Effect of *Bacillus pumilus* CCIBP-C5 on *Musa*–*Pseudocercospora fijiensis* interaction

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Abstract

The effect of antifungal activity of culture filtrate (CF) of *Bacillus pumilus* strain CCIBP-C5, an isolate from a phyllosphere of banana (*Musa*) leaves, was determined on *Pseudocercospora fijiensis* challenged banana plants. The CF was shown to decrease the fungal biomass and induce changes in banana plant. In this sense, at 70 days post inoculation (dpi), a lower infection index as well as a decrease in fungal biomass after 6 dpi was obtained in treated plants with respect to control ones. At the same time, changes in the activities of several enzymes related to plant defense responses, such as phenylalanine ammonia lyase, chitinases, β -1,3-glucanases and peroxidases were observed. These results indicate that *B. pumilus* CCIBP-C5 has a potential role for biological control of *P. fijiensis* possibly due to the production of antifungal metabolites.

Keywords *Bacillus pumilus* · Black Sigatoka · Biological control · Culture filtrate

Introduction

Black Sigatoka, caused by the fungus *Pseudocercospora fijiensis* Morelet, is considered the most destructive banana disease in Cuba and in several other Latin American countries. This fungus is a foliar pathogen that reduces plant photosynthetic area and causes premature ripening of fruits (Castaño Zapata 2006). The disease is mainly controlled with protectant and systemic fungicides sprayed at relatively high frequencies (Churchill 2011). In this sense, extensive research on fungicide resistance, the reduction of beneficial phylloplane and environmental pollution has been carried out to find sustainable alternatives for the management of the disease (Marcano et al. 2016).

The application of microorganisms has been considered as an alternative to chemical fungicides and is thought to be a viable and environmentally friendly approach for improving plant growth and controlling many plant diseases (Radhakrishnan et al. 2017). However, biological control of foliar diseases such as Sigatokas have been less successful

(Guzmán et al. 2013), mainly adjudged to phyllosphere characteristics, which can be considered as a hostile environment for antagonistic microorganisms. Banana phyllosphere is exposed to strong changes in temperature and humidity and has a low and heterogeneous availability of nutrients such as carbohydrates and proteins (Ceballos et al. 2012). Nevertheless, the colonization of these environments by bacteria, which can act as biological control agents have been documented (González et al. 1996; Fu et al. 2010; Gutierrez-Monsalve et al. 2015).

The classic mechanism of plant disease suppression by bacterial species has an antagonistic effect on phytopathogen growth through antibiotic production (Furuya et al. 2011; Malfanova et al. 2012), nutrient competition (Fischer et al. 2010) and the production of extracellular enzymes (Hariprasad et al. 2011). Evidence supporting in vitro antifungal activity of bacterial strains against *P. fijiensis* is numerous and has been related with the production of different active metabolites (González et al. 1996; Cruz-Martín et al. 2012, 2017).

Besides these direct interactions, some microorganisms are also capable of reducing disease development by stimulating inducible defense mechanisms (van Loon and Glick 2004; Compant et al. 2005). Bacterial-mediated induced systemic resistance (ISR) effectively suppresses several fungal, bacterial and viral pathogens in a number of crops

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both in greenhouse and field trials (Kloepper et al. 2004). On the other hand, ISR may activate inducible defense mechanisms in plants frequently associated with the production of pathogenesis-related (PR) proteins like chitinase (CHT), β -1,3-glucanase (GLU) and other proteins such as ascorbate peroxidase (APX), phenylalanine ammonia-lyase (PAL), peroxidase (POD), polyphenol oxidase and superoxide dismutase (SOD) (Chowdappa et al. 2013).

In a recent report, we demonstrate the ability of an antagonist *Bacillus pumilus* CCIBP-C5, isolated from banana phyllosphere, to inhibit the in vitro growth of *P. fijiensis* mycelia (Cruz-Martín et al. 2016). In fact, the application of its culture filtrate (CF) in banana plants delayed disease progress and induces histochemical changes related to defense response when it was applied three days after *P. fijiensis* inoculation (Mena et al. 2015; Cruz-Martín et al. 2017). These results suggested that CF could act against the pathogen through two mechanisms: antibiosis and resistance induction. The aim of the present study was to evaluate the effect of CCIBP-C5 CF on *Musa*–*P. fijiensis* interaction. The main objectives were to investigate the effect of CF on fungal biomass and its influence over different enzymes at early time points of interaction of banana plants with *P. fijiensis* in a controlled environment.

Materials and methods

Bacterial and fungal strains

B. pumilus strain CCIBP-C5 used in this study was obtained from the Microbiological Culture Collection at Instituto de Biotecnología de las Plantas, Cuba. It was isolated from banana phyllosphere (22°26'36.8"N, 79°53'31.8"W) and identified by determining its biochemical activity with an API 50 CHB kit (BioMérieux, Inc.). For CF preparation, an overnight grown culture of strain CCIBP-C5 was centrifuged (10 min at 4 °C and 12,000×g) and cells were adjusted to an OD₆₀₀ nm of 0.1 in deionized water. One milliliter of bacterial suspension was inoculated in 100 mL nutrient broth (NB) culture medium (Fluka, Germany). The strain was grown for 72 h at 30 °C and 120 rpm in an orbital shaker (Gerhardt), centrifuged at 12,000×g for 10 min at 4 °C and filtered with a 0.2 µm membrane (Millipore).

The plant pathogenic fungi *P. fijiensis* strain CCIBP-Pf-83 was isolated from symptomatic leaves of the susceptible cultivar 'Grande naine' (*Musa* AAA) in plantations of small farmers in Santa Clara, Cuba (22°24'48.7"N, 79°57'18.7"W). Its identity was confirmed by the polymerase chain reaction (PCR) amplification of the internal transcribed spacer on the ribosomal DNA according to Johanson and Jeger (1993). This fungus was grown in potato dextrose broth (PDB) culture medium (BioCen, Cuba) for 15 days at 28 °C

on a rotatory shaker at 120 rpm. The fungal suspension was prepared by mixing the mycelium in Ultra-Turrax T25 homogenizer (Rose Scientific Ltd., Canada) for one minute and the concentration was adjusted using a hemacytometer (5×10^5 fragment/mL).

Effect of culture filtrate on plant–pathogen interaction

The effect of CF on *P. fijiensis* biomass and plant response was measured under controlled conditions. 'Grande naine' plants were propagated by tissue culture according to Orellana (1994) and acclimatized during three months in a greenhouse (70–80% humidity, 26–32 °C) until they reached 20 cm of height and more than three developed leaves. The artificial inoculation of plants and symptoms evolution was carried out following the protocol described by Leiva-Mora et al. (2010) and for every experiment, the number of plants used was defined. The first three leaves were inoculated for the abaxial surface of the leaf with the fungal suspension containing 1% gelatin (Spectrum) using a hairbrush. In the same way, as it was mentioned before, CF and NB were applied to banana leaves but at 72 h after *P. fijiensis* inoculation.

Inoculated leaves (3 per plant) were pooled, ground with liquid nitrogen and considered as one biological replicate. In all experiments, the leaves samples at 0 and 3 dpi were taken 2 h after pathogen inoculation and CF application, respectively. A completely randomized design was set up under greenhouse conditions (> 80% humidity, 28 ± 2 °C) during 70 days.

Determination of fungal biomass

A quantitative PCR (qPCR) analysis was used to quantify *P. fijiensis* biomass. Banana leaves of 12 plants were inoculated with the fungal suspension as described above and 5 mL of CF was applied on the abaxial surface of the leaf 72 h after *P. fijiensis* inoculation. An equal amount of plants inoculated with *P. fijiensis* were used as controls. Three of them per treatment were kept for assessing their development and symptom evolution during 70 days. The infection index (II) was calculated by the formula reported by Carlier et al. (2002).

The fungal biomass in leaves of infected banana plants at 0, 6 and 10 dpi was quantified. Three plants per treatment were used as biological replicates and the experiment was repeated twice. DNA from infected plants was extracted (from 0.25 g of powdered tissue) according to the method proposed by Murray and Thompson (1980). DNA from the pure culture of *P. fijiensis* was obtained by Johanson and Jeger (1993) methodology. DNA concentration and quality

were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

The qPCR assay was performed using primers designed from the β -tubulin gene of *P. fijiensis* as described earlier by Arzanlou et al. (2007), in a StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer's instructions. Sequences of the primer for TagMan detection were (MFBF 5'-CGACACAGCAAGAGCAGCTTC-3' and MFBRtag 5'-TTCGAAAGCCTTGGCACTTCAA-3') with an expected amplicon size of ≤ 142 bp. The reactions were conducted in a final volume of 15 μ L including 2 μ L DNA template (50 ng/ μ L), 7.5 μ L 2 $\times$ TaqMan Master Mix, 1.5 μ L TaqMan and 0.5 μ L of both primers. PCR conditions were: one cycle at 50 °C for 2 min; an initial 92 °C denaturation step for 10 min at followed by 40 cycles for 15 s of denaturation at 92 °C and annealing at 60 °C for 60 s. All reactions were set up in triplicate. External standard for *P. fijiensis* quantification in the samples consisted of five concentrations of pathogen DNA serially diluted (1, 1:10, 1:100, 1:1000, 1:10,000). Negative controls (water) were used on every experiment to test the presence of contamination.

Plant response

The enzymatic activities of PAL, CHT, GLU, POD, APX and SOD were analyzed during early stage of *P. fijiensis* infection, to determine the effect of CF in the plant response. The artificial inoculation of banana plants with the pathogen and CF or NB were carried out as described above. In this experiment, five treatments were included: (1) plants inoculated with fungal suspension and NB culture medium (Mf), (2) plants inoculated with *P. fijiensis* and CF applied 72 h after inoculation with the pathogen (CF-Mf), (3) plants only with CF (applied 72 h after inoculation with *P. fijiensis* in other treatments) (CF), (4) non-treated plants kept in same conditions that were used as controls (Control) and (5) plants only with NB (applied 72 h after inoculation with the fungus in other treatments) (NB). Five plants per treatment and for time point were used and the experiment was repeated twice.

Samples were collected at 0, 3, 6, 10 and 14 dpi in all treatments and were prepared as described above. The powder (0.5 g) was homogenized with 2 mL of 0.1 M phosphate buffer (pH 7.0), centrifuged at 15,000 $\times g$ at 4 °C for 20 min and the supernatant was used for enzymatic activity assays.

PAL (EC 4.3.1.24) activity was measured according to the method of Ross and Sederoff (1992), with some modifications. Fifty microliter of the extract was incubated with 300 μ L of 1 mM L-phenylalanine and 250 μ L of 50 mM Tris-HCl (pH 8.8) buffer at 30 °C for 30 min; the reaction was stopped with 2 N HCl. The amount of trans-cinnamic acid produced was determined by absorbance readings at 290 nm using a spectrophotometer (Rayleigh 1601, China).

A calibration curve was drawn using an arithmetic progression of trans-cinnamic acid concentrations.

CHT (EC 3.2.1.14) activity was measured following the method of Tikhonov et al. (2002), with some modifications. The reaction mixture consisted of 500 μ L of the enzymatic extract, 150 μ L colloidal chitin (Sigma) and 100 μ L of 0.1 M sodium acetate buffer (pH 4.7). After 24-h incubation at 40 °C, the reaction was stopped by centrifugation at 12,000 $\times g$ at 4 °C for 5 min. The supernatants were boiled for 5 min with 250 μ L dinitrosalicylate (DNS) and 100 μ L of 5 M NaOH. The absorbance of the solution at 582 nm was measured and CHT activity expressed as units of the enzyme per μ g of protein.

GLU (EC 3.2.1.39) activity was assayed according to the method described by Pan et al. (1991), with some modifications. The enzymatic extract (62.5 μ L) was incubated at 40 °C for 10 min with 62.5 μ L of 4% laminarin (Sigma). The mixture was added to 375 μ L DNS and boiled for 5 min. After cooling, the absorbance of the solution was measured at 500 nm. GLU activity was expressed as glucose units/min per μ g of protein.

POD (EC 1.11.1.7) activity was determined according to the method of Cakmak et al. (1993), with some modifications. Five hundred microliters of supernatant were incubated with 1.5 mL of 0.05% guaiacol and 500 μ L of 1% H₂O₂ and the absorbance was measured during 3 min at 470 nm. One unit of POD activity caused a change of 0.01 absorbance units at 470 nm per min.

APX (EC 1.11.1.11) activity was determined as described by Nakano and Asada (1987), with some modifications. The reaction mixture included 1 mL 0.1 M phosphate buffer (pH 7.5), 5 μ L of 30% H₂O₂, 40 μ L of 10 mM ascorbic acid and 10 μ L of the enzymatic extract. Absorbance of the solution was measured at 290 nm and the activity was expressed as enzyme unit per μ g of protein.

SOD (EC 1.15.1.1) activity was assayed following the method of Ehsani-Moghaddam et al. (2006), with minor modifications. The reaction mixture consisted of 790 μ L buffer solution containing 86 mg/L nitro-blue tetrazolium (NBT), 234 g/L Tris-HCl, 11.4 g/L of ethylenediaminetetraacetic acid (EDTA) (pH 7.5), 10 μ L of riboflavin (44 mg/L) and 400 μ L of enzymatic extract. Riboflavin was added last, the tubes were stirred and the reaction was initiated by placing them under two 15 W fluorescent lamps. The reaction finished after 30 min by switching off the light. Non-illuminated samples served as blank. Absorbance at 560 nm was measured and the volume of enzyme extract corresponding to 50% inhibition of the reaction was considered as one enzyme unit.

The protein content of the extract was determined according to the method of Bradford (1976) using bovine serum albumin as standard. Each enzymatic extract was assayed three times to calculate an average value for the sample.

Statistical analysis

The non-parametric Kruskal–Wallis test was used for data analysis. Treatment means were separated by Mann–Whitney test using SPSS Statistics v. 21.0 for Windows. The significance level for biomass data used was $p < 0.01$ while for enzymes activities and infection index $p < 0.05$ was considered.

Results

The culture filtrate of *B. pumilus* CCIBP- C5 had effect in *Musa*–*P. fijiensis* interaction. In ‘Grande naine’ plants inoculated with *P. fijiensis*, the first symptoms of the disease were observed at 14 dpi for all treatments and the disease progress was developed as previously described by Alvarado-Capó et al. (2003). The inoculated plants (control) completed the disease cycle at 42 dpi. However, the infection on plants treated with CF was low. At 70 dpi the infection index in the CF treatment was significantly lower ($p < 0.05$) than in control with values of 52 and 78%, respectively.

Reduction of fungal biomass by bacterial culture filtrate

The qPCR analysis (Fig. 1) revealed that at 6 dpi the fungal DNA present in the plants treated with CF was 90% lower than in the control plants. Furthermore, a slow growth of the pathogen was observed in the presence of CF. Between 6 and 10 dpi, *P. fijiensis* increased sixfold its biomass in control treatment compared to the CF-treated plants.

Fig. 1 Relative quantification of *P. fijiensis* DNA on artificially inoculated leaves of ‘Grande naine’ (*Musa* AAA). Leaves applied with CCIBP-C5 culture filtrate (CF) 3 days after pathogen inoculation were compared to non-treated control inoculated plants. Parameters of qPCR assay for β -tubulin: $R^2 = 0.997$, $E = 100.86\%$. Columns represent means of six plants ($n = 6$) and the vertical bars indicate standard deviation of biological replicate. Different letters on each time indicate significant differences by Mann–Whitney $p < 0.01$

Effect of culture filtrate on plant defense response

No significant differences ($p < 0.05$) in the activity of the different enzymes evaluated between the NB and control treatments were found, which suggested that the components of the culture medium do not have effect on *Musa* plants.

On the other hand, *P. fijiensis* presence induced changes in plant response. In fact, immediately after inoculation (0 dpi), an increase in the enzymatic activities of PAL, GLU, CHT, POD and SOD was observed in banana plants (Figs. 2, 3). In a similar way, a significant increase ($p < 0.05$) in the activity of these enzymes was observed in inoculated plants where the CF was applied.

At 3 dpi, in CF-Mf treatment, PAL activity was three times higher than in control plants and twice in Mf treatment. Likewise, in CF treatment, PAL values were higher than control and similar to those observed in Mf treatment (Fig. 2a). Besides, at this time point, the values of enzymatic activities for GLU and CHT in inoculated plants where CF was applied were higher than those where it was not, showing a tendency to decrease when compared to initial values (Fig. 2b, c).

By other side, the plant antioxidant response was also influenced by the application of culture filtrates and the presence of the pathogen (Fig. 3). Peroxidase, ascorbate peroxidase and superoxide dismutase activities showed a differential behavior during the time course of the experiment. However, for the three enzymes, the highest activity was obtained immediately after CF application in CF-Mf treatment. *P. fijiensis* also caused a considerable increase in POD and SOD activities after inoculation.

Regarding to APX, an increase of its activity immediately after CF application took place and it was independent of the pathogen presence (Fig. 3b). Later, a faster decrease after 6 dpi was observed for all treatments.

Fig. 2 Effect of application of *Bacillus pumilus* CCIBP-C5 culture filtrate in 'Grande naine' (*Musa AAA*) plants under greenhouse conditions on enzymatic activity of **a** Phenylalanine ammonia-lyase, **b** β -1,3-glucanase and **c** Chitinase. Crude enzyme solutions were prepared from banana leaves and evaluated for enzyme activities. Different letters on each time indicate significant differences by Kruskal-Wal-

lis/Mann-Whitney $p < 0.05$. Control (non-treated plant), CF (plants with culture filtrate of CCIBP-C5), Mf (*P. fijiensis* inoculated plants), CF-Mf (*P. fijiensis* inoculated plants and CF applied 3 days after inoculation). Columns represent means of 10 plants and vertical bars indicate standard deviation

Discussion

Fungal diseases have a great economic significance for many crop plants. At present, their control relies mainly on the application of agrochemicals. However, this practice is becoming less acceptable by the increase of pathogen resistance to systemic fungicides upon their prolonged apply and also raises public concern on the effects of toxic residues on human health and the environment. Safety and more environmentally friendly methods, including the use of natural substances, could have a beneficial effect for disease management.

Numerous *Bacillus* species, considered as harmless to human health, have been isolated and their potential for plant disease control explored (Stein 2005; Perez-Garcia et al. 2011). Besides, antimicrobial secondary metabolites produced by strains of this genus have been studied as biological control against dissimilar phytopathogenic microorganisms (Cawoy et al. 2011; Beric et al. 2012). However, further knowledge of their biocontrol mechanisms is required (Radhakrishnan et al. 2017).

Interestingly, whereas the antifungal properties of bacterial metabolites against *P. fijiensis* have been assessed in numerous in vitro and field studies (González et al. 1996; Ceballos et al. 2012; Gutierrez-Monsalve et al. 2015), the plant response to bacteria/metabolite application has been poorly documented. Here, the effects of CF of *B. pumilus* CCIBP-C5 strain in *Musa*-*P. fijiensis* pathosystem were demonstrated.

The fungal biomass reduction during the first days after inoculation corroborated the direct antifungal effect of CF on *P. fijiensis* mycelium, previously reported by Cruz-Martín et al. (2017). Also, in this experiment, the increase in fungal biomass from 6 to 10 dpi was sixfold lower in the presence of CF with respect to control plants, suggesting a slow development of the pathogen. Up to 70 dpi, a significant reduction in the infection was observed. Similar response has been associated with the induction of plant's defense response by different bacterial metabolites (Yi et al. 2013; Chowdhury et al. 2015). That is why, we can assume the existence of other mechanisms involved in biocontrol such as CF elicitation of plant defense responses. Van Loon (2008)

Fig. 3 Effect of application of *Bacillus pumilus* CCIBP-C5 culture filtrate in 'Grande naine' (*Musa AAA*) plants under greenhouse conditions in the antioxidant plant response **a** peroxidase, **b** ascorbate peroxidase, and **c** superoxide dismutase. Crude enzyme solutions were prepared from banana leaves and evaluated for enzyme activities. Different letters on each time indicate significant differences by Kruskal–

Wallis/Mann–Whitney $p < 0.05$. Control (non-treated plant), CF (plants with culture filtrate of CCIBP-C5), Mf (*P. fijiensis* inoculated plants), CF-Mf (*P. fijiensis* inoculated plants and CF applied 3 days after inoculation). Columns represent means of ten plants and vertical bars indicate standard deviation

has suggested the induction of defense response in plants by non-pathogenic microorganisms or their metabolites as an interesting biological control method. Induced resistance can be local or systemic and are at least two forms of induced, known as systemic acquired resistance (SAR) and ISR. They have been characterized as distinct phenomena based on the types of inducing agents and host signaling pathways that result in resistance expression (Hammerschmidt 2007). However, to determine the type of response observed in this study, additional studies are required.

In this sense, *B. pumilus* strains have been referred to as inducers of plants defense response against several phytopathogenic microorganisms (Kloepper and Ryu 2006; Yi et al. 2013). This specie has also been studied for its antimicrobial activity against several plant pathogens (Pereira de Melo et al. 2009; Yazici et al. 2011). Particularly, lipopeptides have emerged as key players in the induction of plant immunity driven by *Bacillus* (Ongena and Jacques 2008; Falardeau et al. 2013; Rahman et al. 2015).

In fact, in this study, it was found that the CF of *B. pumilus* CCIBP-C5 strain exerts influence on plants artificially inoculated with *P. fijiensis* during the first days after inoculation, even without the presence of the pathogen. The increase of the activity of enzymes such as PAL, CHT, GLU

and POD immediately after CF application (3dpi), suggests a CF-mediated induction of resistance. This response in the presence of strains of *Bacillus* or its metabolites has been reported by some researchers (Yang et al. 2015; Narendrababu et al. 2015). Several authors associate this response with the reduction of the adverse effects of the pathogen infection (Jain et al. 2015; Kang et al. 2015; Yi et al. 2013; Hinarejos et al. 2016). These findings are consistent with the reduction of fungal biomass in artificially inoculated plants in the presence of bacterial CF and with the previous report made by Cruz-Martín et al. (2017) about the reduction of necrotic lesions and the delay of the disease progress after CCIBP-C5 CF application.

'Grande naine' plant response against *P. fijiensis* inoculation was similar to that described by Torres et al. (2012) for the susceptible cultivar 'Williams' (*Musa AAA*). These results demonstrate that there is a plant reaction against the pathogen attack although its magnitude or development over time is insufficient to stop the progress of the fungus. By other side, the response of plants with the application of CF was similar to that observed by these authors in plants of the resistant cultivar 'Calcutta 4' (*Musa AA*) after *P. fijiensis* inoculation; it has been linked to the incompatibility response.

In plants, within the complex network of biochemical processes taking place during pathogen attack, the activation of the phenylpropanoid pathway provides the most important functional defense compounds. Among them, coumarins, lignans, flavonoids and phytoalexins have a variety of structural functions in the cell membrane, the formation of pigments in fruits and flowers, and the development of direct activity against fungal and bacterial infection. That is why, they have been involved in the defense mechanisms of plants (Sgarbi et al. 2003). Therefore, it is possible that the early activation of PAL enzyme by the action of CF promotes the synthesis of secondary metabolites in plant related to the reinforcement of the membrane and/or direct antifungal activity against *P. fijiensis*, thus contributing to decrease the disease progression.

Chitinase and endo- β -1,3-glucanases degrade polysaccharides in the cell wall of the invading pathogen, and in most cases they can be categorized as PR proteins, since their expression is often induced by infection (Rose et al. 2002). According to van Loon et al. (2006), the activity of both enzymes produces a synergistic effect that increases resistance of plants against pathogens. These enzymes have been studied extensively (Van Loon and Van Strien 1999; Ferreira et al. 2007) and some indirect evidence support the hypothesis that they have a protective role (Castro and Bach 2004; Yamamoto et al. 2014). Based on previous information, present results suggest that certain CF compounds are immediately recognized by the plant, triggering changes that involve increased activity of enzymes such as PAL, GLU and CHT with direct effect in the growth and development of *P. fijiensis*.

The peroxidase activity during *Musa*–*P. fijiensis* interaction has been analyzed by other authors (Sánchez-García et al. 2010; Torres et al. 2012). In these sense, an increase of POD activity in inoculated plants with respect to non-inoculated plants has been observed. Peroxidases are a group of oxide-reducing class III enzymes present in the cell wall and vacuoles. These enzymes are involved in the defense response of the plant in processes such as formation of the cell wall through phenols oxidation, suberisation, lignification and metabolism of reactive oxygen species (ROS) (Almagro et al. 2008). According to Caverzan et al. (2012), peroxidases are frequently used as indicators of oxidative stress and are widely distributed in both intra- and extra-cellular environments. The enhanced POD and SOD activities might increase oxidative stress due to increased production of H_2O_2 (Vance et al. 1980; Ślesak et al. 2007). This compound and other free radicals are toxic to diverse microbial pathogens. The presence of ROS by the increase of the activity of these enzymes in CF-treated plants may be one of the factors responsible for the decrease of the *P. fijiensis* biomass observed in this treatment. Also, ROS under stress conditions in the cell

can inactivate enzymes and cause damage on important cellular components (Sharma et al. 2012).

By other side, Shigeoka et al. (2002) reported that APX is the main antioxidant system present in various cellular organelles and takes part in ascorbate–glutathione cycle or Assada–Halliwell–Foyer route. The molecular and enzymatic properties of APX enzymes are different of other peroxidases such as POD. APX have high specificity for ascorbic acid (AsA) as the electron donor and it exerts a very strict control and regulation of ROS production. Considering previous results, the increasing activity of these enzymes suggest an immediate perception of the CF by the plant and the generation of oxidative stress. That is why, the immediate increase of POD activity after inoculation and the application of CF suggests the stress detection by the plant and consequent triggering of defense responses such as strengthening of the cell walls. The increased activity of these enzymes with antioxidant capacity in plants under stress has been referred to as a parameter of stress tolerance (Jiménez et al. 2012).

However, one of the most characteristic properties of APX is its instability in the absence of AsA and under lower levels than 20 μ M, the enzyme activity is rapidly lost (Shigeoka et al. (2002). This instability was not found for a long time in photosynthetic organisms and it seems to be one reason that the activity was not detected after 6 dpi for all treatments.

Furthermore, this result extends the range of plant species in which *Bacillus* spp. and its metabolites act by inducing a defense response previously observed in beans (*Phaseolus vulgaris* L.) (Ongena et al. 2005), tomato (*Solanum lycopersicum* L.) (Ongena et al. 2007), tobacco (*Nicotiana tabacum* L.) (Jourdan et al. 2009), watermelon (*Cucumis melo* L.) (García-Gutiérrez et al. 2013) and beet (*Beta vulgaris* L.) (Desoignies et al. 2013).

These results increase our understanding of the mechanisms used by bacillus strains in plant–pathogen interaction. We can conclude that *B. pumilus* CCIBP-C5 CF has suppressive effects on the fungal disease at greenhouse conditions, which might be attributed to the mechanisms: production of metabolites with antifungal effect and the induction of plant defense resistance in banana leaves. Therefore, *B. pumilus* CCIBP-C5 is a potential biocontrol agent of *P. fijiensis*. However, field trials and a suitable formulation of a bio-product are required to validate its use as a black Sigatoka biocontrol agent.

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Compliance with ethical standards

Conflict of interest The authors declare that they do not have conflict of opposing view of concern.

Ethical standard This article does not contain any researches with human or animals have acted of any of the authors.

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