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ORIGINAL PAPER

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Effect of *Azospirillum* inoculants on arbuscular mycorrhiza establishment in wheat and maize plants

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Abstract Plant growth-promoting rhizobacteria and arbuscular mycorrhizal (AM) fungi represent two main groups of beneficial microorganisms of the rhizosphere. The role of different strains of *Azospirillum* on AM fungi development was evaluated by measuring the percentage of AM colonisation of the root system in durum wheat and maize plants, grown under both greenhouse and field conditions. The effect of wild-type *Azospirillum brasilense* strain Sp245 and genetically modified (GM) derivatives overproducing indole-3-acetic acid was assessed at greenhouse level in (1) three different cultivars of durum wheat, in the presence of indigenous AM fungi and (2) maize plants artificially inoculated with *Glomus mosseae* and *Glomus macrocarpum*. In addition, the establishment of natural AM fungal symbiosis was evaluated using *Azospirillum lipoferum* CRT1 in maize plants at field level. Despite the stimulatory effect of the different *Azospirillum* inocula on root growth, no significant differences in AM colonisation were found, independently of the AM fungus involved, either in wheat or in maize plants. Similarly, GM *A. brasilense*, which strongly stimulates root development, did not affect AM formation. Although these results were obtained in conditions in which the mycorrhization rate was moderate (15–30%), overall considered they indicate that the use of wild-type or GM *Azospirillum* phytostimulators does not alter mycorrhization.

Keywords *Azospirillum* · Arbuscular mycorrhizal fungi · IAA · Wheat inoculation · Maize inoculation

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Introduction

The beneficial effect of plant growth-promoting rhizobacteria (PGPR) as well as arbuscular mycorrhizal (AM) fungi on plants is well documented (Smith and Goodman 1999; Whipps 2001). Extensive genetic, biochemical and ecological studies have been carried out and *Azospirillum* is one of the best characterized genera of PGPR (Kloepper 1995; Bashan and Holguin 1997; Holguin et al. 1999; Steenhoudt and Vanderleyden 2000). Bacterial phytohormone production and nitrogen fixation are recognized as processes involved in plant growth promotion by azospirilli, leading to better root development and enhanced water and mineral uptake (Steenhoudt and Vanderleyden 2000; Dobbelaere et al. 2001). *Azospirillum* inoculant strains with enhanced indole-3-acetic acid (IAA) production ability can be achieved by genetic means, and the phytostimulatory potential of different genetically modified (GM) *Azospirillum brasilense* strains has been examined (Dobbelaere et al. 1999). Preliminary environmental impact assessments of GM *A. brasilense* strains have also been carried out, using field release experiments (Corich et al. 1995; Dobbelaere et al. 2001; Basaglia et al. 2003).

The AM symbiosis, a mutualistic plant–fungus association formed in a vast range of terrestrial environments

(Walker and Trappe 1993), plays an important role in plant mineral nutrition and plant health (Smith and Read 1997; Barea et al. 2002; Ferrol et al. 2002; Giovannetti et al. 2002), with a wide range of applications in sustainable agricultural systems (Schreiner and Bethlenfalvay 1995). Moreover, AM fungi can interact with a wide assortment of microorganisms in the rhizosphere (Giovannetti et al. 1994; Meyer and Linderman 1986; Edwards et al. 1998; Brulé et al. 2001; Barea et al. 2002).

Inoculated or naturally occurring microorganisms in the rhizosphere can affect AM fungi development (Germida and Walley 1996). When assessing the effect in the field of *Pseudomonas* inoculants used for biocontrol of soil-borne fungal pathogens, no effect was found on the percentage of root length that became mycorrhized (Barea et al. 1998). In addition, *Pseudomonas* improved plant growth and nutrient uptake by mycorrhiza. Inoculation with the GM strain *Pseudomonas fluorescens* F113(pCU203), which overproduces 2,4-diacetylphloroglucinol, did not affect the mycorrhization of *Allium cepa* plants (Barea et al. 1998). The effect of bacteria depends on the strains and the mycorrhizal fungi involved (Barea et al. 2002). Moreover, soil parameters, other microbial interactions and plant phenology may influence specific responses (Bashan 1999; Steenhoudt and Vanderleyden 2000; Barea et al. 2002).

Relatively little is known of the effect of *Azospirillum* on AM fungal colonisation and activity (Barea et al. 2002), both for wild-type and GM inoculants. When dealing with GM strains, understanding the ecological impact of functional modifications is important to define usage conditions for these beneficial microorganisms (Barea et al. 1998). Here, we describe the effect of wild-type and IAA-overproducing GM *Azospirillum* phytostimulators on the development of AM symbiosis in wheat and maize crops, grown in the greenhouse and/or under field conditions. The field experiment was done with the wild-type *Azospirillum lipoferum* CRT1, a strain of commercial significance, whereas greenhouse experiments made use of *A. brasilense* Sp245, a key model strain for analysis of IAA metabolism and enhancement of IAA production via genetic means. Particularly, the experimental plan concerned (1) three wheat cultivars in the presence of indigenous AM fungi, which was assessed in a pot experiment under controlled conditions; (2) maize plants inoculated with *G. mosseae* and *G. macrocarpum*, in a pot experiment under controlled conditions; and (3) maize plants in the presence of indigenous AM fungi, in the field.

Materials and methods

Bacteria and their cultivation

The bacterial strains used in this study are listed in Table 1. In the GM strains, the *ipdC* gene encoding an indole-3-pyruvate decarboxylase involved in the biosynthesis of IAA is controlled by the constitutively expressed promoter *pnptII* [in Sp245(pFAJ5002)] or the plant-regulated promoter *psbpA* [in Sp245(pFAJ5005)] (Van Bastelaere et al. 1999). A constitutively expressed *eyfp* gene, which is a enhanced variant of the gene encoding green fluorescent protein, *gfp* (Heeb et al. 2000), transcribed from the *pnptII* promoter, was introduced into the above plasmids to give Sp245(pFAJ0529) and Sp245(pFAJ0535), respectively. The *A. brasilense* strains were grown at 30°C on NfB medium (Day and Döbereiner 1976) supplemented with NH_4Cl (1 g/l), Congo red (25 mg/l) and ampicillin (100 mg/l). To ensure plasmid maintenance, tetracycline (10 mg/l) was added for strains Sp245(pFAJ5002) and Sp245(pFAJ5005), whereas both tetracycline (10 mg/l) and streptomycin (10 mg/l) were added for growth of Sp245 (pFAJ0529) and Sp245(pFAJ0535).

Table 1 *Azospirillum* strains used in this study and relevant traits

Strain	Description	Reference
A. brasilense Sp245	Wild-type strain	Baldani et al. 1986
A. brasilense Sp245 (pFAJ5002)	Tc^r, pLAFR3 derivative containing *ipdC* under the control of *pnptII* constitutive promoter	This work
A. brasilense Sp245 (pFAJ5005)	Tc^r, pLAFR3 derivative containing *ipdC* under the control of *psbpA* plant-inducible promoter	This work
A. brasilense Sp245 (pFAJ0529)	*eyfp*-marked derivative of pFAJ5002	This work
A. brasilense Sp245 (pFAJ0535)	*eyfp*-marked derivative of pFAJ5005	This work
A. lipoferum CRT1	Wild-type strain	Fages and Mulard 1988

Field assessment of the effect of commercial wild-type *Azospirillum* phytostimulator on mycorrhization of maize plants by indigenous AM fungi (experiment 1)

The effect of the commercial wild-type phytostimulator *A. lipoferum* CRT1 on AM development in maize was monitored in two consecutive years in a field experiment located at La Côte St André near Lyon, France. The soil was a luvisol (topsoil, loam; organic matter 2.1%; pH 7.0), and in both years mineral N (half NH_4^+, half NO_3^-) was added at 70 kg N/ha. In 2001, four plots were sown with non-inoculated maize seeds (cv. Eurostar) and four others with seeds inoculated with *A. lipoferum* CRT1 in a commercial (Nitragin) peat-based formulation (3×10^7 CFU added per seed). In 2002, the same treatments were applied again to the same plots (using maize cv. PR38a24), and, in addition, the field trial was repeated nearby (with the same two treatments but smaller plots) in an area grown with non-inoculated maize the year before.

In 2001, samplings were done at 7 (5 June; at 2–3 leaves), 35 (3 July; at 9–10 leaves) and 65 days after sowing (2 August). In 2002, samplings were performed at the first two phenological stages already investigated in 2001, i.e.

at 2–3 (13 May; 18 days after sowing) and 9–10 leaves (21 June; 57 days after sowing) to facilitate comparisons between the 2 years. *A. lipoferum* CRT1 was recovered at 10^6 CFU/g root or more at each sampling, using colony counts on NfB plates followed by colony hybridisations to 16S rDNA-targeted oligonucleotide probes targeting *A. lipoferum* (Kabir et al. 1994) or strain CRT1 (Jacoud et al. 1998), as described (Kabir et al. 1994; Jacoud et al. 1998). Inoculated plants exhibited enhanced rooting depth (at all samplings) and root biomass (at the last two 2001 samplings and the first 2002 sampling) compared with non-inoculated plants. At each sampling, four root systems were collected per plot (most roots were obtained from the first 40 cm), and AM colonisation levels were assessed according to the methods reported by Giovannetti and Mosse (1980), using three root subsamples taken from each of the root systems.

Greenhouse assessment of the impact of wild-type and GM *Azospirillum* on mycorrhization of three wheat cultivars by indigenous AM fungi (experiment 2)

A. brasilense strains Sp245, Sp245(pFAJ5002) and Sp245 (pFAJ5005) were grown overnight at 30°C in Luria-Bertani medium (Difco) supplemented with 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$ until the cells were in the late exponential growth phase. Cells were harvested by centrifugation (10 min at 4,300×*g*, at 4°C), resuspended in 0.9% NaCl solution and adjusted to 10^7 CFU/ml before use as an inoculum. Durum wheat seeds from three cultivars (Iride, Svevo and Baio; Società Produttori Sementi, Argelato, Bologna, Italy) were placed in pots (12 seeds per pot at a depth of 1–2 cm) containing 3 kg of non-sterile sandy loam soil (passed through a 4-mm sieve) from Pisa, Italy (organic matter 1.7%, pH 7.5; naturally infected by AM fungi). For each cultivar, seeds were non-inoculated or inoculated by wetting with 100 µl of cell suspension (10^6 CFU added per seed) of Sp245, Sp245(pFAJ5002) or Sp245(pFAJ5005). Each treatment combination (i.e. wheat cultivar×bacterial treatment) was replicated five times resulting in a total of 60 pots, and the pots were arranged in a randomised complete block design. The pots were placed in a greenhouse at 18–20°C with a 14/10-h light/dark period and 70% relative humidity. Soil moisture was adjusted to approximately 20% and distilled water was supplied when needed. At 60 days after sowing, plants were supplemented with urea (240 mg/pot) and 3 days later with KNO_3 (120 mg/pot).

At 39, 70 and 89 days after sowing, three roots per treatment were analysed for (1) AM colonisation, as reported by Giovannetti and Mosse (1980), and (2) population level of *Azospirillum* inoculants. For the latter analysis, rhizosphere soil was resuspended in 5 ml of 0.9% NaCl solution and cells extracted for 30 min using a rotary shaker at maximum speed. Serial dilutions were plated on semi-selective nitrogen-free NfB medium (Day and Döbereiner 1976) supplemented with antibiotics (when appropriate) and Congo red, and colony numbers were recorded after 3–4 days at 30°C. Since antibiotic resistance could not be used to select the wild-type strain Sp245, putative *Azospirillum* colonies from the Sp245 treatment were further analysed by amplified ribosomal DNA restriction analysis (ARDRA). For cell lysis, a single colony was suspended in 50 µl of lysis buffer (0.05 M NaOH, 0.25% SDS). The suspension was vortexed for 60 s, heated for 15 min at 95°C, centrifuged 10 min at 16,000×*g* and diluted 1/10 with sterile distilled water for polymerase chain reaction (PCR) amplification. The amplification of 16S rDNA was obtained by using the universal primers 27f and 1495r (Lane 1991; Weisburg et al. 1991). Digestion of PCR products (5 µl) was performed overnight at 37°C with a combination of the restriction enzymes *Alu*I, *Hpa*II and *Rsa*I in a total volume of 20 µl. The entire volume was analysed by agarose gel (2% w/v) electrophoresis in Tris–borate–EDTA (TBE) buffer containing 0.5 µg/ml ethidium bromide. DNA molecular marker VI (Boehringer, Mannheim, Germany) was used to determine fragment length. A PCR-based approach was also used to analyse putative GM inoculants by using the primers listed in Table 2. Primer sets VKnpt01/VKnpt02 and Azo211/Azo239 were used for plasmids pFAJ5002 and pFAJ5005, respectively. The PCR mixture contained 1 µl of diluted cell lysate (obtained as indicated above), 0.2 pmol/ml of each primer, 200 µM of each dNTP, 0.5 U Platinum Taq DNA polymerase (Gibco, Invitrogen Corporation, Vienna, Austria) and commercial buffer in a final volume of 25 µl. The PCR conditions used are as follows: an initial denaturation at 94°C for 7 min, 35 cycles with denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 2 min, and a final extension of 7 min at 72°C in a thermal cycler (Perkin-Elmer, Foster City, CA). The PCR products were analysed by agarose gel electrophoresis. Digital images were obtained using ImageMaster VDS (Pharmacia Biotech, Uppsala, Sweden).

Table 2 Primers for PCR analysis

Primer	Specificity	Sequence (5′–3′)	Gene target
27f	Universal primer	GAG AGT TTG ATC CTG GCT CAG	16S rDNA
1495r	Universal primer	CTA CGG CTA CCT TGT TAC GA	16S rDNA
VKnpt01f	*A. brasilense* Sp245 (pFAJ5002)	GGATCCCACGCTGCCGCAAGCACTCAGG	*pnptII*
VKnpt02r	*A. brasilense* Sp245 (pFAJ5002)	GGATCCAAGCTTATCCTGTCTCTTGATCAGATCT	*pnptII*
Azo211f	*A. brasilense* Sp245 (pFAJ5005)	GCCCATGTCTTCTTCGTTCACG	*psbpA/ipdC*
Azo239r	*A. brasilense* Sp245 (pFAJ5005)	ATGCGAGCTCTTTACTCTCCCGACCTTACTCCCG	*psbpA/ipdC*

Greenhouse assessment of the impact of wild-type and GM *Azospirillum* on mycorrhization of maize plants by introduced *Glomus mossae* and *Glomus macrocarpum* (experiment 3)

The experiment was done using the strains studied in the previous experiment, except that GM plasmids pFAJ5002 and pFAJ5005 were also marked with constitutively expressed *yefp*, resulting in pFAJ0529 and pFAJ0535, respectively. Hybrid maize (cv. Rustimax) was treated with bacterial inoculants, as reported above, using 10^6 CFU per plant at sowing and at 7 and 14 days after sowing. Non-treated seeds were used as a control. The mycorrhizal inoculum consisted of soil containing spores, external mycelium and root fragments obtained from pot cultures of *G. mosseae* and *G. macrocarpum* (culture collection of the Department of Agriculture, Chemistry and Biotechnology, University of Pisa, Pisa, Italy), with 17.5 g of pot culture soil in each pot. The seeds were placed in pots (one seed per pot at a depth of 2 cm) containing 2.5 kg of sandy loam soil from Pisa, Italy (organic matter 1.7%, pH 7.5), previously sieved through a 4-mm sieve.

The pots were arranged in a randomised complete experimental design, with ten replicates for each condition. Three plants per treatment were periodically selected at random and the roots analysed for AM colonisation and population levels of *Azospirillum* inoculants. Inocula monitoring was done as reported for the wheat experiment, with the exception that the colonies formed on plates by GM strains were counted under fluorescence using the Leica MZFLIII epifluorescent microscopy (500/20 nm excitation with 535/30 nm long-pass emission). Digital images (Leica Quantimet 500) of root samples (three replicates per sample) were analysed to measure their total profile area A_b defined as $A_b=2RL$, where R is the radius and L is the length. Based on the cylindrical shape of roots, the total root surface area A_r was computed using the formula $A_r=\pi A_b$ (Kokko et al. 1993).

Analysis of mycorrhiza formation

AM infection was assessed as described by Giovannetti and Mosse (1980). The roots were cleared in hot 10% KOH, acidified and stained in trypan blue (0.05% in lactophenol), destained in lactophenol and finally preserved in lactic acid. Each stained root sample was spread out on a square plastic Petri dish (10×10 cm) with a grid of lines on the bottom to form 0.5-cm squares. The presence or absence of infection was recorded through the microscope (magnification up to ×150) at each point where the root intersected a vertical or horizontal line. The calculation of infection is based on the formula of Newman (Giovannetti and Mosse 1980) and the entry-point evaluation was done as reported in Brundrett et al. (1985).

Analysis by confocal laser scanning microscopy

The roots of 2-week-old maize plants (cv. Rustimax) were dipped in the cell suspensions of *yefp*-labelled azospirilli (with an optical absorbance of approximately 0.8–1.1) for 5 min and then planted in potting soil and grown under the same greenhouse conditions as reported above. Three days after inoculation, three plants per treatment were taken for confocal laser scanning microscopy (CLSM) analysis. The roots were washed with phosphate-buffered saline (PBS, Sigma) to remove soil particles and fixed overnight in a 4% paraformaldehyde solution in PBS at 4°C. For long-term storage, the roots were subsequently placed in an ethanol–PBS mixture (1:1) at 20°C. CLSM analysis was done using a LeicaTCS SP2 with an acousto-optical beam splitter (AOBS) and Leica Confocal Software, version 2.5. The fluorescent protein excitation was done at 510 nm.

Data analysis

The data were subjected to analysis of variance (ANOVA). Significantly different means were separated at the 0.05 probability level by the least significant difference test (Snedecor and Cochran 1980). Percentages were transformed in angular values before statistical analysis.

Results

Effect of commercial wild-type *Azospirillum* phytostimulator on mycorrhization of field-grown maize by indigenous AM fungi (experiment 1)

In 2001, entry points were assessed both in control plants and in plants inoculated with *A. lipoferum* CRT1. Inoculation did not affect the percentage of the root length that was mycorrhized, at least during the first 2 months after sowing (46.3±6.3 per root in the control and 52.5±9.6 in the inoculated plants). Indeed, inoculation of maize seeds had no effect on the number of AM entry points in roots at 36 days (28.8±19.7 per root).

In 2002, similar results on the effect of a single inoculation with *A. lipoferum* CRT1 were obtained from an adjacent field experiment started that year. Inoculation did not affect the percentage of the root length that became mycorrhizal during the first 3 months after sowing (Fig. 1). The previous experiment, which was started in 2001, was repeated in 2002 to assess the cumulative effect of inoculations over two consecutive years. Here also, inoculation had no effect on the percentage of the root length that became mycorrhizal during the first 3 months after sowing (Fig. 1).

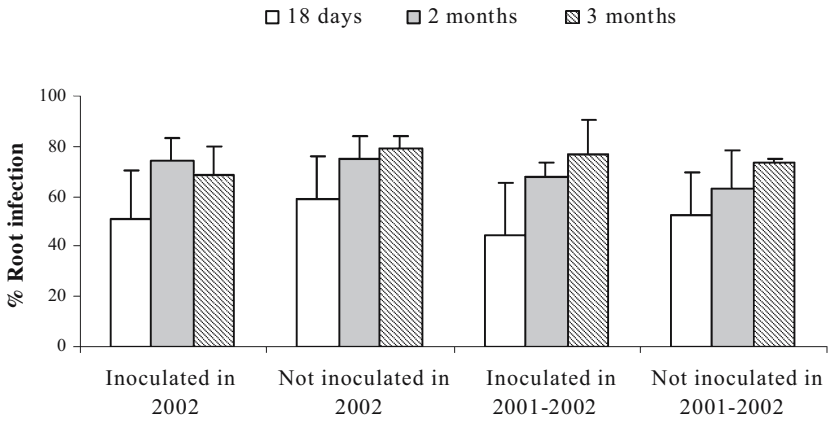

Fig. 1 Effect of *A. lipoferum* CRT1 on establishment of AM symbiosis with field-grown maize inoculated in 2002, analysed by two-way (Inoculated or Not×Inoculation 1 or 2 years) ANOVA. AM symbiosis was not significantly affected by inoculation, regardless of whether inoculation was performed in 2002 only or in 2001 and 2002. Results are the mean of three or more replicates with bars representing standard errors

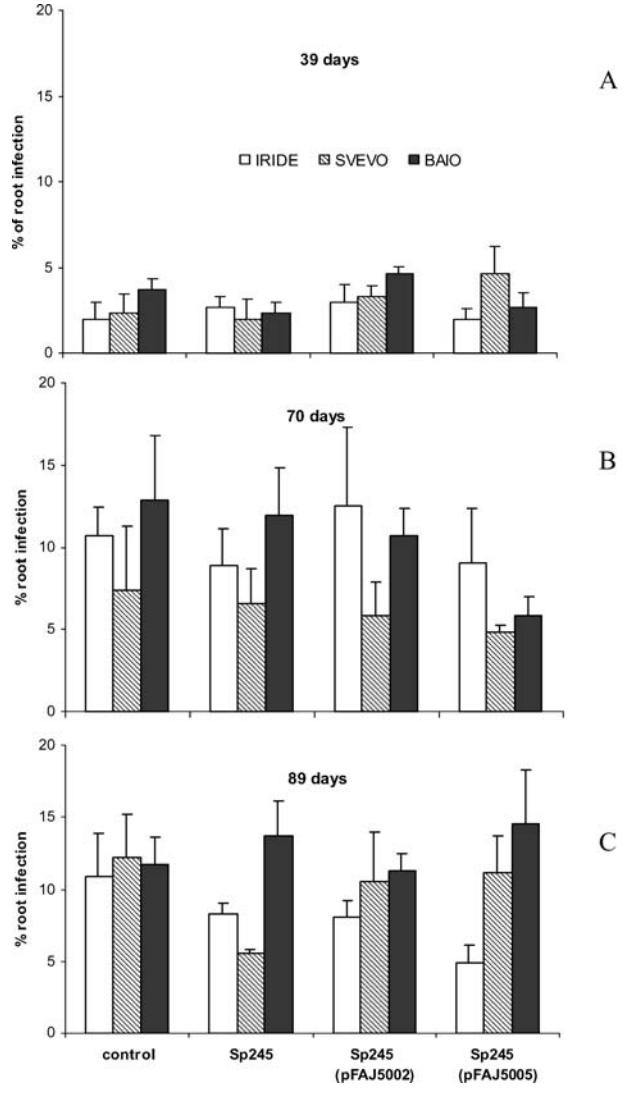

Fig. 2 Effect of *A. brasilense* Sp245 and GM derivatives Sp245 (pFAJ5002) and Sp245(pFAJ5005) on establishment of AM symbiosis with wheat cultivars Iride, Svevo and Baio in natural soil, at greenhouse level. Results are the mean of three replicates with bars representing standard errors

Impact of wild-type and GM *Azospirillum* on mycorrhiza formation of three wheat cultivars by indigenous AM fungi (experiment 2)

For each inoculant, cell number was 5.1 (±0.3)×10^7, 3.0 (±0.8)×10^3 and below 10^2 CFU/g dry root at 7, 14 and 89 days after sowing, respectively. The effects on AM symbiosis of *A. brasilense* strains Sp245, Sp245(pFAJ5002) and Sp245(pFAJ5005) inoculated at 10^6 CFU per seed of wheat cultivars Iride, Svevo and Baio are reported in Fig. 2. There was a clear increase of the percentage of root colonisation at 70 days of analysis with respect to the first sampling data (39 days). However, there was no further increase at the end of the experiment (89 days). A general analysis of the two sources of variability (*Azospirillum* strain and wheat cultivar) was carried out by a two-way ANOVA test (*Azospirillum* strain×wheat cultivar) on mycorrhization data. The results clearly indicate that the two sources of variability did not show any interaction and there was no effect of the strains inoculated on AM infection. The variety showed an effect on AM infection 89 days after

Table 3 Analysis of AM infection (%) of wheat by two-way ANOVA

	39 days	70 days	89 days
Cultivar			
Iride	2.42 a	10.30 a	8.02 a
Svevo	3.08 a	6.20 a	9.85 ab
Baio	3.33 a	10.33 a	12.83 b
Strain			
Control	2.67 a	10.34 a	11.63 a
Sp245	2.33 a	9.14 a	9.17 a
Sp245(pFAJ5002)	3.67 a	9.70 a	9.96 a
Sp245(pFAJ5005)	3.11 a	6.60 a	10.18 a

Percentages reported below were transformed in angular values before statistical analysis. The three (cultivars) or four values (strain) within the same columns followed by the same letters are not significantly different from one another at the 0.05 probability level

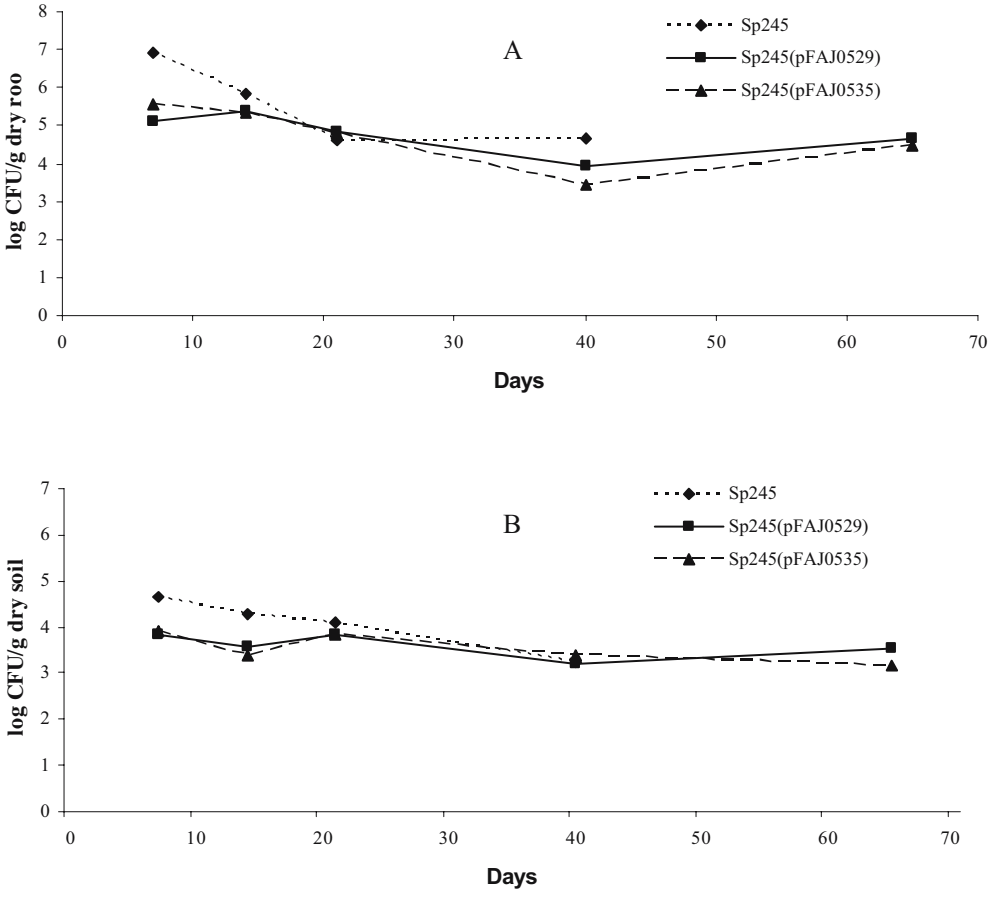

Fig. 3 Maize root (**a**) and soil bulk (**b**) colonisation by *A. brasilense* Sp245, Sp245 (pFAJ0529) and Sp245 (pFAJ0535). Results are the mean of three replicates

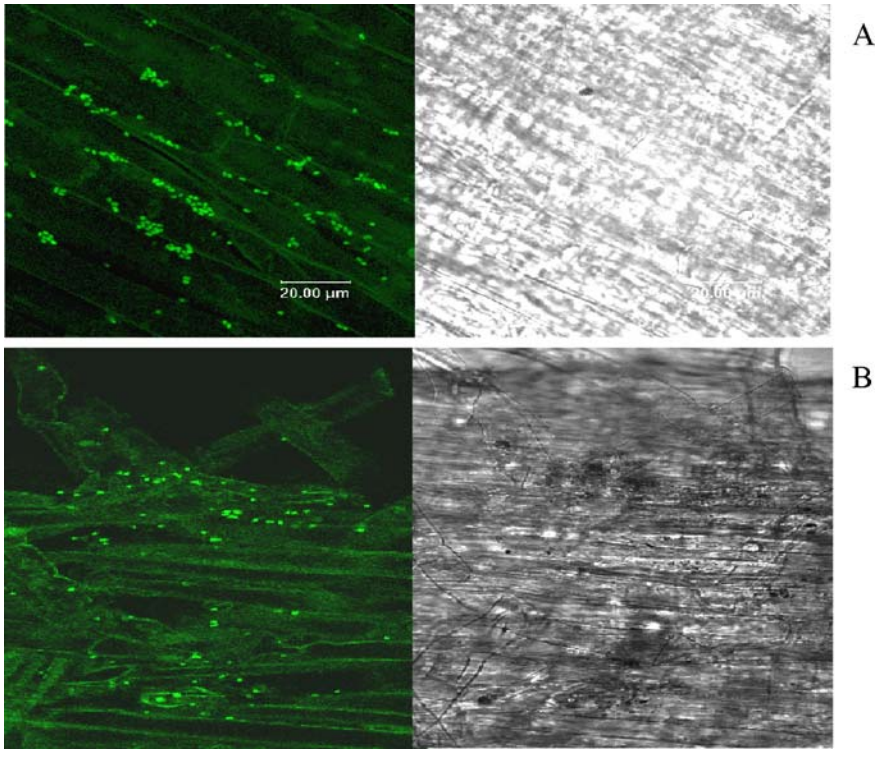

Fig. 4 Confocal laser scanning microscopy images of maize roots colonized by *eyfp*-tagged strain *A. brasilense* Sp245 (pFAJ0535), 14 days after sowing, within depression formed between epidermal cells (**a**) and to the tip and root hair zone (**b**). *Right*, under white light; *left*, under UV light

sowing. As reported in Table 3, in all plant/microbe combinations the percentage of AM infection at each sampling time was unaffected by bacteria inoculation, irrespective of the microbial inoculant used. At 89 days after sowing, differences between cultivars were statistically significant ($P<0.05$) with the highest value of AM mycorrhizal colonisation in cv. Baio (12.8±4.0%) compared with cv. Iride (8.0±2.6%) and Svevo (9.9±4.1%) (Fig. 2).

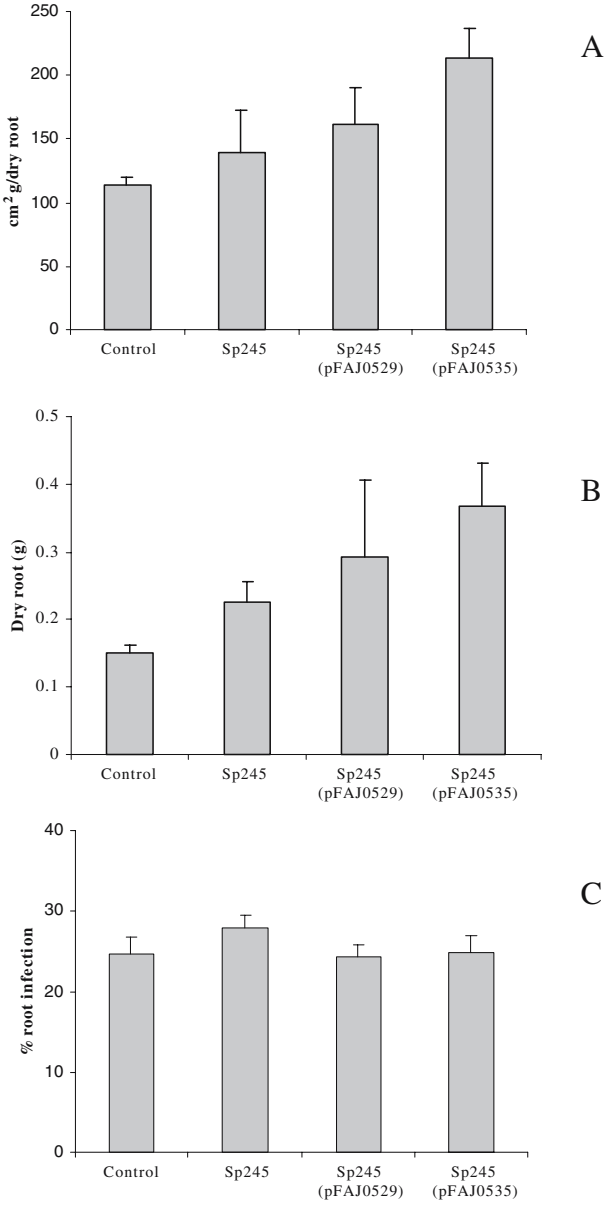

Fig. 5 Effect of *A. brasilense* Sp245, Sp245(pFAJ0529) and Sp245 (pFAJ0535) on (**a**) root surface area, (**b**) on root dry weight and (**c**) on establishment of AM symbiosis in maize, at greenhouse level, 65 days after sowing. Bars represent standard deviation of three independent determinations

Impact of wild-type and GM *Azospirillum* on mycorrhiza formation of maize plants by introduced *G. mossae* and *G. macrocarpum* (experiment 3)

In the second greenhouse experiment, GM plasmids equipped with *eyfp* gene were used to facilitate inoculant monitoring. The GM strains were identical to those used in the first greenhouse experiment with regard to *ipdC* expression. The insertion of the reporter gene as monitoring tool allowed one (1) to observe the adhesion of *Azospirillum* cells to maize roots and (2) to rapidly identify inoculant colonies among those of indigenous bacteria. Fig. 3a shows colonisation of the maize rhizosphere by *A. brasilense* inoculants after repeated bacterial inoculations (three inoculations in total with 10^6 CFU per plant at each inoculation). In all cases the bacteria were able to colonise and persist in the maize rhizosphere at a relatively high population density during the experiment (between 10^4 and 10^5 log CFU/g dry root). Inoculants could also be recovered from bulk soil, at 10^3 to 10^4 log CFU/g dry soil at 65 days (Fig. 3b). When assessed by microscopy, inoculant cells adherent to maize roots were readily found. This was particularly the case at the root tip and in the root hair zone, as well as within the depression formed between epidermal cells (Fig. 4).

The surface area of maize roots (Fig. 5a) and root dry weight (Fig. 5b) at the end of the experiment (65 days) were higher for plants inoculated with *A. brasilense* Sp245 (pFAJ0535) compared with other inoculated plants or non-inoculated plants. At 65 days, the mycorrhizal colonisation of maize roots was 20–30% in all the conditions considered. Indeed, statistical analysis indicated that inoculation treatments had no effect on mycorrhization levels. Significant differences between treatments were determined by least significant differences (LSD) tests at $P<0.05$ (Fig. 5c).

Discussion

In this work, the ecological impact of wild-type and GM *Azospirillum* inoculants on mycorrhization by AM fungi was assessed following a two-step strategy. In the first step, a field assessment was carried out with a wild-type strain of commercial significance, which was delivered to seeds using a proprietary formulation, and the field was managed under standard farming conditions. One of the main commercial strains in Europe is *A. lipoferum* CRT1, which has been proposed mainly for phytostimulation of maize. Results indicated that inoculation had no impact on the percentage of mycorrhization, but since inoculated plants displayed enhanced root development it actually means that the total mycorrhization level, i.e. at the scale of the whole plant, was increased. Several studies have suggested that PGPR do not exert an antagonistic effect against AM fungi (Calvet et al. 1993; Vázquez et al. 2000), a potential concern when dealing with inoculants such as biocontrol

pseudomonads, which produce antimicrobial secondary metabolites (Vázquez et al. 2000). In the case of phytostimulators like *Azospirillum*, the possibility of beneficial effects on AM fungi (e.g. on their activity level) has been shown before (Alarcón et al. 2002). Actually, an indirect effect of *Azospirillum* on mycorrhization can be assumed as a consequence of the positive effect on root growth. In the second part of this investigation, the assessment focused on the effect of GM *Azospirillum* inoculants exhibiting enhanced phytostimulation properties. The prominent model strain in which genetic improvement is being sought is the wheat isolate *A. brasilense* Sp245. Assessment was done under greenhouse conditions because in Europe the use of GM strains in the field is restricted. Results indicated that here again, inoculation with a wild-type *Azospirillum* phytostimulator did not interfere with mycorrhizal formation. More importantly, genetic modifications aimed at optimising production of the phytohormone IAA, which resulted in enhanced phytostimulation of root development and growth, did not have any negative side effects on mycorrhization. These findings were obtained against the background that other biotic factors, here the wheat cultivar, did have an effect on mycorrhization. However, root mycorrhization of wheat at greenhouse level was below 15%, which are values usually found with non-mycotrophic species (Siqueira and Saggin-Junior 2001). This contrasts with data from field-grown maize (almost 80% maize root mycorrhization) and prompted us to seek confirmation of the findings in a maize greenhouse experiment in which AM fungi were inoculated.

In the second greenhouse experiment, AM symbiosis development in maize resulting from inoculated AM fungi was higher than that observed in the previous experiment (25–30% vs 15%). However, impact assessment results were comparable to those of the previous greenhouse experiment, i.e. the enhancement of the phytostimulatory effects on root development and growth resulting from genetic modification of the *Azospirillum* inoculant took place without detrimental effects on mycorrhization. Rather, since the corresponding plants displayed enhanced root biomass, it can be concluded that the total mycorrhization level, i.e. at the scale of the whole plant, was in fact increased. Inoculation of AM fungi had no effect on root colonisation by *Azospirillum* inoculants, and in fact the same colonisation patterns were obtained in soil inoculated with AM fungi and in non-inoculated, AM fungus-free soil (data not shown).

The data obtained in the present study are derived from experiments in which the inoculants colonized the roots at high cell density. This means that *Azospirillum* cells were present also at the root sites where mycorrhizal hyphae interacted with the plants. This interpretation is supported by the CLSM, showing that the bacterial cells were evenly distributed over all the plant root surfaces (see Fig. 4).

Based on these field and greenhouse experiments, it can be concluded that the *Azospirillum*–AM fungus combination seems suitable for sustainable agriculture practices and that both types of microorganisms are compatible with one another for dual inoculation strategies.

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