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Potential of *Pseudomonas putida* PCI2 for the Protection of Tomato Plants Against Fungal Pathogens

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Abstract Tomato is one of the most economically attractive vegetable crops due to its high yields. Diseases cause significant losses in tomato production worldwide. We carried out Polymerase Chain Reaction studies to detect the presence of genes encoding antifungal compounds in the DNA of *Pseudomonas putida* strain PCI2. We also used liquid chromatography-electrospray tandem mass spectrometry to detect and quantify the production of compounds that increase the resistance of plants to diseases from culture supernatants of PCI2. In addition, we investigated the presence of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase in PCI2. Finally, PCI2 was used for inoculation of tomato seeds to study its potential biocontrol activity against *Fusarium oxysporum* MR193. The obtained results showed that no fragments for the encoding genes of hydrogen cyanide, pyoluteorin, 2,4-diacetylphloroglucinol, pyrrolnitrin, or phenazine-1-carboxylic acid were amplified from the DNA of PCI2. On the other hand, PCI2 produced salicylic acid and jasmonic acid in Luria–Bertani medium and grew in a culture medium containing ACC as the sole nitrogen source. We observed a reduction in disease incidence from 53.33 % in the pathogen control to 30 % in tomato plants pre-inoculated with PCI2 as well as increases in shoot and root dry weights in inoculated plants, as compared to the pathogenicity control. This study suggests that inoculation of tomato seeds with *P. putida* PCI2 increases the resistance of plants to root rot caused by *F. oxysporum* and that PCI2 produces compounds that may be involved at different levels in increasing such resistance. Thus, PCI2 could represent a non-contaminating management strategy potentially applicable in vegetable crops such as tomato.

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Introduction

Tomato is the vegetable plant with the highest economic value. Production and commerce of tomato are continually increasing. Consequently, the study of control methods of tomato plant pathogens is of great interest for achieving a more efficient production.

Diseases are major biological constraints for production of vegetables and fruit crops. Many pathogens including fungi, bacteria, viruses, and nematodes are associated with infection of crop plants. Among these, phytopathogenic fungi are one of the major factors limiting crop production [2]. Tomato is susceptible to, among other fungi, subspecific taxa of *Fusarium oxysporum*, which are fungi that grow and survive for long periods on organic matter, in the soil and in the rhizosphere. *F. oxysporum* f. sp. *radicis-lycopersici* (FORL) causes tomato foot and root rot (TFRR) (synonym for crown and root rot), which is a serious disease of worldwide economic importance for tomato production [9], causing significant losses in greenhouses, open field crops, and hydroponic cultures [19]. Chemical pesticides do not efficiently prevent or suppress TFRR [7].

The relatively insufficient activity of chemical control and the absence of resistance in some tomato cultivars have focused interest on the practicality of biocontrol against *F. oxysporum* [6]. The microorganisms designated as plant growth-promoting rhizobacteria (PGPR) increase plant growth through many mechanisms, including protection of

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roots against infection by pathogens [37]. Among PGPR, the fluorescent pseudomonads have been proven to exhibit many traits that make them appropriate as biological control agents [23, 24, 33, 36].

One of the favorable strategies for the environment that can be used for crop protection is the use of plants that are expressing an induced systemic resistance (ISR). The ISR is a mechanism of resistance triggered mainly by pre-inoculation with PGPR [34]. Plants develop an improved defensive capacity against a broad spectrum of plant pathogens after colonization of roots by selected strains of non-pathogenic/biocontrol bacteria. The ISR is phenotypically similar to systemic acquired resistance (SAR), which is triggered by necrotizing pathogens, since the disease caused by a pathogen is reduced. Both ISR and SAR are effective against a large group of pathogens. However, there are differences in the effectiveness of the signaling compounds involved. Interestingly, when ISR and SAR are simultaneously activated, a greater suppression of disease occurs toward pathogens against which the responses mediated by salicylic acid (SA) and jasmonic acid (JA)-ethylene are effective. Such mechanism was proposed for the system *Pseudomonas syringae* pv. *tomato*-tomato [35]. The role of siderophores in ISR has also been established in several systems. For instance, siderophores from *Pseudomonas putida* WCS358 were found to be involved in ISR when this strain suppresses bacterial wilt caused by *Ralstonia solanacearum* in *Eucalyptus urophylla* [25]. To effectively use a promising biocontrol agent, with potential application as a crop protection strategy, it is of interest to study its effect on different host-pathogen systems and the bacterial traits involved in its biocontrol activity.

The compound 1-aminocyclopropane-1-carboxylic acid (ACC) is exuded from plant roots alongside other amino acids. The bacteria that have the enzyme ACC deaminase are able to metabolize ACC and use it as a source of carbon thus reducing the level of ACC in the roots. As a consequence, the production of ethylene by the roots decreases, alleviating the inhibition of root growth [33]. The competence of PGPR that generate ACC deaminase to decrease the levels of plant ethylene, often a result of several stresses, is a main component in the effective performance of such bacteria. The optimal performance of PGPR includes the synergistic interaction between ACC deaminase and auxins, such as indole-3-acetic acid (IAA), from plants and bacteria. These bacteria not only directly promote the growth of plants, but they also protect plants against drought, salinity, organic contaminants, and fungal pathogens, among other stresses [13].

In previous studies, we showed that *P. putida* strain PCI2, isolated from the rhizosphere of a healthy tomato plant, is promising for the control of tomato damping-off caused by *Sclerotium rolfsii*. We also showed that *P. putida* PCI2 produces siderophores, exhibits chitinolytic activity, and synthesizes IAA [20]. In addition, inoculation of tomato plants with *P. putida* PCI2 decreased the percentage of *Alternaria alternata* infected plants by 15 % [21]. Moreover, PCI2 proved to be positive for phosphatase activity, solubilized $AlPO_4$ and hydrolyzed $Ca_3(PO_4)_2$ even in medium with 5 % NaCl [22]. The present work was undertaken to detect production of compounds associated with increased plant resistance and tolerance to stresses and fungal pathogens in *P. putida* PCI2 and to study the potential of the strain as a biocontrol agent in the tomato-*F. oxysporum* system.

Materials and Methods

Bacterial Strain and Culture Media

Pseudomonas putida PCI2 (GenBank accession number GU004535) is a native strain isolated in 2009 from the rhizosphere of a healthy tomato plant located in an orchard in Río Cuarto (33°04′S, 64°38′O), Córdoba, Argentina. Strain PCI2 exhibited inhibition of the fungal phytopathogen *S. rolfsii* [20]. PCI2 was routinely grown at 28 °C on King's B medium [16] and 30 % Tryptic Soy Agar medium and preserved at −20 °C in Tryptic Soy Broth (Britania®) amended with 20 % (v v^{-1}) glycerol.

Assay for Detection of Genes Encoding Antibiotics and Hydrogen Cyanide in *P. putida* PCI2

Total DNA was isolated from PCI2 cells by a standard protocol [28]. Then, PCR reactions were performed to detect the genes involved in the biosynthesis of phenazine-1-carboxylic acid and 2,4-diacetylphloroglucinol in strain PCI2 using the primers and the methodology described by Raaijmakers et al. [23]. Also, following the methodology described by Souza and Raaijmakers [31], we carried out PCR reactions with the specific primers to detect the genes encoding for the production of pyrrolnitrin and pyoluteorin. Additionally, the detection of *hcnAB* genes (involved in the biosynthesis of hydrogen cyanide synthetase) was performed by PCR using the primers PM2-F (5′-TGCGGCATGGGCGCATTGCTGCCTGG-3′) and PM2-R (5′-CGCTCTTGATCTGCAATTGCAGGC-3′) [32]. *Pseudomonas* sp. Phz24 (strain that produces phenazine-1-carboxylic acid and pyrrolnitrin) and *P. protegens* CHA0 (strain that produces hydrogen cyanide, 2,4-diacetylphloroglucinol, pyoluteorin, and pyrrolnitrin) were used as positive production controls.

Quantitative Detection of SA and JA in Liquid Culture of *P. putida* PCI2

Samples of 20 ml each of bacterial cultures (10^{10} CFU ml^{-1}) grown in Luria–Bertani (LB) broth were centrifuged after 72 h of growth at 8000 rpm and 4 °C during 15 min. Supernatants were acidified to pH 3.0 with a solution of acetic acid, additioned with 100 ng ($^2H_4$) SA and ($^2H_6$) JA (OlChemIm Ltd, Olomouc, Czech Republic) deuterated internal standards, and kept at 4 °C for 1 h. The samples were partitioned three times with a 20 ml volume of ethyl acetate. Then, the ethyl acetate was evaporated to dryness at 35 °C and the samples were resuspended in methanol before passing them through a column of DEAE-Sephadex A25 for pre-purification. The fractions (eluted SA and JA) containing the deuterated internal standards were evaporated to dryness and resuspended in 100 µl methanol. The vials were introduced into the autosampler of an Alliance 2695 liquid chromatographer (Waters Inc, USA) and 10 µl were injected. Chromatographic conditions were constant, with acetonitrile:water (65:35) and a rate of flow of 0.2 ml min^{-1}. The samples were further analyzed using a Quattro UltimaTM Mass Spectrometer (Micromass, UK). SA and JA identification was carried out by comparing the retention times of the samples with those of the pure standards, and quantification was performed using the MRM (Multiple Reaction Monitoring) function. For quantification, values were obtained from the calibration curves, previously designed using SA and JA pure standards (Sigma, USA).

ACC Deaminase Activity

The capacity to metabolize ACC as the only nitrogen (N) source derives from the enzymatic activity of the ACC deaminase. First, *P. putida* PCI2 was grown overnight in 30 % TSB and then transferred to a flask containing a medium composed of 1 g K_2PO_4H, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.1 g $SO_4Fe{\cdots}7H_2O$, 1 g $CaCO_3$, 0.2 g NaCl, 5 mg $NaMoO_4{\cdots}2H_2O$, 10 g glucose and 0.3 g of ACC (Santa Cruz Biotechnology, Inc.), instead of $(NH_4)_2SO_4$ as N source, per liter. After inoculation, the culture was incubated at 30 °C on a rotary shaker at 120 rpm for 48 h. Growth was considered (+) when the culture became cloudy. The capacity of PCI2 to use ACC was verified by inoculating the strain in a control flask containing the same medium without any N source. The absence of growth in this last medium confirms the utilization of ACC as N source [10].

Pathogenicity of *F. oxysporum* MR193 and Biocontrol Activity in Vivo by *P. putida* PCI2 in Tomato Plants

The fungal strain used in this study was directly isolated from tomato (*Lycopersicon esculentum* Mill.). Samples of diseased tomato roots were collected from a production zone of Río Cuarto, Córdoba, Argentina. Tissue fragments were surface sterilized with 3 % NaOCl for 3 min, transferred to potato dextrose agar (PDA), and incubated at 25 °C for 7 days. The newly isolated *Fusarium* strains were examined for their morphological characteristics on PDA and Czapek agar and identified using the system described by Nelson et al. [18]. The strains were maintained at 4 °C on Spezieller Nährstoffarmer agar (SNA) medium composed of (in grams per liter): KH_2PO_4, 1 g; KNO_3, 1 g; $MgSO_4{\cdot}7H_2O$, 0.5 g; KCl, 0.5 g; Glucose, 0.2 g; Sucrose, 0.2 g.

Pathogenicity of the *F. oxysporum* isolates was confirmed in a climate chamber with photoperiod. Based on the observation of root rot and death of young seedlings, *F. oxysporum* isolate MR193 was selected as the most virulent isolate and used as target pathogen in the biocontrol experiments in vivo. The inoculum was prepared as follows: conidia and mycelia from 10-day-old colonies of *F. oxysporum* MR193 grown on PDA at 28 °C were harvested by adding sterilized distilled water to the colonies and performing a gentle scraping with a spatula. Ten plates were used, and the final volume was adjusted to 400 ml with sterilized distilled water [15]. The inoculum density was 7×10^6 conidia ml^{-1}, based on hemocytometer counts. The biocontrol experiments were carried out in a climate chamber under controlled conditions for 4 weeks. Seeds of tomato cv. Platense Italiano (Asociación Cooperativa INTA La Consulta, Argentina) were disinfected by soaking in 70 % ethanol for 5 min and subsequently in a 2 % sodium hypochlorite solution for 1 min. The seeds were washed 10 times in sterilized distilled water [14]. Then, 1 g of seeds was soaked for 30 min in 250 µl of a 10^9 CFU ml^{-1} aqueous cell suspension of *P. putida* PCI2. The bacterium was grown under shaking (80 rpm) in KB broth for 24 h at 30 °C. Control seeds were treated with sterile KB broth. Plastic pots (15 cm diameter; 25 cm height) were filled with a sterilized mixture of soil:sand:perlite (at 2:1:1 w/w/w). Three seeds were sown into the soil mix in each pot. Pots were kept in a climate chamber under the following conditions: 16 h light at 28 °C and 8 h dark at 18 °C (with a light intensity of 220°E m^{-2} s^{-1}). After complete emergence at 1 week, seedlings were thinned out to one per pot. The four treatments were as follows: (1) non-bacterized, non-infested

healthy control plants, (2) plants non-bacterized, infested with *F. oxysporum* MR193 1–3 weeks after seeding, (3) plants bacterized with PCI2 and infested with *F. oxysporum* MR193 1–3 weeks after seeding, and (4) plants bacterized with PCI2. Each tomato seedling was infested with a 5 ml suspension of *F. oxysporum* MR193, using the adjusted inoculum density as mentioned above. Arrangement of the pots was made in a completely randomized design with 15 replicates per treatment. At harvest, 5-week-old tomato plants were removed from the pots and the degree of biological control was evaluated. Disease incidence was recorded as the percentage of diseased plants per treatment and was based on the observation of typical root rot symptoms (roots with small to developed lesions). The dry weights (mg) of plant shoots and roots were also recorded. The experiment was repeated twice. The experiments were conducted under the same conditions using the same treatments to ensure reproducibility of results.

Data Analyses

Results from shoot and root dry weights measurements were analyzed for significance after log transformation. Since these results were homogenous, they were pooled together and analyzed by using analysis of variance (ANOVA). When ANOVA showed treatment effect, the Least Significant Difference (LSD) test was applied to make comparisons between the means at $P < 0.05$. Data were subjected to statistical analysis using Statgraphics plus software for Windows V 4.1 (Statistical Graphics Corp., Rockville, MD, USA).

Results

Assay for Detection of Genes Encoding Antibiotics and Hydrogen Cyanide in *P. putida* PCI2

We carried out PCR assays to detect the presence of biosynthetic operons encoding hydrogen cyanide, pyoluteorin, pyrrolnitrin, phenazine-1-carboxylic acid, and 2,4-diacetylphloroglucinol in *P. putida* PCI2. We observed no amplification of fragments of the predicted size from the DNA of strain PCI2. On the other hand, we detected those fragments from the DNA of reference strains.

Quantitative Detection of SA and JA in Liquid Culture of *P. putida* PCI2

Production of SA and JA was measured at 72 h. *P. putida* PCI2 grown in LB medium was used for hormone evaluation by liquid chromatography-electrospray tandem mass spectrometry. Under the conditions tested, *P. putida* PCI2 produced 6.95 and 0.091 μg ml^{-1} of SA and JA, respectively (mean values).

ACC Deaminase Activity

Pseudomonas putida PCI2 was tested for ACC deaminase activity based on the method in which the ACC is used as the only source of N. *P. putida* PCI2 grew well in the liquid minimal medium containing ACC as the only source of N, which was compared with the same medium without a source of N in which *P. putida* PCI2 did not grow (Fig. 1).

Pathogenicity of *F. oxysporum* MR193 and Biocontrol Activity in Vivo by *P. putida* PCI2 in Tomato Plants

We investigated *P. putida* PCI2 as potential biocontrol agent against *Fusarium* root rot of tomato plants, under climate chamber conditions. The incidence of the disease was scored 5 weeks after sowing the seeds. The treatment in which seeds were inoculated with *P. putida* PCI2 1 week before adding *F. oxysporum* MR193 to the potting mixture showed significant biological control. The disease incidence decreased from 53.33 % in the pathogen control to 30 % in the inoculation/infestation treatment (Fig. 2).

In addition, inoculation of tomato seeds with *P. putida* PCI2 increased shoot dry weight of plants by 55 mg and root dry weight by 40 mg over the untreated pathogen control. On the other hand, in non-infested potting mixture, inoculation of seeds with PCI2 increased shoot and root dry weight by 66 and 22 mg, as compared to healthy control seedlings (Fig. 3).

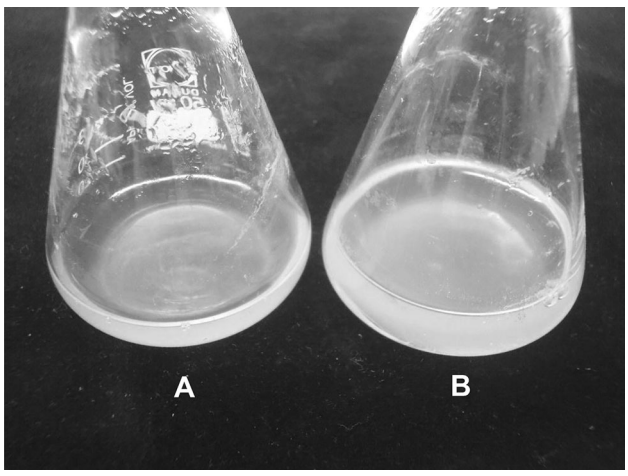

Fig. 1 ACC deaminase activity. **a** PCI2 in control flask containing medium without any N source; **b** PCI2 in medium with ACC as N source

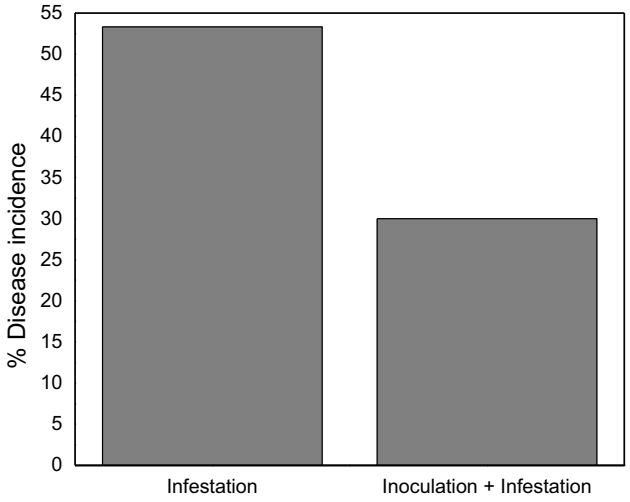

Fig. 2 Disease incidence on 5-week-old tomato roots in the presence of *F. oxysporum* MR193 and *P. putida* PCI2. *Infestation* plants non-bacterized, infested with *F. oxysporum* MR193; *Inoculation + Infestation* plants bacterized with PCI2 and infested with *F. oxysporum* MR193

Discussion

Plants have the ability to acquire a higher level of resistance to pathogens after exposure to biotic stimuli mediated by PGPR. Such capacity has been observed in many species of plants and it would be dependent on the rhizobacteria–plant interaction [34].

The reduction in fungal disease incidence observed in tomato plants pre-inoculated with *P. putida* PCI2 [20] and the non-detection of antifungal metabolites in *P. putida* PCI2 in this work suggest that ISR mechanisms might be involved. ISR has been described for several fluorescent pseudomonads strains in different crop plants and is effective against several plant pathogens [4, 34]. Thus, it seems justified to hypothesize that the SA and JA produced by *P. putida* PCI2, detected by liquid chromatography-electrospray tandem mass spectrometry, would play a role in triggering a systemic resistance.

The role of the SA produced by bacteria is not fully elucidated. SA is a precursor or intermediate in the biosynthesis of certain types of siderophores. Several PGPR have the capacity to produce SA depending on the availability of iron, and it can be detected in the roots of plants. In addition, it is well known that strains of rhizobacteria can induce mechanisms of plant defense ISR [3, 4, 17], and that the exogenous application of SA to plants leads to induced resistance against a wide range of pathogens [1]. The strain WCS374r of *Pseudomonas fluorescens*, producer of salicylic acid, induces resistance in radish but not in *Arabidopsis thaliana*. Conversely, the application of SA leads to induction of resistance in both plant species [26, 34]. It could also be said that jasmonate, salicylate, and their methyl esters are the naturally occurring regulators of higher plants and can induce endogenous levels of secondary metabolites after exogenous application [8].

Ethylene is a gaseous plant growth hormone produced endogenously by almost all plants. It plays a key role in inducing physiological changes in plants at a molecular level. Ethylene has also been established as a stress hormone. Under stress conditions like those generated by salinity, drought, and pathogenicity, the endogenous production of ethylene increases which adversely affects the root growth and consequently the growth of the whole plant. Certain PGPR contain ACC deaminase, which regulates ethylene production by metabolizing ACC (an immediate precursor of ethylene biosynthesis in higher plants) into α-ketobutyrate and ammonia [27]. Plants which grow in association with ACC deaminase-containing PGPR generally have longer roots and shoots and are more resistant to growth inhibition by a variety of ethylene-inducing stresses [13]. For instance, Barnawal et al. [5] demonstrated that protection of *Ocimum sanctum* plants from waterlogging induced damages can be achieved by inoculation of plants with ACC deaminase-containing rhizobacteria and concluded that the ACC deaminase trait of PGPR could therefore be employed as an efficient tool for reducing the waterlogging-induced yield losses by lowering stress ethylene levels, altering biochemical changes with enhanced foliar nutrient uptake and protecting the plants against oxidative stress created by waterlogging conditions. Also, Shakir et al. [29] isolated rhizobacteria from the rhizosphere of wheat plants growing in a semi-arid region and showed that inoculation with the strains selected for their ACC deaminase activity increased root-shoot length, root-shoot mass, and lateral root number of inoculated plants growing under a semi-arid climate. Moreover, Siddikee et al. [30] found that treatment with ACC deaminase-producing halotolerant bacteria reduced ethylene production in salt-stressed (150 mmol NaCl) red pepper plants and caused a significant increase in plant growth. Their results suggest that salt stress enhances ethylene production by increasing enzyme activities of the ethylene biosynthetic pathway and that inoculation plays an important role in ethylene metabolism, particularly by reducing the concentration of ACC. Thus, application of PGPR containing ACC deaminase in agriculture might prove beneficial and could be a sound step toward sustainable crop production and conservation [27]. *P. putida* PCI2 was proven to grow in a culture medium containing ACC as the sole source of nitrogen. Previously, we demonstrated that PCI2 produces IAA [20]. In the present work, we observed that inoculation of seeds with PCI2 increased root dry weight by 22 mg, as compared to control

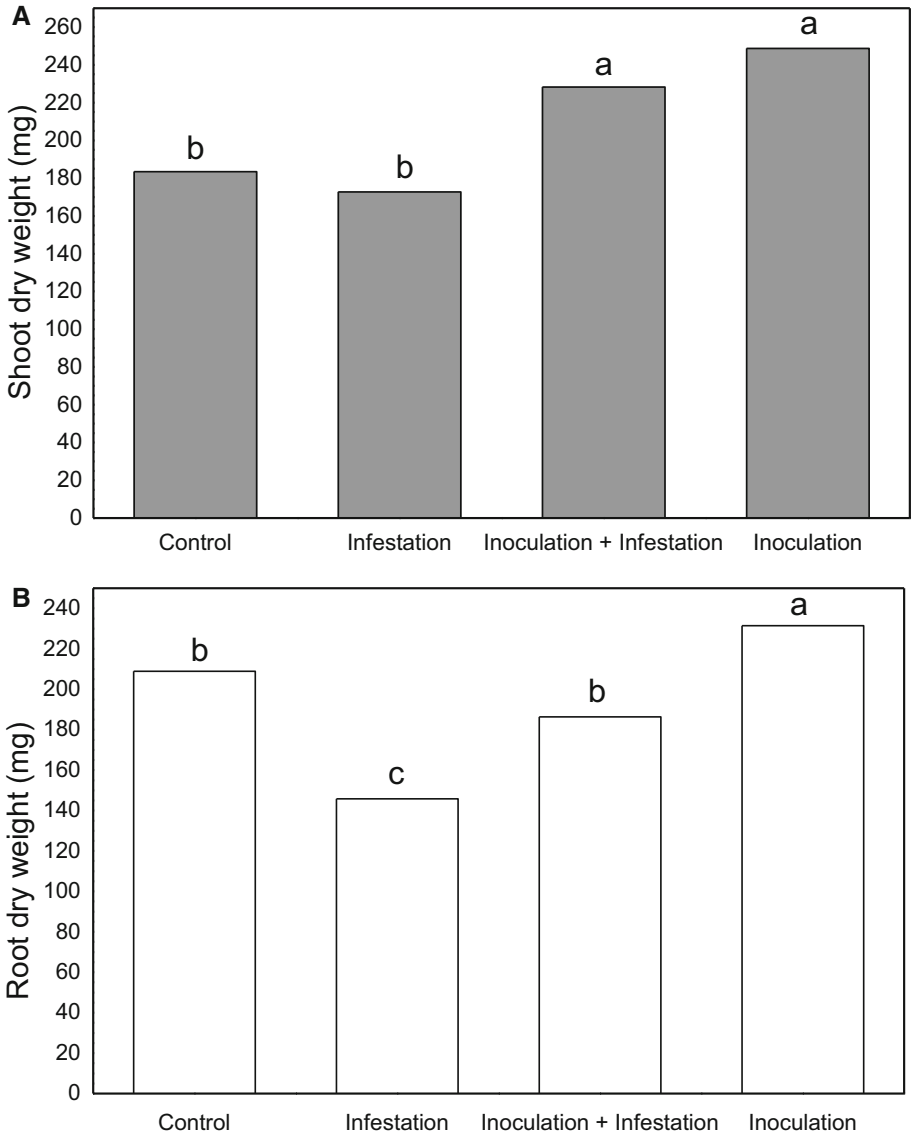

Fig. 3 Effect of *P. putida* PCI2, indicated as dry weight of the plant shoots and roots, against root disease of tomato caused by *F. oxysporum* MR193. *Bars* for each plant fraction with *different letters* are significantly different according to Fisher's least significant difference (LSD) test ($P < 0.05$). *Control* non-bacterized, non-infested plants; *Infestation* plants non-bacterized, infested with *F. oxysporum* MR193; *Inoculation* + *Infestation* plants bacterized with PCI2 and infested with *F. oxysporum* MR193; *Inoculation* plants bacterized with PCI2

seedlings, in non-infested potting mixture. Etesami et al. [12] observed a significant relationship among IAA and ACC deaminase production by PGPR and the rate of root colonization and root length. These authors highlighted that it appears that IAA and ACC deaminase production confer bacteria competitive advantages to colonize plant tissues.

Finally, we explored the possibility of reducing the root rot disease caused by *F. oxysporum* in tomato plants through an alternative that could be efficient, reliable, and safe for the environment. The obtained results suggest that *P. putida* PCI2 could be a potential biological control agent for reducing the impact of *F. oxysporum* on tomato roots. We observed a decrease in disease incidence of 23 % in the treatment in which seeds were inoculated with *P. putida* PCI2 1 week before adding *F. oxysporum* MR193 to the potting mixture. Datnoff et al. [11] evaluated commercial formulations of two beneficial fungi, *Trichoderma harzianum* and *Glomus intraradices*, for the control of root rot of tomato and observed percentages of disease incidences between 48 and 56.6 % in the pathogenicity controls and between 14 and 46.6 % in the inoculated plants. Moreover, these authors evidenced the beneficial effect of inoculation with biocontrol agents prior to exposure to the pathogen.

In summary, the findings from this study together with other already studied attributes of *P. putida* PCI2, related to its antagonistic capacity against other fungal phytopathogens of tomato and its plant growth-promoting activity in the absence of pathogens, suggest that *P. putida* PCI2 is a promising bacterial agent for the formulation of a biofertilizer and/or biopesticide. Here, we show that PCI2 applied to tomato seeds increases the resistance of plants to root rot caused by the fungus *F. oxysporum* MR193 and

that PCI2 produces compounds associated to plant resistance. Certainly, this work suggests that PCI2 represents a non-contaminating management strategy potentially applicable in different agro-ecosystems, particularly in vegetable crops such as tomato.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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