant performance (70 and 73% respectively), compared to other treatments. The percentage of emergence was, for the three isolates, significantly greater than control (Table 4).

Effects of bacterial inoculation on growth and protection from *A. alternata*

Results revealed that Ps168 and Pa15 isolates exerted the best effect on fresh and dry weight of aerial parts of tomato plants (Table 5). The co-inoculation of tomato plants with Ps168 and *A. alternata* supported a good performance of plant growth and development in comparison with those

Table 2 Probability values of Student's t tests for the isolates assays and expression levels of related tomato plants gene transcripts, measured with qRT-PCR

Genes, test times and treatments	*P* values[a]						
	mean ± SE (relative expression)	Pa15	Pa15 + *Alternaria*	Ps155	Ps155 + *Alternaria*	Ps168	Ps168 + *Alternaria*
PR-P2, 24 hpi							
Alternaria	18.5 ± 2.4	0.0017	0.1545	0.0013	0.0648	0.0052	0.0406
Pa15	0.9 ± 0.2		0.0017	0.0398	0.0958	0.0438	0.0122
Pa15 + *Alternaria*	24.0 ± 3.7			0.0015	0.0481	0.0077	0.0130
Ps155	0.2 ± 0.1				0.0820	0.3427	0.0092
Ps155 + *Alternaria*	9.6 ± 5.5					0.1371	0.4606
Ps168	0.1 ± 0.1						0.0283
Ps168 + *Alternaria*	9.0 ± 2.2						
PR-P2, 48 hpi							
Alternaria	2.4 ± 0.8	0.1478	0.2717	0.0598	0.0070**	0.1002	0.0129*
Pa15	0.9 ± 0.1		0.1143	0.0175**	0.0270*	0.0401*	0.0317*
Pa15 + *Alternaria*	1.6 ± 0.3			0.0310*	0.0336*	0.0656	0.0415*
Ps155	5.0 ± 1.2				0.1267	0.4280	0.1878
Ps155 + *Alternaria*	9.6 ± 3.1					0.1171	0.3640
Ps168	4.6 ± 1.5						0.1677
Ps168 + *Alternaria*	8.1 ± 2.5						
ERF, 24 hpi							
Alternaria	12.7 ± 2.4	0.0088**	0.0139*	0.0417*	0.0019**	0.0936	0.0004***
Pa15	0.2 ± 0.02		0.0012**	0.0025**	0.0377*	0.0161*	0.0049**
Pa15 + *Alternaria*	1.3 ± 0.1			0.0092*	0.0401*	0.0285*	0.0057**
Ps155	4.0 ± 0.6				0.0469*	0.1214	0.0083**
Ps155 + *Alternaria*	46.8 ± 19.5					0.0176*	0.2626
Ps168	7.5 ± 1.9						0.0029**
Ps168 + *Alternaria*	35.9 ± 6.9						
ERF, 48 hpi							
Alternaria	3.3 ± 0.3	0.000***	0.0007***	0.0000***	0.0020**	0.0005***	0.0005***
Pa15	22.8 ± 2.8		0.0057**	0.0958	0.3092	0.1505	0.0386*
Pa15 + *Alternaria*	7.7 ± 1.9			0.0060**	0.1524	0.0680	0.1915
Ps155	33.1 ± 5.4				0.0757	0.2166	0.0088**
Ps155 + *Alternaria*	17.9 ± 7.4					0.0909	0.2509
Ps168	51.0 ± 20.5						0.0556
Ps168 + Alternaria	12.0 ± 3.5						
AOS, 24 hpi							
Alternaria	0.5 ± 0.07	0.0045**	0.0040**	0.0053**	0.0209*	0.0014**	0.4082
Pa15	0.1 ± 0.02		0.0024**	0.0138*	0.2624	0.0052**	0.0628
Pa15 + *Alternaria*	1.02 ± 0.16			0.0085**	0.0071**	0.0017**	0.0715
Ps155	0.02 ± 0.01				0.1667	0.2578	0.0720
Ps155 + *Alternaria*	0.19 ± 0.12					0.1137	0.1078
Ps168	0.02 ± 0.01						0.0387*
Ps168 + *Alternaria*	0.5 ± 0.1						
AOS, 48 hpi							
Alternaria	0.06 ± 0.01	0.000***	0.1309	0.0008***	0.0013*	0.0016*	0.0166*
Pa15	0.33 ± 0.08		0.0245*	0.1208	0.4041	0.4757	0.2410
Pa15 + *Alternaria*	0.10 ± 0.04			0.0882	0.0932	0.0960	0.2042
Ps155	0.21 ± 0.06				0.2529	0.2216	0.4715
Ps155 + *Alternaria*	0.29 ± 0.11					0.4394	0.3212

Table 2 (continued)

Genes, test times and treatments	*P* values[a]						
	mean ± SE (relative expression)	Pa15	Pa15 + *Alternaria*	Ps155	Ps155 + *Alternaria*	Ps168	Ps168 + *Alternaria*
Ps168	0.32 ± 0.12						0.2823
Ps168 + *Alternaria*	0.2 ± 0.1						

Second column data are the mean and standard errors of the 6–12 replications, used for all comparisons

[a]Significance levels are also indicated by asterisks ($p \leq 0.05$ =*, $p \leq 0.01$ =** and $p \leq 0.001$ =***)

Table 3 IAA production and Solubilization Index (SI) of the bacterial isolates tested

Isolate	IAA (ppm)	SI/bi-calcium phosphate	SI/tri-calcium phosphate	SI/hydroxyapatite
Ps155	10.7 (±0.15)	1.6 (±0.04)	1.3 (±0.08)	2.1 (±0.12)
Pa15	56.6 (±0.31)	1.2 (±0.02)	1.1 (±0.1)	1.2 (±0.17)
Ps168	17.8 (±0.25)	1.8 (±0.14)	1.2 (±0.02)	2 (±0.07)

inoculated with the pathogen alone. The plants inoculated with both microorganisms showed fresh (11.3 g) and dry weights (0.66 g) similar to plants with Pa15 (12.4 g/0.68 g). The isolates Ps168 and Pa15 also induced an increase in root development. Although the isolate Ps155 was not as effective as the other two tested isolates, it increased tomato length, aerial fresh and dry weights significantly compared to the un-inoculated control. Besides, the interaction of tomato-Ps155-*A. alternata* proved to be effective at improving an increment of the measured parameters compared to the plants infected only with *A. alternata* (Table 5). Moreover, bacteria inoculated plants infected with *A. alternata* showed a lower pathogen disease index and damage compared to the plants infected only with the phytopathogen (Fig. 3).

Expression of selected tomato genes

Significant differences were observed in the expression levels of the genes assayed, among the plants treated respectively, with Ps155, Ps168 and Pa15 alone or challenged with *A. alternata* at 24 and 48 hpi (Table 2). At 24 hpi the tomato plants pre-inoculated with the single bacteria isolates displayed similar levels of *PR-P2* expression, accounting for the healthy condition without any significant change in induction. Conversely, the plants inoculated only with the pathogen showed a strong induction of this gene, as expected

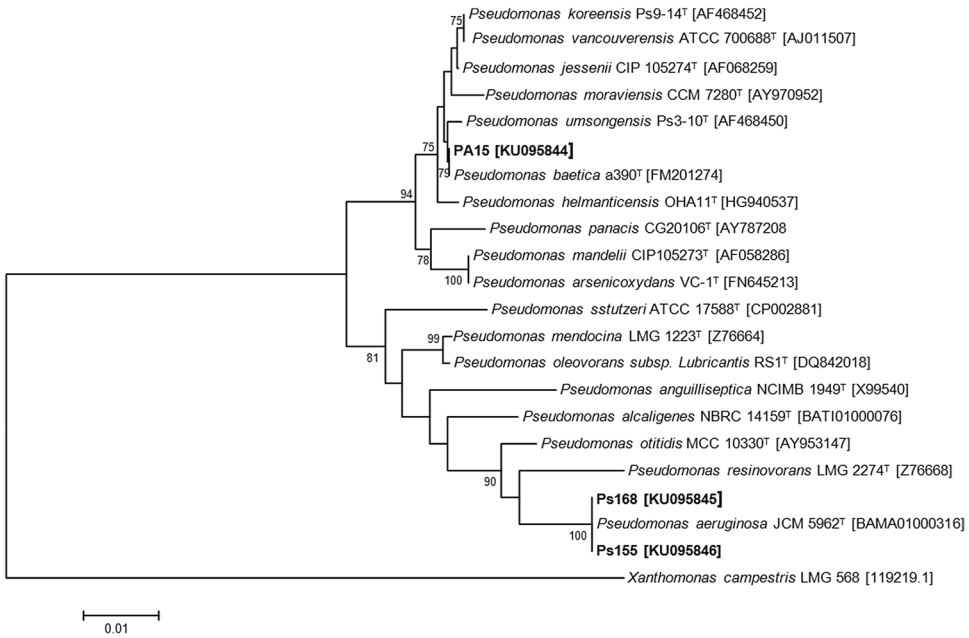

Fig. 1 Neighbour-joining phylogenetic tree analysis based on 16S rRNA sequences. Accession Numbers of isolates in Ps168 (KU095845), Ps155 (KU095846) and Pa15 (KU095844). The tree depicts relationship between the isolates and parenthesis. reference strains from GenBank. The isolates Ps168 and Ps155 are grouped with *P. aeruginosa* with a 100% of identity. Pa15 falls between *P. baetica* and *P. jessenii*. Only bootstrap values greater than 60% are shown (1000 pseudoreplicates)

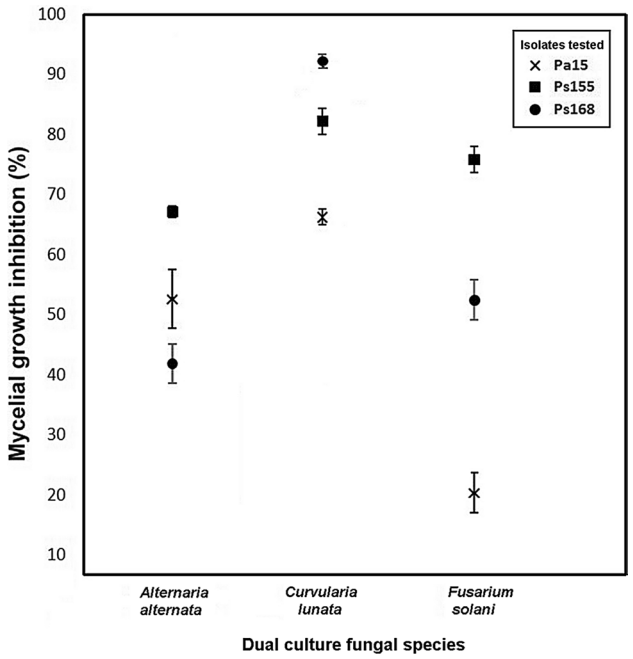

Fig. 2 Radial growth of *F. solani*, C. *lunata* and *A. alternata* mycelia in dual culture tests. Rhizobacterial isolates Ps168, Ps155 and Pa15 were co-cultivated with the fungi in a Petri dish in the dark at 22 °C. The radial growth was determined and percent of growth inhibition compared to control growth. The experiments were replicated three times. Data were analyzed using two-way analysis of variance

Table 4 Germination and emergence percentage of tomato seeds (%) as affected by *Pseudomonas* isolates

Treatments (h)	Germination (%)		Emergence (%)	
	72	96	192	240
Control	52a (±3.85)	65a (±3.85)	56a (±6.80)	61a (±7.97)
Pa15	63ab (±6.67)	73ab (±4.70)	72b (±5.89)	74b (±4.45)
Ps168	70b (±7.70)	78b (±3.87)	74b (±7.50)	78b (±6.56)
Ps155	73b (±6.67)	78b (±3.85)	78b (±6.94)	82 b (±6.86)

*Means with the same letters do not differ significantly ($p < 0.05$) according to the least

significant difference (LSD) tests using Statgraphics Centurion software

(Fig. 4a). The levels of *PR-P2* in plants pre-inoculated with Pa15 and inoculated with *A. alternata*, showed higher transcript accumulation compare to the control with pathogen infection without bacterial treatment. However, Ps155 and Ps168 displayed a lower expression of *PR-P2* in the plants challenged with *A. alternata in* comparison with the plants inoculated with the pathogen alone (Fig. 4a). At 48 hpi the expression of *PR-P2* maintained the same trend in plants inoculated with Pa15 alone, while the plants inoculated with Pa15 in concomitant with *A. alternata* showed a drastic decrease level of *PR-P2* transcript as well as the plants inoculated with the pathogen alone. The other two bacteria, Ps155 and Ps168, pre-inoculated alone showed an opposite effect, with an increase in the transcript level relative to the 24 hpi plants. The transcript expression level of Ps155 and Ps168 treated plants, during the interaction with the pathogen at 48 hpi was the same as at 24 hpi (Fig. 4b).

At 24 hpi, *ERF-2* showed a higher increment on expression in the interaction of Ps155 and Ps168 with *A. alternata* respectively, compared to the tomato plants infected by the pathogen alone (Fig. 4c). Pa15 alone or in combination with *A. alternata* displayed an expression level of *ERF-2* lower than single *A. alternata* inoculated plants or the other plants, pre-inoculated with Ps155 and Ps168 (Fig. 4c). At 48 hpi *ERF-2* was up-regulated in all the plants treated with the three bacteria, in comparison with the pathogen-alone infected plants. The transcripts accumulation were higher during the interaction of Ps155, Ps168 and Pa15 with the pathogen in comparison to plants with the pathogen alone at the same sampling time even if was lowered in comparison with 24 hpi (Fig. 4d).

The expression of *AOS* at 24 hpi showed lower induction in plants infected with *A. alternata* alone, the same trend was observed in Ps168 plus the pathogen, compared to the corresponding healthy plants. The three bacterial isolates showed a minimal expression, relative to the corresponding healthy plants at the same sampling time. The *AOS* transcript, in the case of Pa15 plus *A. alternata* inoculated plants, showed an increased that was twice that of the *A. alternata* infected plant alone at 24 hpi. (Fig. 4e). At 48 hpi the *AOS* transcript level decreased significantly in the *A. alternata* infected plants alone or in combination with bacteria Pa15 and Ps168, keeping almost the same transcript level in Ps155 plus the pathogen, in comparison with the expression showed at 24 hpi. The *AOS* expression increased at 48 hpi in plants treated with isolates Ps155 and Ps168 alone, when compared with 24 hpi (Fig. 4f). The three genes evaluated at 24 hpi, in plants treated with single isolated bacteria, respectively, displayed a minimal expression level, while in the presence of the pathogen was observed an increase in the expression of the tested genes at the same sampling time.

Discussion

The special geographic and climatic conditions of the Andean region and the microbial species richness of its soils make local crops as promising biodiversity spots for search and isolation of beneficial microorganisms with antifungal properties. Microbial communities associated with native

Table 5 Effect of bacterial and fungal inoculation on different growth parameters of tomato plants

Treatments	Plant lenght (cm)	FW aerial parts (g)	DW aerial parts (g)	FW roots (g)	DW roots (g)
CR	30.9a	6.1a	0.2970a	0.1440a	0.01972a
C	42.6bc	8.5b	0.4725b	0.1425a	0.02305a
Ps168R	36.4ab	11.3c	0.6600c	0.4100cd	0.06758bc
Ps168	54.5d	14.2d	0.8829d	0.6786e	0.08143c
Ps155R	39.3b	8.6b	0.5375b	0.1275a	0.05458abc
Ps155	51.7d	13.2cd	0.8175d	0.2250ab	0.01640a
Pa15R	49.7cd	8.6b	0.4580b	0.3525bc	0.03528ab
Pa15	50.2cd	12.4cd	0.6825c	0.4975d	0.05675abc

*CR = plant + *Alternaria alternata*, C = Control, Treatments with R means that they have the treatment and the pathogen (*A. alternata*)

Means with the same letters do not differ significantly ($p < 0.05$) according to the least significant difference (LSD) tests using Statgraphics Centurion software

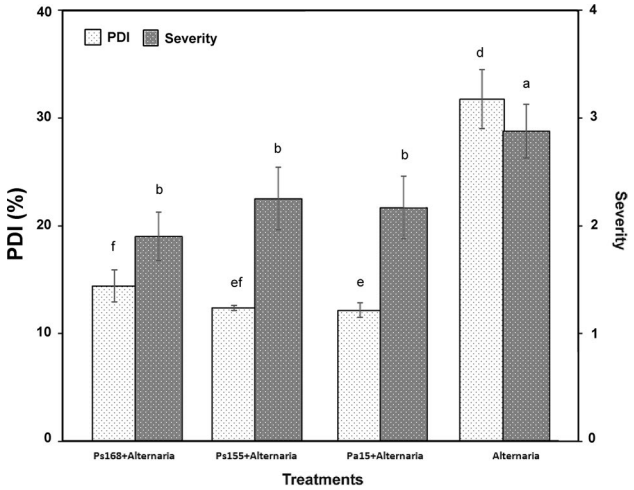

Fig. 3 Pathogen disease index (PDI) and damage level comparing *A. alternata* and *A. alternata*-PGPR inoculated plants (Tuckey Test < 0.05). Vertical bars show SE unit. Control plants were not infected with the phytopathogen

crops can provide new tools useful in plant protection and nutrition, providing efficient microbial isolates underpinning multiple services in the rhizosphere.

Two of the three *Pseudomonas* spp. isolated were identified with high bootstraps and identity as *P. aeruginosa* (Ps168 and Ps155). Isolate Pa15 clustered between *P. baetica* and *P. jessenii*, sharing the highest identity with *P. baetica*. Beneficial microbes, and in particular *Pseudomonas* spp., are well known as important constituents of the rhizosphere bacterial community (Bhakthavatchalu et al. 2013). Although different studies have shown that many *Pseudomonas* isolates exert their beneficial effects, principally by active exclusion of pathogens (Rainey 1999), other studies have pointed out an important role in eliciting induction of systemic resistance. Mechanisms identified included the production of siderophores with antifungal activity, enhanced in some interaction, by the presence of $FeCl_3$ (Sasirekha and Srividya 2016). Antagonistic bacteria residing in soil organic matter also have the capability both to induce systemic resistance in plants and elicit defense mechanisms, likely through a lytic action inhibiting fungal pathogens (Kavroulakis et al. 2010). However, many mechanisms that stimulate the plant response are not yet completely understood (Pieterse et al. 2014). The in vitro and in vivo assays performed confirmed the capability of all isolates to synthesize IAA. Isolate Pa15 showed the best performance in IAA production, which was 3–5 folds higher than that of the other two isolates. IAA, an important phytohormone of the auxin family, is involved in plant growth regulation, controlling many fundamental physiological processes like cell enlargement and division, tissue differentiation and responses to light and gravity (Leveau and Lindow 2005; Spaepen et al. 2007). IAA production appeared variable and depended on species and isolates. Furthermore, there is a strong influence in this pathway, as due to culture conditions, growth stages and nutrient availability (Sarvani and Subhash 2013). It should be noted that the effects of this hormone are not the same in all plants (Vestergård et al. 2007). In many cases, low concentrations of the phytohormone can stimulate plant growth, whereas high concentrations can inhibit and reduce the elongation zone (Hernandez 2002).

Most of the phosphorus present in soil (50–80%) is in organic form, which cannot be directly used by the majority of plants (Richardson 2001). The solubilizing capacity of phosphates by the bacteria tested is an important feature indicating a better use of phosphorus source that, as an essential nutrient, is often immobilized in the soil (Ramazan et al. 2005). Soil bacteria have the capacity of solubilizing phosphate at different extents, this feature representing an important process not only in natural ecosystem but also in organic crops to improve plant growth (Moreno-Ramírez et al. 2015). Bacteria exploit different mechanisms to solubilize phosphate like enzymatic processes or by locally

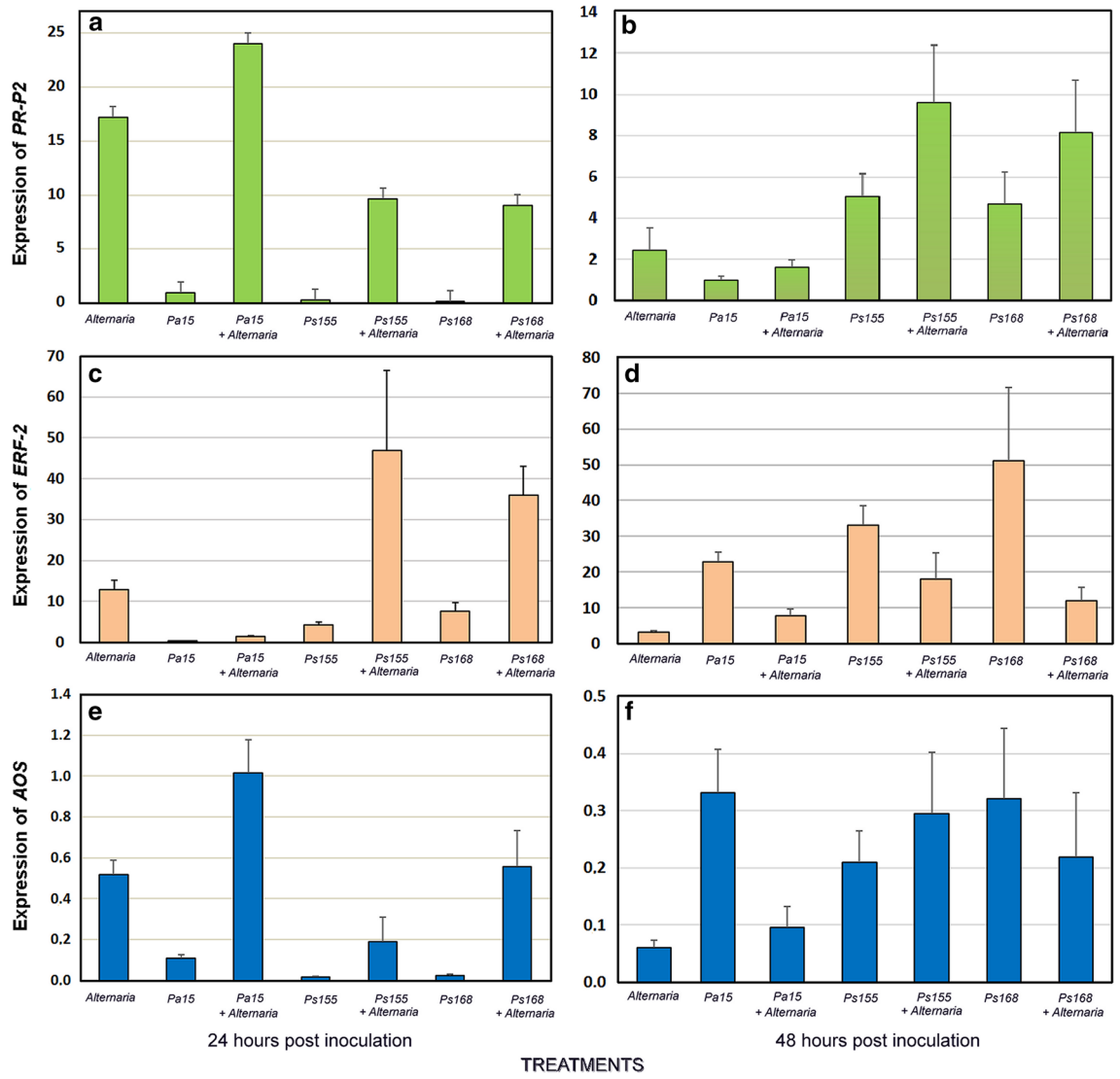

Fig. 4 Relative expression of *PR-P2* (**a**, **b**), *ERF-2* (**c**, **d**) and *AOS* (**e**, **f**) gene transcripts in tomato plants treated with the *Pseudomonas* spp. isolates, alone or in combination with *A. alternata*. The relative expression was measured by qRT-PCR in 21 days old plants of *S. lycopersicum* (var. Río Grande). The expression level for each gene is reported as the fold increased relative to the untreated control plants, 24 (**a**, **c**, and **e**) and 48 (**b**, **d**, and **f**) hpi after challenging with the pathogen

lowering the pH (Halder et al. 1990). The results showed that all tested bacteria could solubilize bi-calcium, but not tri-calcium phosphate. The data fit the observations of Ben Farhat et al. (2009), who reported that certain phosphate solubilizing bacteria metabolize better bi-calcium phosphate, converting a large fraction to its soluble form (P_2O_5), whereas tri-calcium phosphate and hydroxyapatite have a lower conversion rate. All isolates were able to solubilize at least one of the three phosphate sources, however, SI showed low values in all isolates compared to other studies in which it varied between 2 and 7 (Yasmin and Bano 2011; Moreno-Ramírez et al. 2015).

Pseudomonas spp. include several taxa used in biological control of many soil-borne plant pathogens (Whipps 2001). The antifungal activity shown by the isolates tested suggest biocontrol properties, which may result important in the rhizosphere. All the isolates were able to inhibit the growth of phytopathogens tested to different extent, the best performance was showed by isolate Ps155 (Fig. 2). Several *Pseudomonas* spp. have been described that are able to produce an array of compounds inhibiting the growth of many fungal pathogens, as toxins, antibiotic and siderophores, conferring in general a good adaptive metabolism to roots (Thomashow and Weller 1990). Plants assays confirmed that the isolates tested can stimulate seed germination, emergence and growth. Results indicated a favourable response of tomato plant parameters after bacteria inoculation. Positive effects of bacteria inoculation

on plant growth appeared related to fresh and dry root weights, as high development of root matter increased growth parameters and yields through improved micronutrients uptakes (Sharm et al. 2014).

The activation of a local defense response can trigger the production of specific plant hormones such as salicylic acid (SA), jasmonic acid (JA), JA-related oxylipins and ethylene (ET), which take part in various defense signaling pathways (Pieterse et al. 2014; Memelink 2009). Many PGPR are well known for inducing genes that invoke ISR mechanism (Walters et al. 2005).

In the present study, the plant response to beneficial microbes was evaluated during the interaction of tomato inoculated or not with selected beneficial bacteria and challenged or not with the phytopathogenic fungus *A. alternata*. This fungus represents a threat for more than 380 species (Mmbaga et al. 2011) and for this reason was selected for the in vivo infection assay of pre-treated tomato plants. Activation of genes encoding pathogenesis-related proteins active in cellular processes are part of a defensive system deployed by plants against different stresses. These include in particular, pathogen infections, wounds and environmental stresses like heavy metal pollution, UV radiation, salinity, cold and osmotic stress (Oide et al. 2013; Seo et al. 2008). *PR* genes are induced locally in tomato-pathogen interactions as a reaction to pathogen invasion. Their induction is very fast and the increase of expression positively correlates with disease resistance (Seo et al. 2008). This was evident in the tomato plants inoculated with the pathogen alone, that showed at 24 hpi a 15-fold higher transcript level of *PR-P2* relative to the non-infected control (Fig. 4a).

The healthy plants pre-inoculated with the bacteria displayed a lower transcript level than the diseased ones. However, at 48 hpi a higher *PR-P2* gene expression was found when Ps168 and Ps155 were inoculated alone. In the case of the plants pre-treated with the bacteria showed the transcript level from four to fivefold higher than in the plants inoculated with the pathogen alone (Fig. 4b). The capacity of antagonistic bacteria to alter the expression of *PR-P2* suggest a putative protective role involved in the plant response that was maintained to 48 hpi of the pathogen challenging, after the application of beneficial bacteria. This higher expression highlights the synergistic effect exerted by the bacteria, holding up a state of plant vigilance for a longer time. The pre-inoculated plants challenged with *A. alternata* held the expression of *PR-P2* up to 48 hpi, while the single pathogen infected plants showed a largely reduced expression level in comparison with the expression displayed at 24 hpi.

Ps155 and Ps168 strongly enhanced the expression of *ERF-2* after inoculation with the pathogen. ISR is usually triggered by contact with non-pathogenic microorganisms. The mechanism does not appear to be SA-dependent, but seems regulated by JA and ethylene (Pu et al. 2014). The data herein presented indicates a possible role of ethylene.

SA has been suggested to suppress the expression of the JA biosynthetic enzymes lipoxygenase-2 and allene oxide synthase (Mur et al. 2006). The allene oxide synthase, encoded by *AOS*, is the second enzyme in the biosynthesis pathway of JA, according to the model proposed by Sun et al. (2011), and one of the key enzymes involved in the JA pathway. In the present study, the expression level of this gene at 24 hpi was very low in almost all treatments. At 48 hpi the *AOS* transcript level suffers a decreased in the plants inoculated with the pathogen alone. The comparison between the pathogen alone and the single inoculants reveal a four–sixfold differential expression of the *AOS* transcript, suggesting that beneficial microorganisms hold the endogenous level of the JA pathway higher than the infected plant alone. A noteworthy fact is that *AOS* expression level was at overall low, results that are congruent with data showed in Tucci et al. (2011), corroborating the behavior of JA genes as less responsive. In the case of the pathogen-inoculant interactions, *AOS* transcript level decrease ten times in the case of Pa15 and 2.5 times in Ps168, keeping almost the same level in the case of Ps155 in comparison with the single pathogen inoculated plant. Overall, data indicate that the defense response induced by inoculants can be controlled at the molecular level by multiple genes expressed by the tomato plant.

In the present study the selected bacteria showed a potential to improve tomato plant growth, sustaining their performance during the pathogens attack. Data indicated that all isolates tested, and in particular Ps168 and Ps155, strengthened the plant development, reinforcing their tolerance towards the phytopathogenic fungi tested. More studies are needed to elucidate the molecular basis of these relationships, aiming at disclosing their use as PGPR promoters and bioinoculants, for management of fungal diseases that are endemic in Andean food crops.

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