Rhizobacteria *Pseudomonas fluorescens* and *Azospirillum* sp. association enhances growth of *Lactuca sativa L.* under tropical conditions

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Abstract

The selection of microorganisms that enhance plant growth and confer biotic and abiotic tolerance to crops constitutes a biotechnology currently gaining importance on a global scale. The aim of this investigation was to evaluate the effects of inoculating rhizobacteria to lettuce (*Lactuca sativa L.)* on seed germination and vegetative development in order to use isolates as potential biofertilizers under tropical conditions. Five isolates of *Pseudomonas fluorescens* (Pf) and one of *Azospirillum sp.* (Az) were inoculated to seeds using a bacterial suspension of $1.5*10^8$ CFU*mL^{-1}. *In vitro*, none of the isolates promoted germination. *In vivo*, isolates promoted growth and acted as stress alleviators by conferring tolerance to high temperatures (≥ 30 °C). The highest seedling emergence percentages were induced by the association of *P.fluorescens* with *Azospirillum.* This association also promoted the highest leaf-area in 25 d seedlings and exhibited a significantly higher dry-weight in 40 d plants compared to the control (P≤0.05) supporting the advantages of bio-consortiums over individual strains. The strains were able to produce dependent L-tryptophan indole-3-acetic acid (IAA), to solubilize phosphorous *in vitro* and tolerated at least 5%-salt stress. The results indicate that isolate Pf (26) and Az possess plant growth promoting rhizobacteria (PGPR) traits and should be further assessed. This study suggests that *P. fluorescens* and *Azospirillum* act synergically and are able to trigger an induced-tolerance mechanism in lettuce under abiotic stress.

Keywords: auxins, biofertilizers, lettuce, plant growth promoting rhizobacteria, plant stress

Introduction

The biotechnological application of soil microorganisms is becoming a relevant alternative to costly inorganic fertilizers in a context of high demand for food products, depletion of water resources, climate change and accelerated rates of agricultural soil degradation worldwide.

The rhizosphere is the narrow region of soil directly influenced by plant roots. Exudates from roots influence microbial activity, creating a complex, dynamic and unique microenvironment critical for plant development (Vessey, 2003). Beneficial free-living soil bacteria are collectively referred to as Plant Growth Promoting Rhizobacteria (PGPR), (Kloepper and Schroth, 1978). Although PGPR represent a minority group within the rhizosphere community, they play a key role in plant development by increasing the bioavailability of nutrients and biosynthesizing growth regulating compounds such as auxins, cytokinins and gibberellins (Vessey, 2003). Furthermore, they play an important role in the fixation of nitrogen (Ahemad and Kibret, 2014) and the solubilization of phosphates (Gyaneshwar et al., 2002) among other direct beneficial effects. Other favorable but indirect actions of PGPRs on the rhizosphere include the biocontrol of phytopathogenic microorganisms via the production of substances like cianhidric acid (HCN), siderophores and antibiotics (Bakker et al., 2007). PGPR are also able to modulate plant stress by induced systemic tolerance (Jain et al., 2014) when plants are subject to abiotic adverse conditions such as high salinity, high temperatures and hydric stress. Some particular PGPRs such as *Azospirillum sp.* and *Pseudomonas fluorescens*, are able to produce and secrete plant hormones (secondary metabolites) which are utilized as plant growth promoting substances by the host plant (Abbas-Zadeh et al., 2010). Plant growth promotion by *Azospirillum sp.* involves the production of regulatory substances, being the auxin indole-3-acetic acid (IAA) the most important phytohormone (Steenhoudt and Vanderleyden, 2000). Likewise, *P. fluorescens* enhances growth in many different plant species, with advantages over other rhizobacteria. For instance, *P. fluorescens* bacteria are able to use a broad range of nutritional sources, producing high-affinity Fe^{+3} binding siderophores, thus restricting the growth of other rhizosphere bacteria under conditions of low iron concentrations. They can also remain viable for long periods of time under very adverse conditions, making them suitable as biofertilizers (Weston et al., 2012).

Considering lettuce (*Lactuca sativa* L.) is an important model in plant ontogenesis studies (Scheibe and Lang, 1969; Nascimento, 2003) and represents an economically important crop, the aim of this work was to evaluate the effects of inoculating lettuce seeds with rhizobacteria *P. fluorescens* and *Azospirillum sp.* on morpho-physiological parameters, from germination to harvest time, in order to identify potential isolates to be used as biofertilizers under tropical conditions.

Materials and methods

Experiments were performed at the bacteriology facilities of the Central University of Venezuela - Faculty of Agronomy (UCV-FAGRO), located in Maracay at 450 m a.s.l. (67°36´37´´ W and 10°16´10´´ N; Aragua, Venezuela), during the period April – June 2014, with average temperatures of 30.5 °C and average relative humidity of 65%.

The experiments were conducted using *Lactuca sativa L.* var. *Crespa Grand Rapids,* Tip Burn Resistant (TBR) seeds from ISLA®- Sao Paulo, Brazil, 2014. For greenhouse experiments Sunshine® Mix # 5 substrate (Agawam, MA, USA) composed of plug-grade Canadian sphagnum peat moss, plug-grade perlite and dolomitic limestone, with an electrical conductivity of 1900 µS cm^{-1} and pH = 5.7, was used. Chemical composition provided by the manufacturer was (in ppm): N (160); P (40); K (150); Ca (140); Mg (60); Fe (40); Zn (0.3); Cu (≤0.5); Mn (0.1); B (≤0.6).

Six isolates from the collection of the Bacteriology Laboratory, UCV-FAGRO collection were used: *Azospirillum sp.* (Az), *P. fluorescens* (24), *P. fluorescens* (26), *P. fluorescens* (17), *P. fluorescens* (03), *P. fluorescens* (b) and the association *Azospirillum (Az) + P.fluorescens* (26). *P.fluorescens* 26 was selected to be combined with *Azospirillum* based on previous biocontrol capacity tests against plant pathogenic bacteria and fungi.

Phenotypic characterization of bacterial isolates

Biochemical assays and electron microscopy were performed to verify some key features of the isolates used. Morphology, motility, and Gram-type were observed in a phase-contrast microscope (Zeiss, Oberkochen, Germany). The type of flagellation and cell dimensions were determined by transmission electron microscopy (TEM) using negatively stained preparations (Lowy and Hanson, 1965).

Biochemical assays performed were: Gram-reaction, Cytocrome oxidase (discs impregnated with dimethyl *p*-phenyl-enediamine), catalase activity (1.5% H_2O_2), Hugh & Leifson assay, production of fluorescent pigments under UV light in King's medium B (Schaad et al., 2001) and particularly for A*zospirillum* sp., growth of typical colonies in *Nfb* (Nitrogen free broth) and Congo red agar (Cáceres, 1982). All reagents, otherwise stated, were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

Indole-3-Acetic Acid (IAA) production, phosphate solubilization and salt tolerance assays were performed to verify the activation of these specific growth promotion mechanisms in the bacterial isolates.

Electron microscopy

Bacteria were grown on falcon tubes containing 5 mL of Luria Bertani (LB) broth and incubated at 30 °C, 36 h, 60 rpm on a rotary shaker. TEM preparations were made using copper grids (300 mesh) covered with a thin film of 2% collodion and dried at 60 ˚C for 24 h. Negatively stained preparations were made by delicately pipetting 1 µL of suspension containing motile bacteria into each grid, withdrawing it after 2 min and replacing it with a drop of distilled water. The washing fluid was eventually removed and immediately replaced with 1 µL of 2% uranyl acetate. After 2 min the stain was withdrawn and the preparation allowed drying for 24 h at room temperature. A FEI Tecnai Spirit G2 (Hillsboro, Oregon, USA) transmission electron microscope was used to visualize the samples.

PGPR capacity tests *in vitro*

Indole-3-acetic acid (IAA) production was assessed through a colorimetric method adapted by Cattelan (1999). Bacteria were seeded by the streak plate method in Petri dishes (15 mm*60 mm) containing TSA (Tryptic soy agar) medium enriched with 1.021 g of L-tryptophan, covered with nitrocellulose membranes and incubated at 28-30 °C for 24 h. Afterwards, nitrocellulose membranes were transferred to another Petri dish and covered with *Salkowski* solution (1 mL of 0.5 M $FeCl_3 \cdot 6H_2O$ diluted in 50 mL 35%, v/v, $HClO_4$). A reddish halo zone in the corresponding membrane was taken as a positive result.

Phosphate solubilization was performed through a method adapted by Cattelan (1999). Bacteria were seeded by the streak plate method in Petri dishes (15 mm*60 mm) containing TSA medium enriched with $CaHPO_4$ and incubated at 28-30 °C for 7 d. Positive results could be seen as a clear halo zone around the bacterial colony.

Negative controls consisted of *X. axonopodis* pv*. manihotis* and sterile distilled water for both IAA and Phosphate solubilization assays.

In order to test tolerance to high NaCl concentrations, yeast extracts (5 $g*L^{-1}$) with NaCl (2%, 3%, 5%, 7% and 10%) were used. Pure bacterial cultures (24 h, 10 µL, 10^8 $CFU*mL^{-1}$) were added to test tubes containing 10 mL of each of the yeast-NaCl solutions. The resulting suspensions were shaken at 120 rpm (24 h, room temperature). Positive results were detected by visualizing bacterial turbidity of the medium.

Growth promotion assays

Protocols to test the capacity of bacterial isolates to promote growth of lettuce seeds were conducted as described by Mariano and Silveira. (2005). Germination *in vitro* and emergence and growth parameters *in vivo* were evaluated. Bacterial isolates were separately cultured to exponential phase and then inoculated to lettuce seeds before seeding. To assess germination promotion *in vitro* the parameters germination percentage, hypocotyl and radicle length were determined. Experiments of emergence and growth *in vivo* were carried out under greenhouse conditions from 0-40 d post emergence (DPE) of lettuce seeds, using sterile peat-moss substrate. The parameters assessed during lettuce early ontogenesis (0 to 25 DPE: nursery stage) were emergence percentage, number of leaves per plantlet, leaf area, primary root length and number of lateral roots. For the evaluation of growth *in vivo* 25 to 40 (DPE) the parameters determined were fresh and dry matter production of aerial parts and roots. Destructive analysis of plantlets was used. Temperature and relative humidity were recorded with a hygrothermograph (225-5020-A Hi-Q, USA) during the whole experiment.

Bacterial cell culture

P. fluorescens were cultured in King's medium B and *Azospirillum* sp. in Congo red agar by the streak plate method. The bacteria were incubated at 25 °C, 24 h (12 h light - 12 h dark), a period long enough for the typical colonies to appear.

Azospirillum sp. colonies were scarlet red, Gram negative, curved rod shaped bacteria showing lipoidal droplets, while *P.fluorescens* colonies were fluorescent green-blue colored (under UV light), Gram negative, rod shaped, smooth edged and convex aspect bacteria, white colored on Nutrient Agar (NA) medium. Typical colonies were isolated and reseeded in NA medium, allowing them to grow for 36 h (exponential growth stage). At this time, they were transferred to Erlenmeyer flasks by previously pouring sterile distilled water on top of the culture media containing the bacterial colonies and scraping them out. Bacterial concentration was adjusted to $1.5*10^8$ CFU*mL^{-1} by spectrophotometry (λ: 535 nm, Absorbance (A): 0.42) in a spectrophotometer VIS –UNICO, model S-1200-E (Dayton, NJ, USA).

PGPR germination assay *in vitro*

Each of the six bacterial isolates at exponential growth phase was inoculated to lettuce seeds in test tubes and then transferred to Petri dished and stored at a germination chamber at 20 °C. Briefly, seeds were disinfected with sodium hypochlorite 1% for 2 min and then washed with sterile distilled water. Seeds (400 per treatment) were submerged for 20 min in the specific bacterial suspension (in Tween-80, 0.05%) in tests tubes. Afterwards, they were placed with the help of sterile lab tweezers on Petri dishes, on top of wet filter paper (1 mL sterile distilled water each) and transferred to a dark growing chamber at a controlled temperature of 20 °C and 12 h light - 12 h dark cycle for 7 d. Parameters associated to germination were estimated according to RAS (Seed Analisis Rules: Ministry, 2009) and through digital images with ImageJ software (Schneider et al., 2012).

PGPR early ontogenesis assay *in vivo* 0-25 DPE

Bacterial suspensions were inoculated as described for germination assay *in vitro*, but this time the seeds were placed on polyethylene trays with sterile substrate instead of Petri dishes, and kept inside a greenhouse. The germination trays consisted of 25 cells of 2.4 cm^3 (100-mm depth). When more than one seedling emerged on the same cell only the strongest one was allowed to grow and the rest were torn out. Three seeds were planted per cell in autoclaved Sunshine-5 substrate (121 °C, 1 atm, 1 h). The plantlets were homogeneously irrigated daily with 2-3 applications of demineralized water through a pressurized irrigation system. Temperature at the greenhouse had a daily range of 22-40 °C and humidity of 57-70% (photoperiod: 12 h light - 12 h dark). All plants were fertilized during this period according to Freitas et al. (2003), with the following formula (in mg per plant): N (8.68); P (0.80); K (5.80); Ca (6.93); Mg (0.91) y S (0.924). Micro-elements solution (in mg per plant): B (0.01); Cl (1.81); Cu (0.002); Mn (0.03); Mo (0.04), Zn (0.008) and traces of Fe ≤0.001. At transplant age (25 DPE), 25 plantlets per treatment were randomly chosen in order to evaluate the parameters described at this stage.

PGPR growth assay *in vivo* 25-40 DPE

The roots of 25-days-old plantlets were sunk in the specific bacterial suspensions for 20 min and then potted in clean polyethylene plant pots (1 L) containing Sunshine #5 substrate. Ten days after transplant plantlets were re-inoculated with the specific treatments with a syringe (5 mL of bacterial suspension per pot). Plants were irrigated 30 d post emergence with Hoagland solution 0.25X (as a source of macro-elements) and a micro-elements solution with the following formula (in mg per plant): B (0.01); Cl (1.81); Cu (0.002); Mn (0.03); Mo (0.04), Zn (0.008) and traces of Fe ≤ (0.001). Photoperiod was 12 h light and 12 h dark. Plants were harvested 40 DPE, early in the morning and immediately dried in a heating chamber with forced air circulation at 72 ºC during 48 h in order to estimate the total dry matter of the roots and aerial parts.

Experimental design

All experiments were carried out using a completely randomized design. *In vitro* experiments consisted of 7 treatments (6 bacterial strains and distilled water as control) and 8 replications. 400 seeds per treatment were used, as recommended by ISTA (1996) and the experimental unit consisted of a Petri dish (100*15 mm) containing 50 lettuce seeds. *In vivo* experiments (greenhouse) consisted of 8 treatments (6 bacterial strains, the combination of Pf+Az and control) and evaluations were divided in two periods, 0-25 DPE, and 25-40 DPE. Plantlets were transferred to pots at day 25. For the first period 4 replications were used and the plot was one tray of 25 cells (2.4 cm^3) and for the latter 5 replications and the plot consisted of 5 randomly chosen pots (1 plant each). Experimental units were randomly re-arranged in the physical space during evaluation time to guarantee that each one occupied all possible positions. Destructive analysis of plants was used.

Statistical analysis

The statistical analysis was performed by one-way-ANOVA using the GLM procedure and comparisons were done with Tukey HSD test in SPSS statistics software (Released 2008, SPSS Statistics for Windows, Version 17.0. Chicago: SPSS Inc). Significant differences were considered at probability of P≤0.05. Data normality and homoscedasticity were examined prior to ANOVA. Bacterial isolates with best PGPR traits under the study conditions were identified.

Results and discussion

Bacterial characterization

All the isolates used in the present work were Gram-negative, oxidase-positive and grew under aerobic conditions. Colonies showed the standard morphology of the specific bacterial types. Cells of *Azospirillum sp*. were curved rods (some s-shaped), (0.8 -1 µm * 3.2-4.2 µm), with lipoidal droplets. A single polar flagellum was observed when cells were grown on liquid medium. *Azospirillum* sp. generated typical red scarlet colonies in Congo red agar and grew in Nfb medium under microaerophilic

conditions. Cells of *P. fluorescens* were motile with one polar flagella, rod shaped (1.2 -1.3 µm * 2.5 - 2.9 µm), catalase-negative and UV fluorescent on King's B medium. The colonies showed smooth edges with convex aspect and white coloured when cultured in Nutrient Agar (NA). The results support the identification of bacterial isolates as *P. fluorescens* and *Azospirillum sp.* that belong to the UCV-FAGRO collection (Figure 1).

All isolates were able to produce IAA in the presence of L-tryptophan. Based on the halo size, Pf (24) appeared to produce higher quantities of IAA, while overall, the rest of the isolates produced relatively less amounts of this auxin.

Both *P.fluorescens* and *Azospirillum* sp. were able to solubilize phosphates, as evidenced by colonies exhibiting a whitish halo around the bacterial cells. Halos were more intense and greater in size in *Pseudomonas* spp. compared to *Azospirillum* sp. which suggests a higher capacity to solubilize this compound. As expected, controls with *Xanthomonas axonopodis pv. manihotis* and without bacteria resulted negative for IAA and Phosphate tests.

As phosphate-solubilizing microorganisms (PSM) have been identified as the best eco-friendly means for Phosphorus nutrition of crops (Sharma et al., 2013), the strains evaluated on this study are promising prospects, provided further field testing.

Salinity stress tolerance is another key trait of PGPR that could make them suitable for soil salinity alleviation (Shrivastava & Kumar, 2015). On this study all strains were able to tolerate 5% NaCl and the isolates Pf (24), Pf (17) and Pf (03) even grew at 7% NaCl, demonstrating their high potential to withstand high saline medium stress *in vitro*. Likewise, all isolates were able to grow in macro-elements formula (08-12-24/4) at a dose of 1000 ppm, indicating that conventional fertilizers with the doses used are apparently compatible with PGPR use. However, higher doses could possibly affect soil bacteria negatively.

Effect of rhizobacteria inoculation on lettuce seeds germination *in vitro*

Rhizobacterial isolates tested showed remarkable differences regarding lettuce seed germination and seedling development. Germination percentage of lettuce seeds under control conditions *in vitro* exceeded the commercial seed germination standard (Nascimento et al*.,* 2012) of 80%. Treatments Pf (17) and Pf (26), had germination percentages similar to the control ($P>0.05$), while treatments Pf (03), Pf (24) and Pf (b) performed significantly lower than the control ($P<0.05$). Interestingly, none of the seeds inoculated with *Azospirillum* germinated during the 7 d experiment. Under the conditions used, none of the bacterial strains were able to promote higher germination percentages than the control (about 85%), (Table 1).

The results showed significant differences in the germination promoting capacity of the strains tested. Seed germination *in vitro*, was negatively affected by inoculation with some of the strains of *P. fluorescens*. When *Azospirillum sp*. was inoculated no germination occurred. These inhibitory effects could be attributed to quantitative or qualitative differential expression of phytohormones by the strains involved. For instance, the alteration of Abscisic acid (ABA)/Gibberelins balance is known to play a major role in seed primary dormancy (Miransari and Smith, 2014). ABA can prevent germination by adversely affecting the genes of chromatin assembly and modification

of the cell wall, in this way positively affecting the genes that regulate gibberellins catabolic pathways (Miransari and Smith, 2014).

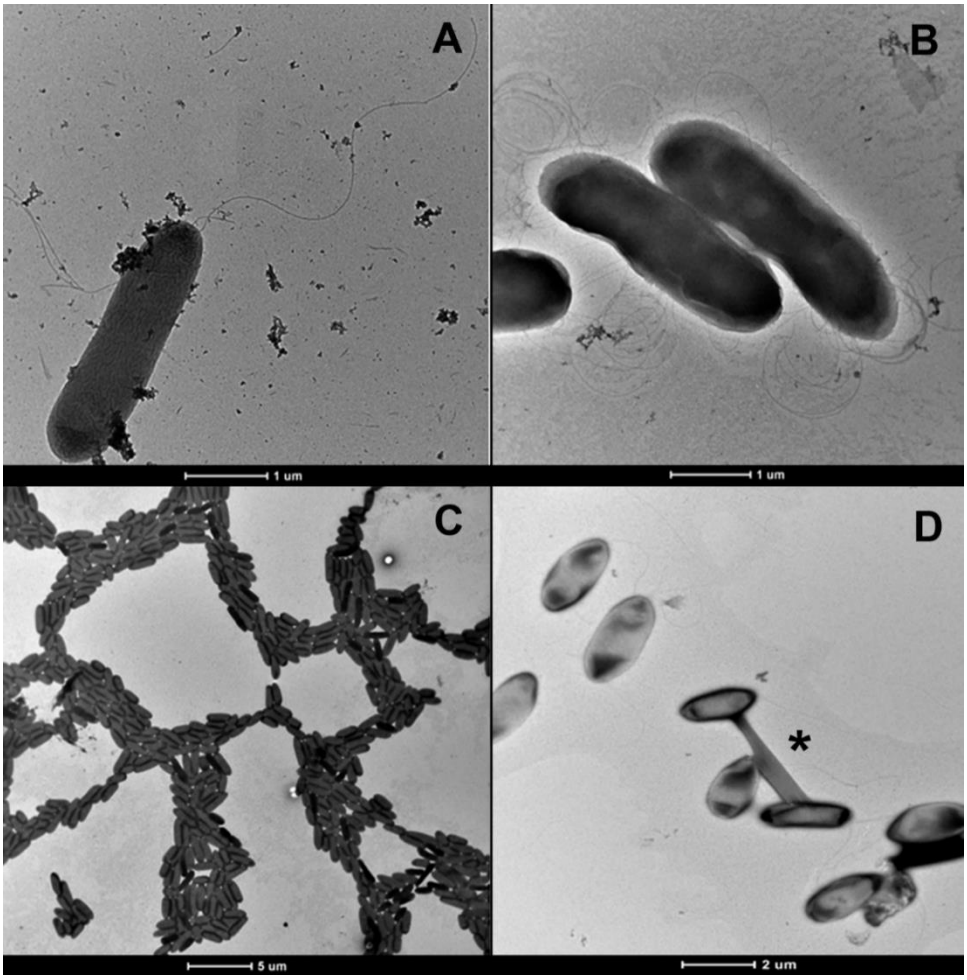

Figure 1. Transmission Electron Microscopy images of (A) *Pseudomonas fluorescens* (B) *Azospirillum sp*. (C) *Pseudomonas+Azospirillum* (D) *Pseudomonas.* (*) Nanotube during bacterial conjugation

Hypocotyl length was significantly higher than the control and the rest of the treatments, when using the inoculum Pf (17), ($P<0.05$). Lowest values were with Pf (03), Pf (b) and Az (Table 1). This result shows that bacterial inocula affect the hypocotyl development as well. In this respect, cytokinins are known to control cytokinin response factors which in turn affect hypocotyl and root growth, among other tissues (Santner et al., 2009). Ethylene also plays an important role in early plant development. Abeles (1986) suggested that ethylene promotes seed embryonic hypocotyl cell expansion during lettuce germination and this could probably explain the superiority of Pf (17) treatment.

Pseudomonas strains Pf (17), Pf (b) and Pf (03) exhibited significantly longer radicles than the control ($P<0.05$) and Pf (024) significantly shorter ones (Table 1). Miransari and Smith (2014) pointed out that ethylene and gibberellins affect radicle growth, with gibberellins being the most important hormone. High concentrations of the auxine (IAA) could also be responsible for the lower radicle growth induced by Pf (24) and

this effect has been previously reported (Schlindwein et al., 2008). Fargasová (1994) suggested that auxins in general possess herbicide action (at specific concentration ranges) and this could possibly be involved in the adverse effect observed on germination parameters.

Table 1. Germination 7d post-inoculation with rhizobacteria *in vitro*

Treatments	Germination (%)	HL	RL
Control	85.1±1.5^{a}	2±0.07^{b}	0.42±0.01^{b}
Pf24	73.9±1.9^{b}	1.6±0.04^{bc}	0.35±0.01^{c}
Pf26	83.4±1^{a}	1.7±0.05^{b}	0.41±0.01^{bc}
Pf17	86.3 ±0.9^{a}	2.6±0.05^{a}	0.59±0.01^{a}
Pf03	75.8±1.5^{b}	1.4±0.08^{c}	0.55±0.03^{a}
Pfb	70.8±2^{b}	0.8±0.01^{d}	0.56±0.01^{a}
Az	0±0	0±0	0±0

HL = hypocotyl length (cm). RL = radicle length (cm). Different superscripts indicate significant differences by Tukey's HSD test (P<0.05). The results are presented as the mean ± SEM

Effect of rhizobacteria inoculation *in vivo* on lettuce early ontogenesis (0-25 DPE)

Under nursery conditions (green house), seedlings inoculated with rhizobacteria had emergence percentages statistically higher than the control (P<0.05) (Table 2), even when inoculated with *Azospirillum sp.*, which had produced negligible germination rates under *in vitro* conditions (P<0.05). Needless to say, it is clear that the environment is a key factor controlling bacterial performance and an evidence of this is that *in vitro* results did not match *in vivo* data regarding seedling emergency. *Az* alone and Az + Pf (26) promoted the highest germination percentages *in vivo*. Control seedlings significantly underperformed all other treatments regarding emergence percentages (P<0.05). It is usually acknowledged that temperature is a critical factor for lettuce seed germination and most cultivars do not germinate above 30 °C (Nascimento, 2002). Khan and Prusinski (1989) reported that high temperature appears to inhibit the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene in lettuce seeds and Nascimento (2003) mentioned that addition of ACC has been associated with an increased activity of endo-β-mannanase (a cell-wall enzyme that weakens the endosperm) allowing lettuce seeds to germinate at high temperature. Production of ACC deaminase (ACCD), a pyridoxal phosphate-dependent enzyme, by *P. fluorescens* and *P.putida* enhances plant growth under stress conditions (Miransari and Smith, 2014). It is possible that rhizobacteria in the inoculation treatments were able to alter ethylene levels via ACCD among other factors and this allowed overcoming the relative thermoinhibition of the seeds induced by tropical high temperatures in the present work.

Az+Pf (26) produced remarkably greater values of total leaf area (TLAP) than all other treatments (including the control), followed by Az (P<0.05), (Table 2). All the *Pseudomonas* strains used, except for Pf (03), produced higher values for this parameter than the control (Table 2). A higher TLAP can be associated with plant

growth promotion. It is well known that plant growth is significantly affected by phytohormone cross-talk. Among plant promoting phytohormones, gibberellins are known to influence leaf growth through the regulation of the activity of lactoylglutathione lyase (Hattori et al., 1995) and probably were expressed by treatments involving Az on the present work. Both strains in *Az+Pf* (26) association seemed to mutually produce synergistic signaling dialogues resulting in higher leaf area values. This further demonstrates the advantages of bacterial associations over individual strains, as previously reported (Wang et al., 2012). Similarly, Neyra et al. (1995) found a synergistic effect while co-inoculating *Azospirillum sp.* with *Rhizobium* in mung bean (*Vigna radiata*), resulting in significantly higher nodulation and plant growth. Additionally, Rodelas et al. (1999) using *Azospirillum-Rhizobium* and Ahmad et al. (2008) using the bio-consortium *Rhizobium-Pseudomonas,* found that such associations could induce superior growth promoting effects than the independent strains.

Regarding the number of leaves per plant (NLP), another indicator of plant growth, seedlings inoculated with *Az* showed significantly higher values than the control ($P<0.05$), while one of the Pseudomona strains, Pf (03), had the lowest ($P<0.05$), (Table 2). Although not statistically significant, the effect of Az+Pf(26) on this parameter was very close to that of Az. Evidence in support of these findings can be found in the work of Hokmalipour et al. (2013), who also observed a high number of leaves per plant and leaf area index generated from seeds primed with a combination of *Azospirillum* sp. and *Azetobacter sp.*

Primary root length (PRL) of the seedlings treated with Az+Pf (26), Pf (03) and Pf (24) was significantly higher than the control ($P<0.05$), followed by Pf (b) and Az ($P<0.05$), (Table 2). Strains Pf (17), produced short primary roots (significantly shorter PRL than the control, $P<0.05$). In general, PGPRs are able to stimulate root growth. They regulate ethylene production in plantlets via ACC-deaminase, through the hydrolysis of α-cetobutirate and ammonium, consequently promoting root growth. Hence, when post-germination ethylene levels are too high, root elongation can be inhibited (Glick et al., 2007) and this could possibly be the explanation for the lower performance of Pf (17).

The number of lateral roots (NLR) was positively influenced by *Az* and its association with a strain *P. fluorescens* 26, Az+Pf (26), exhibiting significantly higher values for this variable compared to the control and the rest of the treatments ($P<0.05$), (Table 2). *Azospirillum* species have been widely reported to induce the formation of lateral roots and abundant radical pili (creating higher rizhospheric volumes) in several plant families by biosynthesis of growth promoting substances (Marks et al., 2015).

Effect of rhizobacteria inoculation *in vivo* on the vegetative growth of lettuce plants (25-40 DPE)

At 40 DPE (plant harvest), fresh weight (FW) and dry weight (DW) of the aerial part and the radical system were significantly higher for the plants inoculated with Az+Pf(26) compared to all other treatments ($P<0.05$), (Table 2). The increase in biomass could be possibly associated to more developed root systems, as shown with root development parameters evaluated during the period of plant growth up to 25 d. This better root development possibly resulted in greater absorption of nutrients

available over equal periods of time compared to the rest of the treatments. Nitrogen is the fundamental element for the increase in the dry mass production of lettuce, the absence of which considerably reduces plant growth (Resende et al., 2010). It was hypothesized that a greater uptake of nitrogen by the plants inoculated with Pf (26)+Az or Az could have occurred, which consequently could have promoted the highest DW of the aerial part. This aspect could also be qualitatively evidenced given the darker green color of the leaves of the *Azospirillum* inoculated plants, while with all other treatments, leaves showed a considerably lighter green color, possibly resulting from lower concentrations of N. In this respect, *Azospirillum sp.* belongs to the non-symbiotic group of N fixing organisms (Ahemad and Kibret 2014).

Based on all the evaluations performed, the isolates Az and Pf (26) combined are a promising association that could be further evaluated under field conditions to be used as a potential biofertilizer for lettuce and probably other plants under tropical conditions.

Rhizobacteria Induced tolerance to heat

Results obtained *in vivo* suggest that P. *fluorescens* and *Azospirillum* are able to confer tolerance to lettuce plants under high temperature stress (mean temperatures ≥ 30 °C) since bacterized plants had better performance in all parameters evaluated compared to the control ($P<0.05$) and plants were exposed in a prolonged way to heat stress during the evaluation period with temperatures that even reached 40 °C. Some studies have suggested that 1-aminocyclopropane-1 carboxylate deaminase (ACCD) activity plays a key role in the protection of plants from the deleterious effects of abiotic stress such as heat (Glick et al, 2007; Singh et al, 2015). Cohen et al., (2009) suggested that *Azospirillum* sp. helps corn plants (*Zea mais*, L) to tolerate water stress, which can be induced by high temperatures, probably through production of Abscisic Acid (ABA). Salomon et al., (2016) found that PGPR trigger the accumulation of terpenes in leaves of *Vitis vinifera* L. that confer protection to cells against photo-oxidation, by decreasing oxygen consumption associated to the amino acid tryptophan. Other research suggested that salicylic acid (SA) is the key component of heat tolerance and that H_2S might be the novel downstream signal molecule in SA-induced heat tolerance, since SA enhances the activity of L-cysteine desulfhydrase (L-DES),which is a key enzyme in H_2S biosynthesis (Li et al, 2013; Li et al., 2015). Further investigation needs to be carried out to elucidate the biochemical pathways involved in heat tolerance induction by PGPR and thus take advantage of these mechanisms. PGPR Induced tolerance represents a promising tool in agriculture for coping with global warming.

Table 2. Parameters of lettuce plant development under the influence of bacterial strains (*P.fluorescens* and *Azospirillum sp.*)

	Germination *in vivo*					Growth *in vivo*			
						Fresh weight		Dry weight	
	E%	TLAP	PRL	NLP	NLR	AP	RS	AP	RS
Control	30±3^{a}	254±6^{a}	82.3±5^{ab}	3.6±0.2^{ab}	6.1±0.5^{ab}	683.9±22^{a}	39.1±1^{a}	33.4±1.1^{a}	1.8±0.1^{ab}
Pf24	54±3^{bc}	337.2±5^{b}	141.4±7^{c}	4±0.2^{ab}	7±0.5^{ab}	929.3±36b^{c}	49±3^{a}	47.3±1.9^{bc}	2.4±0.1^{c}
Pf26	51±6^{bc}	359.9±6^{bc}	67.1±5^{ab}	3.6±0.2^{ab}	6.8±0.7^{ab}	843.6±47^{b}	47±2^{a}	41.4±2.3^{ab}	2.1±0.1^{bc}
Pf17	42±3^{ab}	325.8±3^{b}	59.4±5^{a}	3.4±0.2^{ab}	5.4±0.6^{a}	747.5±31^{ab}	41.7±3^{a}	39.4±1.1^{ab}	2±0.1^{b}
Pf03	38±3^{ab}	263.6±6^{a}	136.1±7^{c}	3.3±0.2^{a}	5.7±0.4^{a}	572.1±26^{a}	37.9±2^{a}	27.6±1.3^{a}	1.4±0.1^{a}
Pfb	57±6^{bc}	307.2±7^{b}	107±5^{b}	3.5±0.2^{ab}	6.3±0.5^{ab}	721.7±2^{ab}	43.8±2^{a}	34.9±1.1^{a}	2.1±0.1^{bc}
Az	61±3^{c}	393.2±1^{c}	109.3±7^{b}	4.2±0.3^{b}	10.2±0.6^{b}	961.7±16^{bc}	50.9±3^{b}	47.6±0.8^{bc}	2.3±0^{bc}
Az+Pf26	71±4^{c}	465.6±11^{d}	140.4±6^{c}	4.1±0.2^{ab}	9±0.6^{b}	1029.1±29^{c}	72.8±4^{c}	52.8±1.6^{c}	2.5±0.1^{c}

Phase II = 0-25 DPE; Phase III = 40 DPE (harvest time). Different superscripts indicate significant differences by Tukey's HSD test (P<0.05). The results are presented as the mean ± SEM. E% = percentage of seed emergence; TLAP = total leaf area per plant (mm^2); PRL = primary root length (mm); NLP = number of leaves per plant; NLR = number of lateral roots; AP = Aerial parts; RS = Radical system. Weights are in mg per plant.
Pf = Pseudomonas fluorescens; Az = Azospirillum sp.

Conclusions

Rhizobacterial strains tested positively affected growth parameters of lettuce (*Lactuca sativa L.*) var. Crespa Grand Rapids, ($P<0.05$) under the conditions studied. The association *P. fluorescens* and *Azospirillum* sp. significantly promoted leaf development of lettuce and this suggests that this bacterial association act synergistically. Phosphorus solubilization, IAA dependent L-tryptophan production and 5%-salt tolerance were evidenced as PGPR traits of the strains evaluated. The rhizobacterial strains were apparently able to modulate the stress caused by high temperatures and this suggests that PGPR may be an alternative to partially overcome the effect of this abiotic stress.

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