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ORIGINAL PAPER

Assessment of bacterial inoculant formulated with *Paraburkholderia tropica* to enhance wheat productivity

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Abstract

Paraburkholderia tropica is an endophytic nitrogen-fixing bacterium isolated from the rhizosphere, rhizoplane, and internal tissues of sugarcane and corn plants in different geographical regions. Other plant-growth-promoting abilities, such as phosphate solubilization and antifungal activity, have also been reported for this bacterium. With an aim at investigating the potential use of *P. tropica* as an inoculant for improving the performance of wheat crop, in this work we evaluated an experimental inoculant formulated with *P. tropica* MTo-293 with respect to root colonization, the practical aspects of its application, and the effects under field conditions when applied to wheat seeds. Bacterial colonization was monitored by culture dependent techniques and the wheat yield determined by quantifying the total grain production in two different seasons. Rhizoplane and endophytic colonization in wheat roots was achieved efficiently (on average, 8 and 4 log colony-forming units/g fresh weight, respectively) even at relatively low concentrations of viable bacteria in the inoculum under controlled conditions. *P. tropica* was compatible with a widely used fungicide, maintained viability for 48 h once applied to seeds, and was also able to colonize wheat roots efficiently. Furthermore, we were able to formulate an inoculant that maintained bacterial viability for relatively long time periods. Preliminary field assays were realized, and even though the average yields values for the inoculated treatments remained above the uninoculated ones, no significant effects of inoculation were detected with or without fertilization. The correct physiologic behavior of *P. tropica* suggests the necessity to continue with field experiments under different conditions.

Keyword Endophytes · *Paraburkholderia* · Plant growth promotion · Seed treatment · Wheat

Introduction

Since decades ago, many studies have been focussed on beneficial plant–microbe interactions in order to improve the productivity of agricultural systems (Vessey 2003). Therefore, the use of microbial inoculants has emerged as a biotechnological alternative aligned with the principles

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of sustainable agriculture (Bashan 1998). Bacterial inoculants are usually called plant-growth-promoting bacteria, or PGPB. In general terms, PGPB are a heterogeneous group able to confer beneficial effects on the growth of plants through direct or indirect actions (Glick 2012) such as: biologic-nitrogen fixation; the solubilization of key nutrients, especially P, and the enhancement of their availability to the plants; antibiosis and other mechanisms indicating the use of PGPB as biofertilizers and phytostimulation and/or biocontrol in order to reduce the use of chemical fertilizers and pesticides that produce a negative impact on the environment (Adaime et al. 2014). PGPB confer beneficial effects on plant physiology including: an increase in performance, a reduction of pathogen infection, and a decrease in biotic and/or abiotic plant stress (Glick 2012). Most of the PGPB have the ability to colonize the rhizosphere and/or the rhizoplane (Compant et al. 2005a), and in addition, some are able to enter the roots and establish themselves as endophytes, such as species belonging to the genera *Gluconacetobacter, Azoarcus*, and *Burkholderia* (Brader et al. 2017; Compant et al. 2010; Hardoim et al. 2015). The *Burkholderia* genus contains species with a wide range of hosts suggesting a great metabolic and physiologic adaptability (Coenye and Vandamme 2003; Estrada-de los Santos et al. 2013). Phylogenetic clustering has recently validated the division of the genus *Burkholderia* into two genera: a genus retaining this name and including animal and plant pathogens as members, and the genus *Paraburkholderia* containing so-called environmental bacteria with promising candidates for biotechnologic applications (Oren and Garrity 2015).

Paraburkholderia tropica was isolated from the rhizosphere, rhizoplane, and internal tissues of the stem and root of corn plants and from the tissues of sugarcane in different geographical regions (Reis et al. 2004). *P. tropica* possesses different abilities to promote in vitro-plant growth, such as a biologic-nitrogen-fixation capability, an organic-acid production facilitating phosphate solubilization, and a production of antifungal substances (Bernabeu et al. 2016; Bolívar-Anillo et al. 2016; Caballero-Mellado et al. 2007; Tenorio-Salgado et al. 2013). Bernabeu et al. (2015) have reported that *P. tropica* MTo-293 is able to increase fruit yield in tomato plants by about 15% as compared to uninoculated controls under greenhouse conditions, but the in vivo plant-growth-promoting mechanisms were not discussed. Despite the attention given to this diazotrophic species in recent years, only little information is still available concerning in vivo assays with *P. tropica* as an inoculant. In order to investigate the potential use of *P. tropica* as inoculant in cereal crops, the ability to colonize those plants (Compant et al. 2010) and a characterization of the beneficial effects of its action need first to be determined. Moreover, certain essential practical aspects of the use of *P. tropica* must also be studied, namely: the inoculum concentration and the ability to survive on the surface of the inoculated seeds, the possibility of achieving a prolonged bacterial viability in the liquid formulations usually used to apply PGPB, and the tolerance to the agrochemicals commonly used by farmers, among others.

The aim of the present work was thus to evaluate an experimental inoculant formulated with *P. tropica* MTo-293 with respect to the colonization pattern, the practical aspects mentioned above, and the effects of the experimental inoculant under field conditions after application to wheat seeds.

Materials and methods

Organisms, maintenance, and culture conditions

The bacterial strains used in this study were *P. tropica* MTo-293 (ATCCBAA 569; Reis et al. 2004), kindly provided by Dr. Jesús Caballero-Mellado (Centro de Ciencias Genómicas, Cuernavaca, Morelos, México) and its tetracycline (Tc) resistant (Tc^r) derivative strain *P. tropica* MTo293/pFAJGFP (Bernabeu et al. 2015). Microorganisms were maintained at 4 °C in Luria–Bertani medium (Sambrook et al. 1989) for monthly subcultures and in LGI medium (Stephan et al. 1991) with 20% (v/v) glycerol at − 80 °C. The *P. tropica*-derivative strain was grown on solid medium containing 15 µg ml^{-1} of Tc. Both the wild-type and derivative strains can otherwise be considered as phenotypically equivalent (Bernabeu et al. 2015), but in this work the wild type was employed in the field experiments. This strain was grown in flasks containing LGI liquid medium with different sources of carbon (10 g l^{-1} glycerol or glucose) and nitrogen (2.64 g l^{-1} $(NH_4)_2SO_4$ or 1.00 g l^{-1} yeast extract) at an initial pH of 6.00 to select a growth condition that provided final concentrations of approximately 1×10^9 colony-forming units (CFU) ml^{-1} and producing the least change in the pH of the medium. The culture media were inoculated with an appropriate volume of centrifuged, washed, and resuspended bacteria to an initial optical density at 600 nm of 0.05 and then incubated on a rotatory shaker at 200 rpm and 30 °C. Samples were taken every 2 h for 48 h and growth parameters such as the specific growth rate determined (Zwietering et al. 1990). Three biological replicates were performed.

Wheat seeds (*Triticum aestivum)* cv. Baguette 9 (Nidera) were used in all the experiments.

Growth-chamber experiments

Two different experimental protocols were performed with *P. tropica*-inoculated wheat seeds. Disinfected seeds were used to evaluate the efficiency of root colonization by the bacteria under gnotobiotic conditions, whereas nondisinfected seeds were employed to study the practical aspects

of the inoculant under the conditions normally used in field experiments.

Colonization efficiency

Wheat seeds were disinfected with 10% (v/v) sodium hypochlorite, washed three times with phosphate-buffered saline (PBS) (8 g l^{-1} NaCl; 0.2 g l^{-1} KCl; 1.44 g l^{-1} Na_2HPO_4; 0.24 g l^{-1} KH_2PO_4), and then immersed in an inoculum containing the Tc-resistant strain for 20 min. The inoculated seeds were placed on semisolid Fähraeus medium containing 0.5% (w/v) agar at pH 6.00 in a controlled-growth chamber under a 16/8-h light/dark cycle at 28 °C. Sterile distilled water instead of bacterial inoculum was used as a negative control. Rhizoplane and endophyte populations of *P. tropica* were quantitatively evaluated at days 4, 7, 11, 15, and 17 postinoculation (PI) by cultivation on LGI solid medium containing Tc as previously described (Luna et al. 2010). At each time point, the mean CFU g^{-1} were plotted for each of three replicate root samples. Three biological replicates were performed at 24 plants per treatment.

In order to confirm the identity of the bacteria isolated from the root tissues DNA preparations were analyzed by the polymerase-chain reaction (PCR) through the use of the following specific primers for the amplification of a region of the 16S rRNA gene of *P. tropica* according to Wong-Villarreal and Caballero-Mellado (2010): Btrop-F: 5′-TAA TACATCGGAACGTGTCCT-3′ Btrop-R: 5′-GGCCATATT AGGACCAAGGAT-3′. The protocol to obtain the total DNA of the bacterial isolates and that of pure cultures of *P. tropica* (the positive control) was performed according to previously established techniques (Sambrook et al. 1989). After the PCR amplification, the samples were analyzed in 1.5% (w/v) agarose gels containing 0.5 µg ml^{-1} ethidium bromide. Electrophoresis was run for 30 min at 100 V. The identity of the isolated bacteria was confirmed as *P. tropica* if a 356-bp band was obtained upon observation of the gel under ultraviolet illumination.

Inoculum concentration

Serial dilutions of *P. tropica* MTo293/pFAJGFP with sterile distilled water were used to inoculate wheat seeds for the evaluation of the inoculum concentration that was necessary to reach an efficient colonization. The dilutions investigated were: (1) culture without dilution; then dilutions of (2) 1×10^{-2}, (3) 1×10^{-4}, and (4) 1×10^{-6}. The exact concentrations were determined by plate counting in LGI medium. Nondisinfected seeds (250 g) were placed in polyethylene bags (a bag for each bacterial dilution) and inoculated with each dilution in a proportional volume to reach a final total dose of 600 ml of inoculum per 100 kg of seeds. Water was added as described below. The bags were stirred until all the seeds were impregnated. In order to determine the efficiency of bacterial colonization at the different inoculum concentrations, the inoculated seeds were placed on semisolid Fähraeus medium (Fähraeus 1957) and the rhizoplane and endophytic colonization quantitatively evaluated at days 4, 8 and 15 PI by serial plating on selective medium as described above. Three biological replicates were performed.

Bacterial survival on the seeds

Eight treatments were performed in an aim at evaluating *P. tropica* MTo293/pFAJGFP viability on seeds after inoculation in the presence of fungicide and protectant in the proportions commonly used in field trials with other PGPB (Naiman et al. 2009). The inoculum volume was calculated to provide initially approximately 10^6 CFU $seed^{-1}$. The proportions and additional components inoculated were: the bacterial inoculum alone (I) at 600 ml (100 kg)$^{-1}$ of seed, the fungicides (F) at 18.8% (w/v) carbendazim + 18.8% (w/v) thiram in water at 250 ml (100 kg)$^{-1}$ of seed, the protectant (P) 80% (w/v) sucrose plus glucose syrup (1:1, v/v) at 140 ml (100 kg)$^{-1}$ of seed, and sterile distilled water (W) at up to 2.0 l. The different treatments were: Treatment 1, I; Treatment 2, I+F; Treatment 3, I+P; Treatment 4, I+F+P; Treatment 5, W; Treatment 6, W+F; Treatment 7, W+P; Treatment 8, W+F+P.

Nondisinfected seeds (250 g) were placed in polyethylene bags (a bag for each treatment). The bacterial inoculum, water, and protectant were mixed before being applied to the seeds. The bags were stirred until all the seeds were impregnated. In Treatments 2, 4, 6, and 8, the fungicide was added first and left a few min to aerate before applying the other additives. Once all the components were added, some of the seeds were used immediately to determine the initial bacterial number present, while the rest were left to aerate before transfer to the paper bags where they were kept until the analysis.

The bacteria on the seeds were determined at different times PI (0, 4, 24, and 48 h). At each time, 10 g of seeds (approximately 250 seeds) were taken from each bag and placed in flasks containing 100 ml of saline solution (0.85% (w/v) NaCl) plus 0.1% (v/v) Tween 80 that were then shaken for 30 min at 250 rpm to remove the bacteria adhering to seeds. The appropriate dilutions of bacterial suspension were plated on LGI solid medium with Tc to evaluate the number of viable cells as described above. Likewise, seeds were taken from each bag at the same time PI and placed on semisolid Fähraeus medium to analyze the efficiency of colonization of surviving bacteria on the seeds as outlined above. Three biological replicates were performed.

Bacterial survival in aqueous formulations

Paraburkholderia tropica MTo293/pFAJGFP was grown in modified LGI medium (with glycerol and yeast extract according to the previous results) and grown at 30 °C and 150 rpm for 48 h. The final culture was packaged in sterile polyethylene bags (approximately 150 ml per bag) for preservation under different conditions as follows: (1) WA: *P. tropica* without additives; (2) PVA: *P. tropica* + polyvinyl alcohol (Sekisui Speciality Chemical, Japan) at 10 g l^{-1}; (3) XG: *P. tropica* + xanthan gum (Sidley Chemical Company, China) at 3 g l^{-1}; (4) Gel: *P. tropica* + gelatine (Parafarm, Argentina) at 10 g l^{-1}.

Samples of the aqueous formulations were taken at different times (0, 3, 5, 8, 10, and 12 months) to determine the number of viable cells (CFU ml^{-1}) by plate counting. In addition, at each time analyzed, disinfected seeds were inoculated by immersion in each suspension to evaluate the efficiency of root colonization at 7 days PI as described above. Two biological replicates were performed.

Field experiments

Experimental design

Field experiments were performed at the Estación Experimental Agropecuaria Integrada Barrow (INTA), Ruta 3, Km 488, Tres Arroyos, Province of Buenos Aires, Argentina (38° 19′36″S; 60°14′55″W). The experimental design was a completely randomized block with three replicates per treatment and eight treatments. 1-m^2 sampling units were randomly chosen within a uniform 100 m^2 area within each treatment plot. Before sowing, the seeds were treated with fungicide and with the admixture of *P. tropica* MTo-293 and protectant as described above (Treatment 4). The seeds were sown in July of 2012 and 2015 and the plants harvested in December of those respective years. The soil type at the experimental site was a clay-loam texture. In 2012, the nutrient composition of the soil at the sowing was: 10.2 ppm of available P (0–20 cm Bray-1 method), 4.6% organic matter (Walkley and Black 1934), pH 6.30, and 38 kg ha^{-1} of available N. The average rainfall was 655, 221 mm higher than the historical average (434 mm). In 2015, the nutrient composition of the soil was: 13.5 ppm of available P, 4.5% organic matter, pH 6.20, and 22 kg ha^{-1} of available N. The average rainfall was 793, 358 mm higher than the historical average. Field experiments with different N and P fertilization levels were carried out as follows: Treatment 1, uninoculated control without fertilization (–I –N –P); Treatment 2, control with N fertilization (130 kg urea ha^{-1}; –I +N –P); Treatment 3, control with P fertilization (90 kg diammonium phosphate ha^{-1}; –I –N +P); Treatment 4, control with N and P fertilization (–I +N +P); Treatment 5, inoculated with *P. tropica* without fertilization (+I –N –P); Treatment 6, *P. tropica* with N fertilization (+I +N –P); Treatment 7, *P. tropica* with P fertilization (+I –N +P); Treatment 8, *P. tropica* with N and P fertilization (+I +N +P). The P and N fertilizations were performed at sowing time and at tilling, respectively.

Crop-yield evaluation

The crop yield was defined as the grain yield (in kg ha^{-1}) at the time of harvest. The grain yield was expressed as the average of the total measurements per replicate for each season.

Statistical data analysis

Data were processed using Infostat software (Di Rienzo et al. 2018) for performing the analysis of variance (ANOVA) as described below. One-way ANOVA with four inoculation levels was applied to analyzed the data of *Inoculum concentration* experiment; factorial ANOVA with two factors (time and additional components) and four levels each was applied to analyzed the data of *Bacterial survival on seeds* experiment; factorial ANOVA with two factors (time and additives), six and four levels respectively, for analyzing *Bacterial survival in aqueous formulates;* and factorial ANOVA with three factors (inoculation, N fertilization, and P fertilization) and two levels each was applied to analyzed the *Field trials* data. Comparisons of means were conducted using LSD test and p values ≤ 0.05 were considered statistically significant. Statistically analyzed experimental data were included in supplementary material.

Results

Bacterial cultures and inocula

Paraburkholderia tropica grew on all the carbon and nitrogen sources tested at a specific growth rate (μ) of 0.42 ± 0.04 h^{-1}, reaching a final bacterial concentration of around 1×10^9 CFU ml^{-1} in 48 h. The culture pH decreased down to a value of 3 in the first 15 h in all cultures except those with glycerol and yeast extract. Under that condition, the pH increased to 7 during this time and thereafter remained constant at that value until the end of the experiment. On the basis of these results, LGI with glycerol and yeast extract was the medium selected for growing the bacterial inocula in all the experiments.

Root colonization under gnotobiotic conditions

Uninoculated controls did not manifest any growth of microorganisms when macerated wheat roots where plated on LGI medium. No plant-growth inhibition occurred or macroscopically visible disease symptoms appeared in the inoculated plants. Moreover, no bacterial growth was observed in LGI solid medium containing Tc overlaid with surface-disinfected roots, and bacterial growth was rarely observed when the wash solution from that last tissue rinse was placed on LGI, suggesting that the method of disinfection was sufficient to eliminate the surface-associated bacteria.

We were able to observe rhizoplane and endophyte populations of the inoculated bacteria in plants growing under the gnotobiotic conditions (Fig. 1). The rhizoplane population of *P. tropica* reached values ranging from 1×10^{8} to 1×10^{10} CFU g^{-1} thus demonstrating that a large number of bacteria were able to colonize the root surface. We also found an efficient establishment of *P. tropica* as endophyte, reaching populations with values ranging from 1×10^{4} to 1×10^{5} CFU g^{-1} in the wheat roots, thus evidencing the ability of this microorganism to colonize the internal tissues of the wheat plant. The numbers of both the rhizoplane and the endophytic bacteria were moderately stable throughout the experiment, with the endophytic bacteria remaining at 2–4 orders of magnitude lower than those of the rhizoplane.

The identity of the microorganisms was checked not only in this manner by growth on LGI plates supplemented with Tc but also by obtaining a characteristic PCR-amplification product of 356 bp after carrying out a specifical PCR of the isolated colonies (not shown).

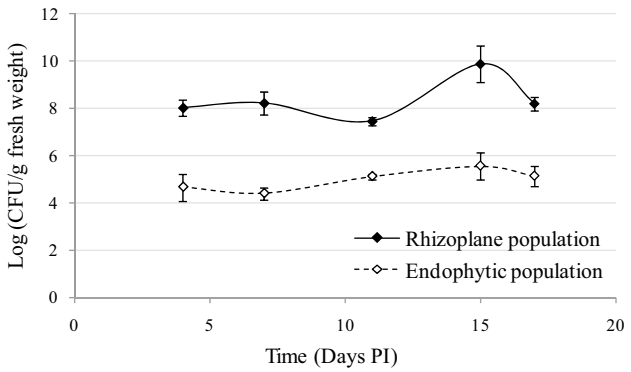

Fig. 1 Root populations of wheat plants growing under gnotobiotic conditions after seed inoculation with *P. tropica*. In the figure, the log(CFU) per g of root fresh weight of the rhizoplane (solid lines with closed diamonds) or endophytic (broken lines with open diamonds) bacteria is plotted on the *ordinate* as a function of time in days postinoculation (PI) on the *abscissa*. The data are the means of three biological replicates with the brackets representing the standard errors of the means

These results suggest that *P. tropica* efficiently colonized the roots of wheat plants growing under gnotobiotic conditions after the inoculation of disinfected seeds.

Inoculum concentration

Nondisinfected seeds were inoculated with different inoculum concentrations to assess how many inoculated bacteria would be necessary to achieve an efficient colonization (Table 1).

Root colonization by *P. tropica*, both internal and external, was equally efficient for all times PI when the inoculant concentration insured > 100 bacteria per seed (concentrations 1, 2, and 3 in Fig. 2; dilutions 1, 2, and 3 in Table 1). When, however, the number of bacteria per seed was lower than this threshold—i.e., approximately 10 bacteria per seed (concentration 4 from dilution 4 in Table 1)—both the surface and the endophytic populations exhibited values of CFU g^{-1} significantly lower than those of the other concentrations tested; and indeed at the shortest time investigated (i.e., 4 days PI) the inoculated bacteria had not yet been able to enter into the internal tissues. Despite this delay, by 8 days PI endophytic bacteria became detectable in the roots from those seeds inoculated with the lowest concentration. The number of viable bacteria present in that low inoculum, which value corresponded to the number of CFU $seed^{-1}$, affected the endophytic population, but after longer time periods *P. tropica* was able to enter and colonize the internal tissues even at that concentration.

Bacterial survival on the seeds

The survival of *P. tropica* attached to seeds was evaluated at different times PI (Fig. 3). Regardless of the treatment, bacterial viability decreased significantly through-out the time of the experiment, as was expected, as a consequence of the stress probably from the resulting desiccation on the seed surface (Herridge et al. 2001; Singleton et al. 2002). The initial concentrations of CFU ml^{-1}, as assessed from the number of inoculated bacteria removed from the seeds before storage (0 h in Fig. 3, the symbols on the *ordinate*

Table 1 Inoculum concentration and number of bacteria per seed after inoculation of nondisinfected wheat seeds

Inoculum dilution	CFU ml^{-1}	CFU $seed^{-1,a}$
1: None	3.1×10^{10}	7.7×10^{6}
2: 1×10^{-2}	2.5×10^{8}	2.3×10^{4}
3: 1×10^{-4}	7.4×10^{5}	1.8×10^{2}
4: 1×10^{-6}	5.1×10^{3}	< 10

[a]Data calculated for an inoculum dose of 600 ml per 100 kg of seeds and at 250 seeds per g

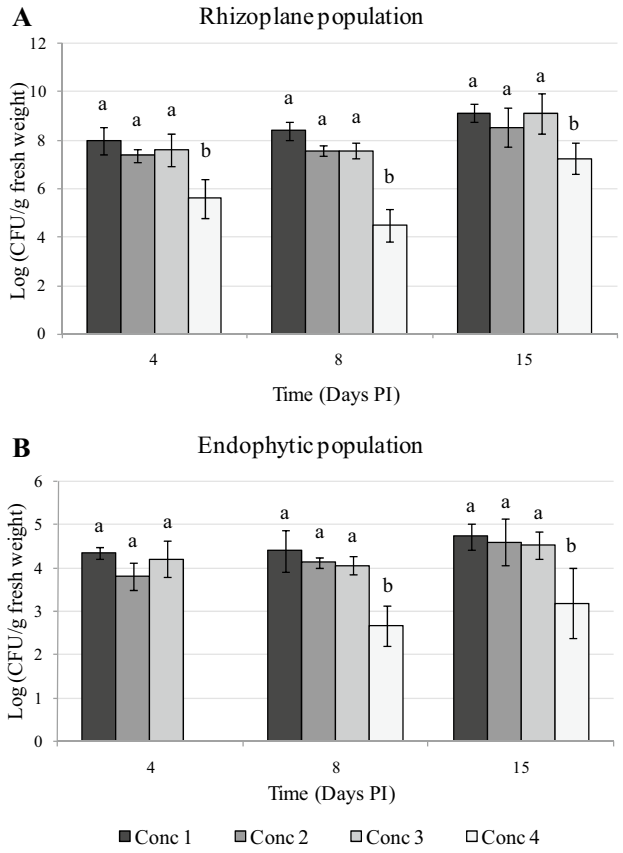

Fig. 2 Root populations of wheat plants growing under controlled conditions after seed inoculation with different inoculum dilutions of *P. tropica*. In the figures, the log(CFU) per g of root fresh weight of the rhizoplane (**a**) or endophytic (**b**) bacteria is plotted on the *ordinates* for each of the days postinoculum (PI) indicated on the *abscissas* for plants grown from seeds containing the following concentrations of bacteria (data from the inoculum dilutions indicated in Table 1): concentration (Conc) 1, 7.7×10^6 CFU seed^{-1} (black bars); Conc 2, 2.3×10^4 CFU seed^{-1} (dark-gray bars); Conc 3, 1.8×10^2 CFU seed^{-1} (medium-gray bars); Conc 4, <10 CFU seed^{-1} (light-gray bars). The data are the means of three biological replicates with the brackets representing the standard errors of the means. Different letters above the bars signify significant differences in the values at $p\leq0.05$

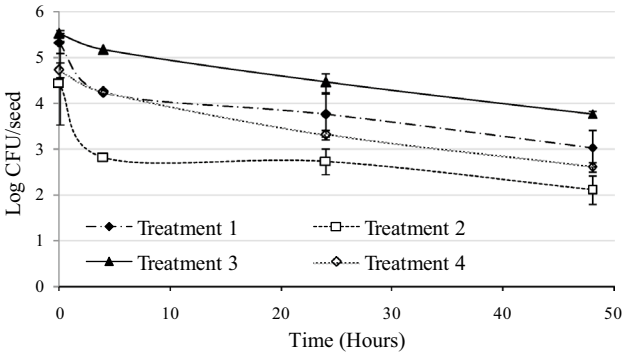

Fig. 3 Viability of *P. tropica* on seeds with different treatments. In the figure, the log(CFU) per seed is plotted on the *ordinate* as a function of the time in h on the *abscissa* after the addition of the bacteria to the seeds with or without the following indicated additives: *P. tropica* alone (Treatment 1, broken line with closed diamonds); *P. tropica* + fungicide (Treatment 2, dashed line with open squares); *P. tropica* + protectant (Treatment 3, solid line with closed triangles); *P. tropica* + fungicide + protectant (Treatment 4, dotted line with open diamonds). The data are the means of three biological replicates with the brackets representing the standard errors of the means. I: inoculum. F: fungicide. P: protectant. Treatments: 1, I; Treatment 2, I+F; Treatment 3, I+P; Treatment 4, I+P+F

axis), were different among the three treatments possibly because the protectant and fungicide both acted immediately in their respective positive and negative manner. From that time on during the first 4 h, the viability of *P. tropica* on seeds decreased approximately by one order of magnitude in samples without protectant compared to that of the bacteria with protectant, where that drop was minimal (Fig. 3, Treatments 1 and 3). The addition of protectant enhanced significantly the bacterial survival on the seed, as indicated in Fig. 3, over that occurring upon the addition of the bacteria either alone or in the presence of fungicide without or with protectant (*cf.* Treatment 3 compared to 1, and Treatment 4 compared to 2). Thus, the presence of fungicide decreased significantly the viability of the microorganisms, as might have been expected; whereas the protectant contributed notably to the maintenance of the cell viability even in presence of fungicide (*cf.* Treatment 4 compared to Treatment 2 in Fig. 3), as had been observed with other PGPB (Salema et al. 1982). Although the presence of fungicide affected the viability of bacteria on the seed surface, the results indicated that *P. tropica* can nevertheless be applied to seed in conjunction with the fungicides widely used in the conventional cultivation of wheat crops.

To assess efficiency in the colonization of the inoculated bacteria on seeds with the different treatments, the rhizoplane and endophyte populations were quantified in plants at 7 days postgermination on semisolid Fähraeus medium, as detailed above. The results indicated that, regardless of the treatment used or the reduction of bacteria on the seeds after 48 h PI, the viable bacteria on the seed surface were enough to efficiently colonize root tissues. Furthermore, normal values of superficial and endophytic root populations were found in all the samples analyzed. No growth, however, was observed in the uninoculated controls with sterile distilled water having been added instead of *P. tropica* (Treatments 5, 6, 7, and 8; data not shown).

Bacterial survival in aqueous formulations

Survival studies of *P. tropica* in aqueous suspensions with different additives packed in polyethylene bags were carried out by plate counting performed sequentially at different times postpackaging (Fig. 4). Approximately

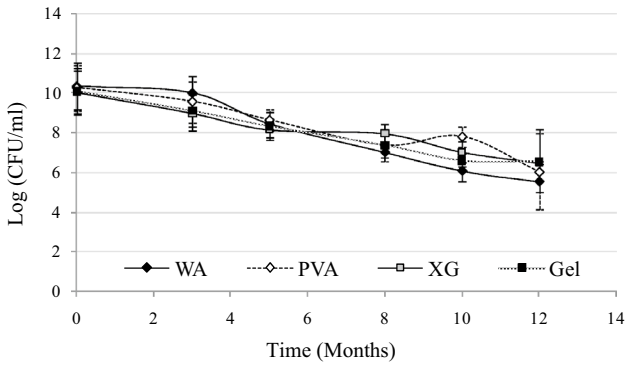

Fig. 4 Viability of *P. tropica* in aqueous formulations. In the figure, the log(CFU) per ml is plotted on the *ordinate* as a function of the time in months on the *abscissa* of storage in the formulations containing bacterial culture (WA, solid line with black diamonds) or the additives polyvinyl alcohol (PVA, dashed line with white diamonds), xanthan gum (XG, solid line with gray squares), or gelatine (Gel, dotted line with black squares). The data are the means of two biological replicates with the brackets representing the standard errors of the means

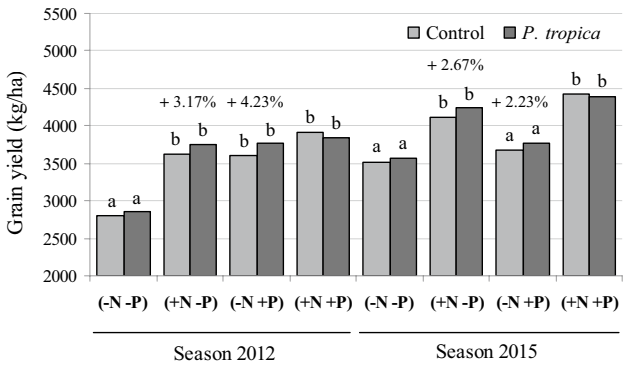

Fig. 5 Effect of *P. tropica* on wheat-grain yield after seed inoculation. In the figure, the grain yield in kg per ha is plotted on the *ordinate* during the two growing seasons (2012 and 2015) for each of the soil-fertilization conditions indicated on the *abscissa*. The percentages above the pairs of bars denote the increases in grain yield associated with the presence of *P. tropica*. Different letters above the bars indicate significant differences in the values at $p \leq 0.05$. Each period was analyzed separately. Control treatments, gray bars; Inoculated treatments, black bars; N, nitrogen fertilization; P, phosphorus fertilization Results are expressed as means of the data from three biological replicates for the total production per ha for each treatment during the evaluation period (two seasons)

1×10^8 CFU ml^{-1} of viable cells were retained at 6 months postpackaging for all formulations, as commonly occurs with commercial inoculants. In the final analysis, the additives tested did not significantly improve bacterial viability compared to the bacterial inoculums (WA). Nevertheless, an adequate concentration of viable bacteria could be maintained in a potential commercial product for a number of months under any of those conditions. In addition to this continued maintenance, because an efficient rhizoplane and endophytic colonization was achieved under controlled conditions at all those times tested (data not shown), we can conclude that the observed decrease in the viability of *P. tropica* over these time periods did not affect the ability of the bacterium to colonize plant tissues.

Field experiments

Figure 5 summarizes the wheat yields obtained expressed as grain production per hectare. The ANOVA data obtained from both crop seasons showed that there were no interaction between the different analyzed factors and that only fertilization had a significant effect on performance. In the 2012 season, the grain yield was significantly lower ($p \leq 0.05$) in treatments without fertilization (with or without *P. tropica* inoculation) than when the soil was fertilized with N and/or P, as expected since the periodic fertilization with N and P is currently recommended. Nevertheless, in the 2015 season, the yields in the absence of fertilization were, for their part, higher than in the 2012 season so that only the two fertilization protocols involving N produced grain yields that were significantly higher, with the increment in yield upon P fertilization alone not being statistically significant. Even though the average values of the yields for the inoculated treatments remained above the uninoculated ones, no significant effects of inoculation on the grain yield were detected with or without fertilization.

Discussion

The use of PGPB as inoculants on nonleguminous crops is a promising way of achieving sustainability in agriculture (Ahemad and Kibret 2014; Bashan et al. 2014; Oliveira et al. 2017). Seed treatment with diazotrophic and phosphate-solubilizing bacteria as PGPB to partially substitute for the demands for N and P fertilizers is a practice with numerous previous examples of application to cereal crops (Díaz-Zorita and Fernández-Canigia 2008; Cassán and Diaz-Zorita 2016; Oliveira et al. 2017). Nevertheless, the lack of widespread adoption of inoculation with PGPB as a regular agricultural practice in nonleguminous crops is attributable to the scarcity of scientific understanding regarding the characteristics of the plant-bacteria interaction in these systems. The necessity of improving the quality of the inoculants in order to obtain an efficient plant colonization for attaining an optimal response to the inoculation is also clearly evident (Naiman et al. 2009). A high-quality inoculant needs to insure physiologically suitable cells and a sufficient concentration to resist the environmental adversities to which bacteria are exposed once attached to seeds (Oliveira et al. 2017). Furthermore,

increases in performance in cereal crops resulting from the action of PGPB effects are in general only about 10%, nevertheless that increment is considered a significant contribution for the farmer (Cassán et al. 2015). In addition, with respect to such evaluations, the substantial number of observations needed to describe significant differences between treatments because of the large variability in the responses of crops to inoculation with PGPB (Cassán et al. 2015), would make the accuracy in determining the true degree of impact of these beneficial bacteria on crop yields somewhat questionable.

The work presented here has defined certain practical aspects of the use of PGPB through the acquisition of data related to the *P. tropica*-root colonization and persistence capabilities that become necessary to consider in the use of *P. tropica* in agriculture in order to enhance wheat-crop yields after seed inoculation.

The results obtained in this study have indicated five principal conclusions:

1. *P. tropica* was able to efficiently colonize superficial and internal root tissues of wheat plants after seed inoculation under gnotobiotic growth conditions, and it showed a behavior commonly presented by other endophytic bacteria growing under similar conditions (Andreote et al. 2009; Compant et al. 2005b; De Souza et al. 2016; Luna et al. 2012). Although, certain publications have described associations of wheat with different PGPB (Baig et al. 2012; Naiman et al. 2009; Verma et al. 2014), to the best of our knowledge, the present report is the first one involving wheat colonized by *P. tropica*. These results suggest that wheat-root exudates provided enough nutrients to sustain the growing bacterial population and constituted the proper environment for the expression of the epigenetic program necessary for entering and establishing themselves as endophytes even at the lowest inoculum used. Endophytic colonization enables the bacteria to establish a more direct interaction with the plant so as to facilitate an efficient transfer of nutrients and other elements (Ryan et al. 2008), and, once internalized, those bacteria could spread from the root to the aerial tissues. Other techniques, such as confocal laser-scanning microscopy will be necessary to determine the nature of the specific colonization sites inside the plants.
2. Both rhizoplane and endophytic colonization were achieved efficiently at relatively low concentrations of viable bacteria in the inoculum when the plants were grown under control conditions, however, the inoculum concentration affected the rate of endophytic root colonization. These results could be attributable to the existence of a certain threshold-population density of bacteria that is needed to colonize the root surface (probably by quorum-sensing signals) before entering the internal tissues (Whitehead et al. 2001).
3. *P. tropica* maintained its viability for 48 h once applied to seeds, and the bacteria were able to colonize the rhizoplane and the internal tissues of roots after this time.
4. *P. tropica* was compatible with a fungicide widely used in wheat crops.

   Although root colonization was efficient when plants were grown under control conditions, even at a low number of bacteria per seed, as well as in presence of a fungicide; nevertheless, a control of the physiologic aspects of root colonization by *P. tropica* in wheat plants growing in soil is still necessary because, in addition to all the aspects considered above, the efficiency of PGPB colonization and subsequent action depends on the chemical, physical, and biologic features of the agricultural environment at the time of its application (Bashan et al. 2014). Some of those environmental parameters could decrease the efficiency of *P. tropica* colonization, leading to a deficient response to the inoculation in field trials with *P. tropica* applied to wheat seeds. Conversely, should root colonization by *P. tropica* prove efficient in field trials, a number of other conditions, related mainly to the particular experimental design, might have been conducive to those results. Even commercial inoculant formulations with nonsymbiotic diazotrophic bacteria such as *A. brasilense* have been associated with a high variability in plant response under field conditions (Oliveira et al. 2017).
5. We were able to formulate an inoculant that maintained bacterial viability for relatively long periods of time. Nevertheless, the continued testing of formulations with other additives (or admixtures of them) for extending the life of microorganisms in the packaging until the time of application is still necessary.

The correct physiologic behavior of *P. tropica* characterized in this investigation suggests the necessity to further our understanding of the conditions effecting the bacterial competitiveness required for successfully achieving plant–PGPB interactions along with the need to continue with field experiments involving this microorganism under different fertilization conditions, in different seasons, under other edaphoclimatic conditions, and with additional wheat genotypes (Oliveira et al. 2017).

The findings reported here contribute to our understanding of beneficial plant–microbe interactions in nonlegumes—and in particular those of an endophytic bacterium such as *P. tropica*—in order to enhance wheat production.

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