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In vitro and *in vivo* inoculation of four endophytic bacteria on *Lycopersicon esculentum*

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Four bacteria selected on the basis of their capability of fixing atmospheric nitrogen, stimulating plant-growth, and protecting the host plant from pathogens – *Azospirillum brasilense*, *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae*, *Burkholderia ambifaria* – were inoculated on tomato seeds either singularly, in couple and in a four bacteria mixer. Aim of this research was to evaluate: (1) effect of single and mixed cultures on the inoculated plant – plant growth, dry weight, root length and surface, number of leaves, among others; (2) colonization and interactions of the bacteria inside the host plant; (3) localization inside the host of single bacterial strains marked with the *gusA* reporter gene.

The results obtained indicate that all selected microbial strains have colonized *Lycopersicon esculentum* but in a different way, depending on the single species. *A. brasilense*, *G. diazotrophicus* inoculated *in vitro* singularly and together were the best plant colonizers. *In vivo* essays, instead, *B. ambifaria* and the four-bacteria mixer gave the best results.

It was possible to localize both *A. brasilense* and *H. seropedicae* inside the plant by the *gusA* reporter gene. The bacterial strains occur along the root axis from the apical zone until to the basal stem, on the shoot from the base up to the leaves. The four bacteria actively colonize tomato seeds and establish an endophytic community inside the plant.

This review gives new information about colonization processes, in particular how bacteria interact with plants and whether they are likely to establish themselves in the plant environment after field application as biofertilizers or biocontrol agents.

Introduction

A large number of studies demonstrated that all plants harbor inside – the xylematic vessels in particular – an abundant and various microflora, composed mostly by bacteria and fungi, which carry out roles for their host nutrition, so-called endophytes [1,2]. Endophytes are microorganisms that spend most of their life cycle inside plants. Various endophytic nitrogen-fixing bacteria have been identified associated with crop plants. These endophytes do not cause damage to the host organism or humans [3–6]; on the contrary they promote plant growth [7,8].

Such plant growth-promoting bacteria (PGPB) or plant growth-promoting rhizobacteria (PGPR) can, without conferring pathogenicity, stimulate plant growth, increase yield, reduce pathogen infection, as well as reduce biotic or abiotic plant stress, by one or more factors: production of substances which directly or indirectly stimulate plant growth such as auxines, cytochinines, giberellins, production of molecules actives against pathogens such as siderophores, antibiotics, among others, and supply of biologically fixed nitrogen [9–18]. It has been shown in many researches that these plant beneficial microorganisms are of interest for application in agriculture either as biofertilizers or as well as for phytoremediation application, to reduce or substitute chemical fertilizers input in agriculture [14,19–21].

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Concerning *B. ambifaria*, previously ascribed to the *Burkholderia cepacia* complex (Bcc), the U.S. Environmental Protection Agency (EPA) as a result of the clinical relevance of Bcc species and their close interspecies relatedness, severely restricted the biotechnological applications of all Bcc species [22]. However, recent advances in the taxonomy and ecology of the *B. ambifaria* species deserve reconsideration. *B. ambifaria* is a typical rhizosphere species with an important role in protection against attacks by pathogenic fungi on the plant, and it is among the species found with infinitesimal frequency in patients with CF (a disease in and of itself rare) and, moreover, as an occasional, never pathogen, guest. Strain PhP7, in particular, utilized throughout the present work has never been found in CF patients [23].

Plant cultivation interferes with the equilibrium which would establish with the associated microorganisms in natural growth conditions. Soil tillage, treatments against pests, continuous removal of organic matter from soils, monocultures, are agricultural practices hampering the preservation of the agro-ecosystem because they lower biodiversity, the microbial one in particular [24]. To break this negative chain which reaches the ecosystem level, it is possible to act in different ways, increasing biodiversity: the vegetable one by increasing plant species, but particularly the microbial one, for example by inoculating bacteria selected for properties useful for the crop. Differently from the past, the problem is afforded now more holistically: instead of substituting a chemical product with a single microorganism (a single nitrogen-fixing bacterium instead of combined nitrogen, a single bacterium against a single pathogen) the whole plant/microorganisms ecosystem is taken into consideration and is tentatively reconstructed. However, application to sustainable agriculture is still largely at the experimental stage.

The biodiversity of plant-associated microbial species is a basic condition to establish plant fitness in each environment. Effective stimulation of plant growth, biological fixation of atmospheric nitrogen and its supply to the host depend on an efficient colonization [25].

In the present work four bacteria, selected on the basis of their capability of fixing atmospheric nitrogen, stimulating plant-growth, and protecting the host plant from pathogens – *Azospirillum brasilense* [25–27], *Gluconacetobacter diazotrophicus* [28,29,38], *Herbaspirillum seropedicae* [30–33], *Burkholderia ambifaria* [34–36], – were inoculated on tomato seeds either singularly, in couple and in a four bacteria mixer, to demonstrate their beneficial effects in tomato plant. Tomato crop need high nitrogen, phosphate, potassium inputs and, in addition, it is frequently affected by numerous pathogens. It thus requires large amount of pesticides the cost of which, combined to the cultural interventions, makes this production very expensive.

Following this approach, the aim of this study was: (1) the evaluation in a gnotobiotic system *in vitro* and *in vivo* – an unsterile system – of the effect of single and mixed cultures on the inoculated plant – plant growth, dry weight, root length and surface, number of leaves, among others; (2) colonization and interactions of the bacteria inside the host plant; (3) the detection inside the host of two bacterial species marked with the *gusA* reporter gene.

Materials and methods

Bacterial strains and media

In this study four endophytic bacteria were used (Table 1): *A. brasilense* strain Cd [37] originally provided by Y. Okon the Hebrew University of Jerusalem, Israel; *G. diazotrophicus* strain Pal5 provided by the late J. Döbereiner, C.N.P.B.S., Embrapa, (Rio de Janeiro, Brazil). This bacterium was previously classified as *Acetobacter diazotrophicus* [37], and successively renamed *G. diazotrophicus* [39]. *H. seropedicae* strain Z67 was also provided by J. Döbereiner. *B. ambifaria* gv VII strain PHP7 [40], previously *B. cepacia* [41] originally provided by T. Heulin, C.P.B., CNRS (France).

The bacteria were routinely grown at 30°C on their specific cultural medium: *A. brasilense* in OK medium [42]; *G. diazotrophicus* in LGI medium [43] and in PDA (Potato dextrose agar – Oxoid) [44]; *H. seropedicae* was grown in J-NFb medium [45]; *B. ambifaria* in KB medium [46] and in PCAT medium [47]. For the four bacteria inoculum, all bacteria were separately grown on a common medium, T4, which was specifically set up with the following composition per L^{-1}: KH_2PO_4 1 M 80.8 mL, K_2HPO_4 1 M 19.2 mL, $MgSO_4$ 0.2 g, NaCl 0.1 g, $CaCl_2$ 0.02 g, Na_2MoO_4 0.001 g, $MnSO_4$ 0.002 g, NH_4Cl 1 g, FeEDTA 2 mL (1.64%, w/v $FeCl_3$), yeast extract (Difco) 0.05 g, pH 6.4. Fructose, 10 g per L^{-1}, was chosen as carbon source because it is the only sugar well utilized by all strains. Fructose was 30 min steam-flowing sterilized, phosphates were separately sterilized (at 120°C for 20 min) and both were added after the medium cooling.

Germination, inoculation and growth of seedlings

In *in vitro* experiment seed of tomato (*Lycopersicon esculentum* cv. Guadalete), supplied from Peto Seed Seminis (Vegetable Seed Italia s.r.l., Parma, Italy), were surface sterilized by immersion in 70% (v/v) ethanol for 3 min followed by incubation in NaClO 5% for 5 min, by shaking. Seeds were subsequently washed six times with sterile distilled water by shaking (15 min each). The treated seeds were placed on a disc of filter paper, moistened with 1.5 mL sterile distilled water in a sealed Petri dish and germinated in the dark at 22°C. After 96 hours uniform seedlings were selected and inoculated in tubes containing 10 mL of washed bacterial cultures 10^8 cells mL^{-1}, in different combination (single bacteria, couples and four together) for 20 min. All four bacteria were grown in T4

TABLE 1

Endophytic diazotrophic strains utilized and source of isolation

Name	Strain	Tissue associated	Plant
***Burkholderia ambifaria* (Ba)**	PHP7	Root	*Zea mays*
***Herbaspirillum seropedicae* (Hs)**	Z67 ATCC35893	Root	*Sorghum bicolor*
***Gluconacetobacter diazotrophicus* (Gd)**	Pal5 ATCC49037	Stem	*Saccharum officinarum*
***Azospirillum brasilense* (Ab)**	Cd ATCC29729	Root	*Cynodon dactylon*

medium, collected at the beginning of the stationary phase, were washed and diluted or concentrated at 10^8 cells mL^{-1} with physiological solution. Inoculated seedlings were transferred to glass tubes containing 10 mL of a sterile nutrient medium MS [48] (Duchefa) agarized with 3.5 g L^{-1}. Subsequently the seedlings were randomly placed in a growth chamber for 30 days at 24°C, a photoperiod of 16 hours (bulb FLUORA L 30W/77, 18,000–20,000 lux), and relative humidity of 65%; seeds inoculated with autoclave killed bacteria (same amount of living cells) were used as controls and were grown in the same conditions.

In *in vivo* experiment, uniform seedlings obtained by unsterilized seeds germinated as previously described, were selected and placed in tubes containing washed bacterial cultures (10^8 cells mL^{-1}) in different combinations (single bacteria and four together) for 20 min. Inoculated seedlings were positioned in 2 L pots containing a mixture of unsterilized [49,50] soil and sand (4:1). Controls were inoculated with the same amount of autoclave killed bacteria. Plants were grown in a greenhouse for about 60 days, at 25°C during the day and 16°C at night, a photoperiod of 16 hours and were irrigated daily with droppers.

Analysis on plants

After 30 days for *in vitro* experiment and 60 days for *in vivo* experiment, plants were collected and the effects of inocula were evaluated: root and aerial part length, number of leaves and of lateral roots, dry weight.

For the *in vitro* experiment it was possible to evaluate the colonization and interactions of the bacteria, in single and mixed inocula, inside the host plant. All plant sections, roots, stems and leaves were cut, weighed and homogenized in sterile physiological solution. Then the homogenates were serially diluted and plated on their specific cultural medium. Bacterial colonies were counted after 2–3 days incubation at 30°C.

Experimental design and statistical analysis

Each treatment was replicated six times, and three pots served as a single replica. Data from the three pots were combined and the entire experiment was analyzed by 'Statistica' program utilizing the Duncan's test at $P < 0.01$ and $P < 0.05$. All experiments were repeated at least three times.

Gus A fusion

To recognize single bacterial strains inside the host plant both *A. brasilense* and *H. seropedicae* have been constitutively marked by a plasmidic vector with the reporter gene *gusA* [51]. This gene, promoting the utilization of glucuronic acid, allows to colorimetrically evidenciate bacterial cells *in situ*. The plasmids utilized in this research were pFAJ 31.13 (promoter of *gusA* gene + plasmidic vector pFAJ 31) [52] and pRK 20.13 [53], supplied by J. Vanderleyden, Leuven University, Belgium.

The plasmid pFAJ 31.13 (Tc^r) including in *Escherichia coli* HB101 was inserted in *A. brasilense* and *H. seropedicae* strains by conjugation. The 'helper' strain was *E. coli* pRK20.13. The transformant strains, carrying *gusA* fusion, were selected on OK medium containing 50 μg mL^{-1} 5 Br-4 Cl-3indolyl-β-D-glucuronide (X Gluc) and tetracycline, 10 μg mL^{-1} for *A. brasilense* ad 100 μg mL^{-1} for *H. seropedicae*, respectively. It was not possible to introduce pFAJ 31.13 plasmid in *B. ambifaria* and *G. diazotrophicus*.

Tomato seeds were sterilized, according to the procedure previously described, were then inoculated with *A. brasilense* and *H. seropedicae* strains harboring the *gusA* gene [54], and were grown in glass tubes containing 10 mL of a sterile MS medium. After 10 days, aerial parts (stems and leaves) and roots were washed twice in phosphate-buffered (PBS: K_2HPO_4 1.24 g L^{-1}, KH_2PO_4 0.40 g L^{-1}, NaCl 8.80 g L^{-1}, pH 7.2) and incubated for 24/48 hours in staining solution consisting of 100 mM K-phosphate, 0.5 mg mL^{-1} 5Br-4Cl-3indolyl-β-D-glucuronide (X Gluc), 0.33 mg mL^{-1} $K_3[Fe(CN)_6]$ and 0.42 mg mL^{-1} $K_4[Fe(CN)_6]$, in the dark at 37°C. All plant sections were dipped for 10 min in a solution of ethanol 20%, formaldehyde 5%, acetic acid 5% (v/v) [55], and then washed for 2 min in ethanol 50% (v/v), and 2 min in ethanol 100%. Finally aerial parts and roots were washed with distilled water [52,56].

Results

In vitro *experiment*

In previous experiments, conducted both in the field and in the greenhouse, we selected a mixture of four bacteria with strong capability of competing with indigenous microflora and of colonizing several crop plants. This mixture, the one we utilized in this study, composed by *A. brasilense* Cd, *H. seropedicae* Z67, *B. ambifaria* PHP7, and *G. diazotrophicus* Pal5, showed both nitrogen fixation activity, production of auxine compounds, plant crop promotion and protection against some tomato pathogen (unpublished data).

In this work we studied in a gnotobiotic environment the different interactions inside the plant among the different bacteria singularly, in couples and four together. We monitored the plant growth promotion and the localization of the different bacteria inside.

Initially we studied the interactions of the bacteria inside the host plant in a gnotobiotic conditions. The response to an efficient colonization of tomato plants with the four bacteria, in different combination – single bacteria, couples and all together – were, in comparison with uninoculated controls: (1) enhanced root length (Fig. 1a) particularly for plants inoculated with *A. brasilense* (Fig. 2) and with the couple *A. brasilense* plus *G. diazotrophicus*; (2) increased number of lateral roots and enhancement of root hair number and size (Fig. 1a and b) especially in plants inoculated with *A. brasilense*, *A. brasilense* plus *G. diazotrophicus*, *A. brasilense* plus *H. seropedicae*, *A. brasilense* plus *B. ambifaria*, *G. diazotrophicus* plus *H. seropedicae* and with the four bacteria together, (3) increased number of leaves and a general enhanced aerial part (stems and leaves) (Fig. 1a and b); (4) general increase of dry matter in each inoculum tested (Fig. 1c). This is a favorable feature because an increase in the crop canopy enhances photosynthesis and productivity, thus capability of supporting associated microflora.

Table 2 shows the level of colonization of roots, stems and leaves from single bacteria, couples and four together.

A. brasilense strain was recovered in high number from roots (10^7 CFU/g dry weight), stems and leaves (10^5 CFU/g dry weight). This bacterium produces beneficial effects on the host plant, particularly on the roots area, either single or in couples with *G. diazotrophicus* and *B. ambifaria*.

Among the four bacteria, *G. diazotrophicus* was the best colonizer of the whole tomato plant. A high number of bacteria was recovered both inside roots (10^8 CFU/g dry weight) and inside stem and leaves (10^5 CFU/g dry weight).

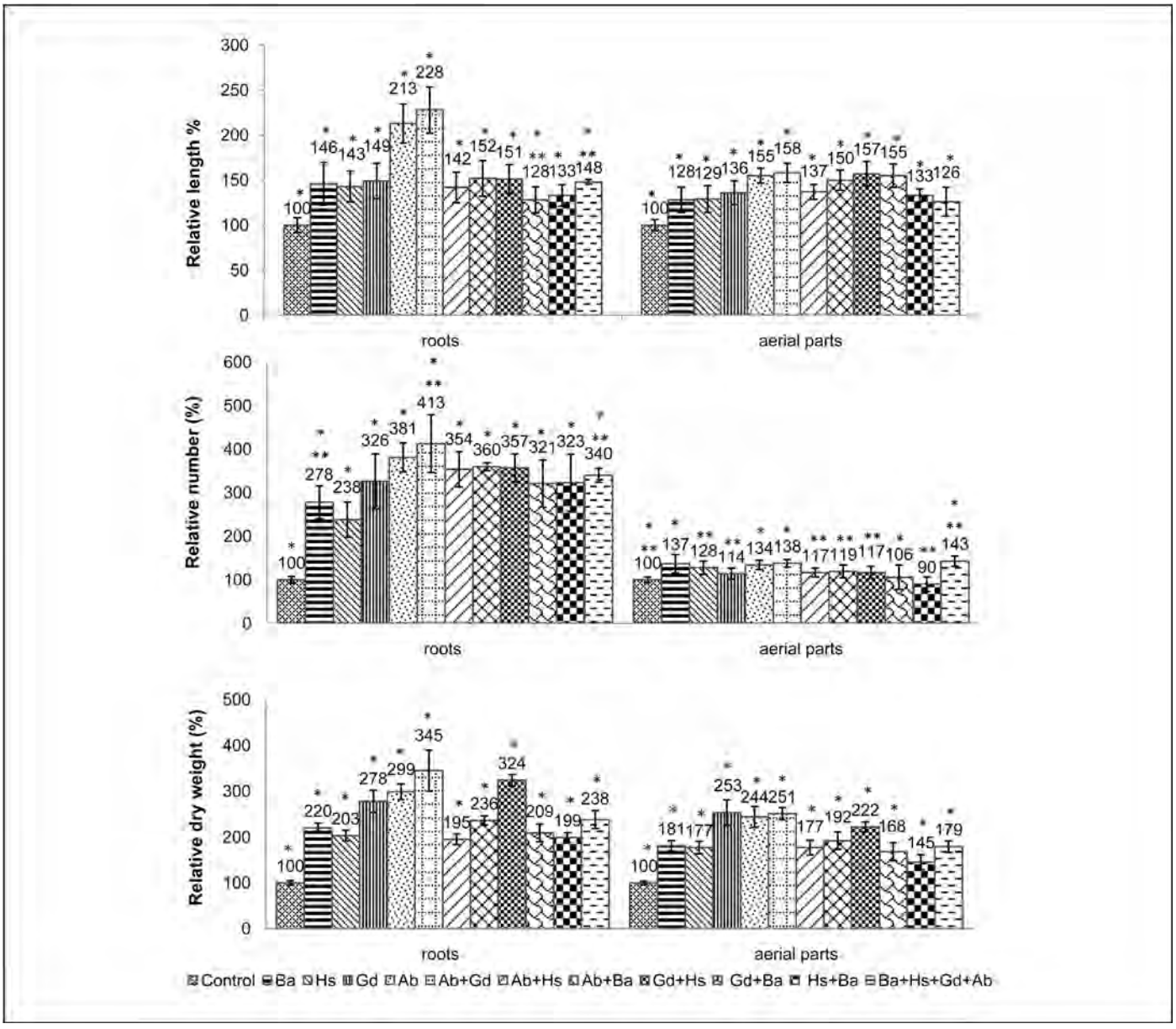

FIGURE 1

Comparison of the plant growth-promoting effects of the bacteria on *L. esculentum in vitro* experiments. Tomato plantlets 30 days after seed inoculation. **(a)** Percentages of relative length of the root part (left) and aerial part (right); **(b)** percentage of relative number of lateral roots (left) and leaves (right); **(c)** percentage relative of dry weight of root (left) and aerial part (right). Error bars indicate standard deviation. Different numbers of asterisks indicate significant differences (*$P < 0.01$ and **$P < 0.05$, Duncan test).

H. seropedicae colonized tomato plant in a lower concentrations with respect to the other bacteria, its levels ranging between 10^5 CFU/g dry weight in the root and 10^3 CFU/g dry weight in the stem and in the leaves. In combination with the other bacteria *H. seropedicae* showed a lower colonization of the different plant sections, mainly stem and leaves.

B. ambifaria showed a good colonization of all sections of the tomato plant, with concentration levels ranging between 10^7 CFU/g dry weight in the root and 10^5 CFU/g dry weight in the stem and in the leaves. Its positive effects can be observed mostly in the aerial parts. Among the different combinations, the more effective were those with *A. brasilense* and with *G. diazotrophicus*.

The four bacterial strains inoculated together, were all able to colonize the tomato plant inducing a positive effect on its growth, with respect to the control plants, particularly increasing leaves number.

β-Glucuronidase (GUS) staining

In an *in vitro* experiment we also followed the colonization of tomato plant both by *A. brasilense* and *H. seropedicae* harboring a constitutively expressed *gusA* gene. Gus-staining revealed an efficient overall colonization of tomato plants by both bacteria, showing preferential regions of association.

The use of the gus-staining has confirmed the presence of the bacteria inside tomato plants, as showed from plate counts of

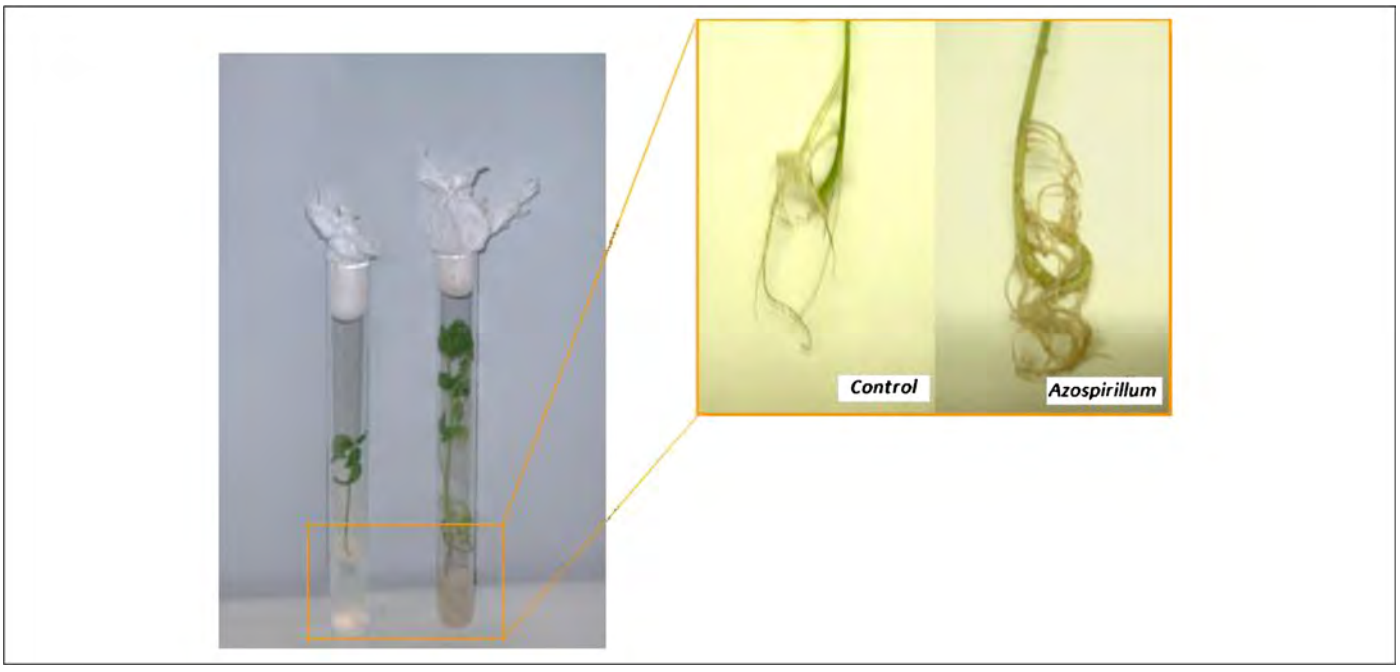

FIGURE 2

On the left control plant and on the right plant inoculated with *A. brasilense*: detail of roots, in the *in vitro* experiment.

bacteria present inside the host plant after surface sterilization (Fig. 3).

In vivo *experiment*

In this work, we also evaluated the effects of this selected bacterial pool inoculated on tomato plants in *in vivo* experiments (Fig. 4).

Differently from the *in vitro* experiment, *in vivo G. diazotrophicus* gave poor results in stimulating plant growth.

Concerning the effect of the single bacteria: *A. brasilense*, when inoculated on tomato plants *in vivo* experiments, stimulates their growth in a significant way (Fig. 4a and b). This growth, anyway, is far lower than that observed *in vitro* experiments.

Unlike what happened in the *in vitro* system, the inoculation with *H. seropedicae* produced good effects on the growth of tomato plants, especially on the roots (Fig. 4a and b).

B. ambifaria showed favorable effects on the plant growth, mainly in the aerial part (Fig. 4a and b).

The four bacteria, inoculated together, were able to produce good effects on the global plant growth (Fig. 4a and b) and in particular as for the dry matter (Fig. 4).

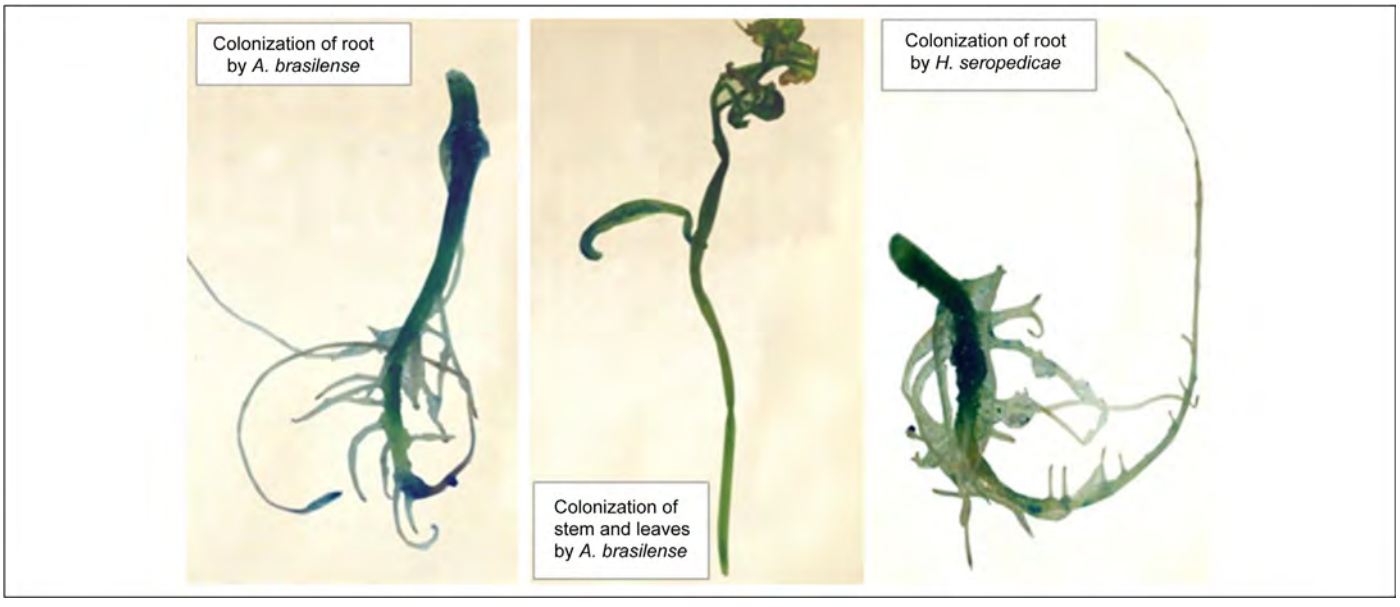

FIGURE 3

Colonization of tomato plant by *A. brasilense* and *H. seropedicae* harboring a constitutively expressed *gusA* gene. The bacteria are concentrated mostly on the side root emerging points, on the root hairs, root cap, root tip. Intensive blue staining was obtained on root hairs and sites of lateral root emergence.

TABLE 2

Endophytic population of micropropagated tomato plants in roots, stem and leaves after seed inoculation with single, couple or all four bacteria. Values are mean ± SD of at least three replicates. bd: number of cells below the minimum level of detection

Species recovered (log number of CFU/g dry weight)

	Ba			*Hs*			*Gd*			*Ab*		
	Root	Stem	Leaves	Root	Stem	Leaves	Root	Stem	Leaves	Root	Stem	Leaves
Control	bd	bd	bd	bd	bd	bd	bd	bd	bd	bd	bd	bd
Ba	7.1 ± 1.2	5.4 ± 0.8	5.4 ± 1.0	bd	bd	bd	bd	bd	bd	bd	bd	bd
Hs	bd	bd	bd	5.6 ± 0.5	3.8 ± 0.6	3.7 ± 0.9	bd	bd	bd	bd	bd	bd
Gd	bd	bd	bd	bd	bd	bd	8.4 ± 1.2	5.7 ± 1.2	5.6 ± 0.7	bd	bd	bd
Ab	bd	bd	bd	bd	bd	bd	bd	bd	bd	7.6 ± 1.0	5.2 ± 0.8	5.2 ± 1.0
Ab + Gd	bd	bd	bd	bd	bd	bd	8.2 ± 1.9	5.5 ± 1.0	5.4 ± 0.8	7.4 ± 1.8	5.3 ± 1.2	5.1 ± 0.8
Ab + Hs	bd	bd	bd	5.50 ± 1.4	3.2 ± 0.8	3.06 ± 1.0	bd	bd	bd	6.5 ± 0.3	3.4 ± 1.0	3.1 ± 0.6
Ab + Ba	7.3 ± 1.0	4.3 ± 0.9	4.3 ± 1.0	bd	bd	bd	bd	bd	bd	bd	bd	bd
Gd + Hs	bd	bd	bd	5.4 ± 0.8	3.2 ± 0.3	3.1 ± 0.7	6.4 ± 1.5	6.4 ± 1.5	4.2 ± 1.0	7.2 ± 1.4	4.5 ± 0.7	4.3 ± 1.2
Gd + Ba	6.5 ± 1.2	4.2 ± 0.6	4.1 ± 1.2	bd	bd	Bd	6.7 ± 1.0	4.3 ± 0.7	4.2 ± 0.6	bd	bd	bd
Hs + Ba	6.2 ± 0.5	3.7 ± 0.8	3.5 ± 0.3	5.5 ± 0.6	3.6 ± 1.0	3.3 ± 0.9	bd	bd	bd	bd	bd	bd
Ba + Hs + Gd + Ab	6.6 ± 1.4	4.4 ± 0.7	4.2 ± 1.0	5.8 ± 0.6	3.4 ± 1.2	3.3 ± 1.4	7.4 ± 0.6	4.3 ± 1.0	4.2 ± 0.5	6.8 ± 1.0	3.4 ± 0.8	3.3 ± 0.5

Discussion

The present study demonstrated that *A. brasilense*, *B. ambifaria*, *G. diazotrophicus* and *H. seropedicae* can form sustaining endophytic populations in roots, stems and leaves on *L. esculentum* plantlets [57].

In an *in vitro* experiment we also followed the colonization of tomato plant both by *A. brasilense* and *H. seropedicae* harboring a constitutively expressed *gusA* gene. Gus-staining revealed an efficient overall colonization of tomato plants by both bacteria, showing preferential regions of association. The use of the gus-staining has confirmed the presence of the bacteria inside tomato plants, as showed from plate counts of bacteria present inside the host plant after surface sterilization.

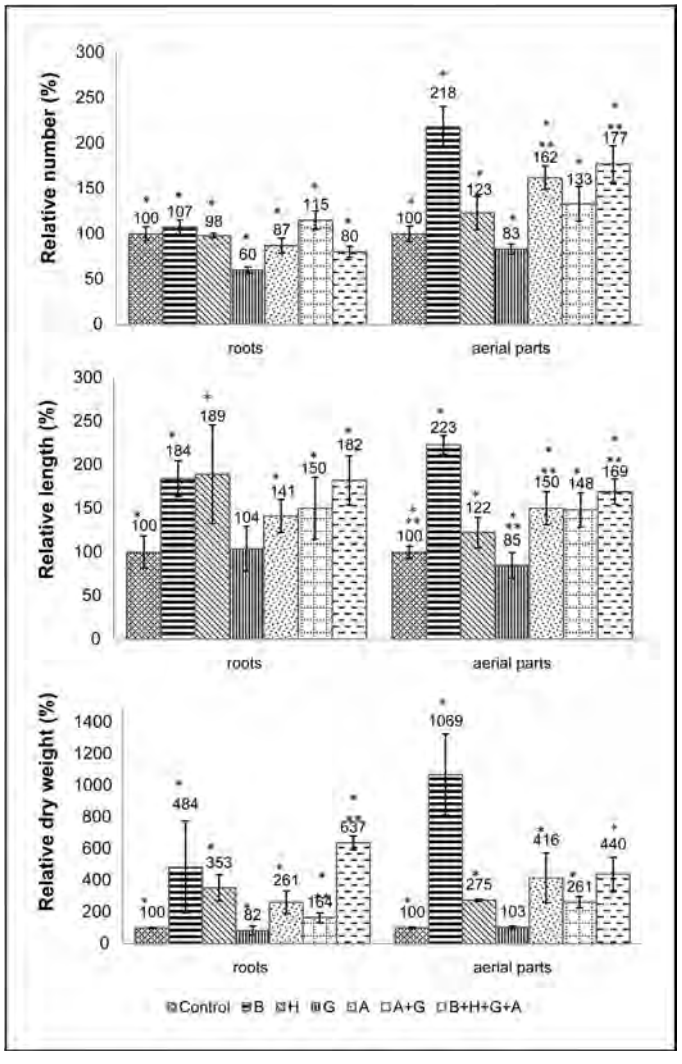

FIGURE 4

Comparison of the plant growth-promoting effects of the bacteria on *L. esculentum in vivo* experiments. Tomato plantlets 60 days after seed inoculation. **(a)** Percentages of relative length of the root part (left) and aerial part (right); **(b)** percentage of relative number of lateral roots (left) and leaves (right); **(c)** percentage relative of dry weight of root (left) and aerial part (right). *Legend*. B: *Burkholderia* spp; H: *Herbaspirillum* spp; G: *Gluconacetobacter* spp; A: *Azospirillum* spp. Error bars indicate standard deviation. Different numbers of asterisks indicate significant differences (*$P < 0.01$ and **$P < 0.05$, Duncan test).

A key feature of all plant-beneficial bacteria is efficient colonization of root surfaces [57,58]. After this initial colonization step on root surfaces, certain bacteria are able to colonize roots internally through [59]: (1) via lateral root emergence sites (Fig. 3), suggesting a colonization route similar to that of sugar cane [60,61] could occur in sorghum and wheat plantlets; (2) via root hairs (Fig. 3), that would indicate that both bacteria could also enter plants through them, as has been reported by Hallmann et al., [1]; and (3) via root tips in cells of the root cap and meristem which is considered a possible way of entry for other endophytic microorganisms like *Azoarcus* sp. [63].

In our study, intensive blue staining was obtained on root hairs and sites of lateral root emergence (Fig. 3). The presence of *A. brasilense* and *H. seropedicae* at lateral root emergence sites suggested that crack entry colonization occurred in tomato plantlets, similar to the phenomenon previously observed with potato [54,61]. Moreover, the occurrence of a blue color at root tips after *A. brasilense*::*gusA* and *H. seropedicae*::*gusA* inoculation also supports the possibility of entry via root tips [56,62,64–66].

In the gnotobiotic experiments, the inoculated strains could be re-isolated from the roots and aerial parts of the plant as also demonstrated by Baldani et al. using the same gnotobiotic approach applied here [67–69].

A. brasilense, *B. ambifaria*, *G. diazotrophicus* and *H. seropedicae* were able to colonize aerial parts of tomato plants; as already reported for other endophytic population CFU/g were always lower than found in roots (Table 2) [59,70].

The results obtained with the inoculation of the four bacteria together – in an *in vitro* gnotobiotic experiment – are extremely interesting, particularly concerning the ecological aspects: an inoculated plant is a microcosm in which five biological components live together in an extremely constrained and artificial environment. In this microcosm all five components reach a biomass in equilibrium with the others. This supposes a microbe–microbe and microbe–root/plant communication which should be studied more in detail [71].

The results obtained indicate that all selected microbial strains have colonized *L. esculentum* but in a different way, depending on the single species. *A. brasilense* and *G. diazotrophicus* inoculated *in vitro* singularly and together give the best results in stimulating plant growth. As reported by Dobbelaere et al., the inoculation effect of *Azospirillum* sp. on the growth of some agriculturally important plants gives a significant increase in the dry weight of both the root system and aerial parts of the PGPR inoculated plants, resulting in better development and flowering [26].

Plant studies have shown that the beneficial effects of *Azospirillum* on plants can be enhanced by co-inoculation with other microorganisms. Co-inoculation frequently increase growth and yield, compared to single inoculation, provide the plants with more balanced nutrition, increase availability of minerals and nutrients, and improve nitrogen and phosphorus economy [12,24,72,73].

In dual strain inoculum *A. brasilense* plus *G. diazotrophicus* reach a syntrophic metabolism, probably due to the invertase excreted by *G. diazotrophicus* [42] which breaks the sucrose present in the MS medium in glucose and fructose: glucose is preferably used by *G. diazotrophicus*, whereas fructose by *A. brasilense* which, instead, is unable to utilize either sucrose or glucose [74,75,84].

The scaling up from the *in vitro* experiments to *in vivo* ones can cause various problems because of the complexity of the soil-system and because of the numerous environmental factors that slow down or hamper the effects of the inoculation [74–76]. At the moment it is difficult to forecast the destiny of microorganisms inoculated in the soil, and it is difficult to evaluate the effects on them of chemical and physical features of the soil and of the agricultural activity. The successful use of microbial inoculants in agriculture depends essentially on a deeper knowledge of the rhizosphere.

Concerning the effect of the single bacteria: *A. brasilense* and *B. ambifaria* when inoculated on tomato plants *in vivo* experiments, stimulates their growth in a significant way; *G. diazotrophicus* and *H. seropedicae*, instead, reduce the yield with respects to the controls, lightly in the case of *H. seropedicae*. The four bacteria, inoculated together gave a synergetic effects in term global plant growth and development, compared to uninoculated plant [77,78]. This effect, however, is less visible in comparison with *in vitro* experiments. Some of the factors that may affect the performance of an endophyte *in vivo* are: nitrogen content of the soil [79,80], soil type [81] and host plant age and cultivar [81–83], sterile or unsterile soil, as well as if the support is coming from a previously inoculated culture [85,86]. For example, high-nitrogen fertilized soil reduced colonization of sugarcane by *G. diazotrophicus* and *H. seropedicae* [79,80,85,86]. Even the presence of any concentration of Ca_2^+ and $(PO_4)^{3-}$ above 50 mM in the media had a derogatory effect on the rate of *Azospirillum* adsorption on wheat root surface [87].

In summary, the present study clearly demonstrated that *A. brasilense*, *B. ambifaria*, *G. diazotrophicus* and *H. seropedicae* can colonize roots, stems, and leaves and increase the raise of *L. esculentum* cv. Guadalete plantlets.

B. ambifaria, in particular, has shown a positive role in the interaction. The very positive role that the strain may have in the spread of biocontrol and in avoiding – these really toxic – antifungal pesticides could warrant a reconsideration of the regulatory status of the individual Bcc species and a relaxation on the restriction on the use of selected Bcc strains also in the U.S. [6].

We plan to insert molecular markers also in this strain and in *G. diazotrophicus* to identify the plant-colonization dynamic, the location of both bacteria inside the host plant and to better understand the interactions between the four bacteria in their natural environment.

The present work wants to increase knowledge on plant–microbes interactions, and to develop an ecofriendly-nutrient source for tomato plant and restoring the agrosystem balance so as to contrast the causes of biodiversity decrease due to traditional agricultural practices.

We are conducting further experiments (unpublished results) of inoculation in the field to assess the effects of inoculation in a natural environment: the results are very encouraging and we do envisage a possible wider use of these strains in a short time.

We believe it may be feasible to convert the potential of this association into a standard inoculation practice in agriculture.

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