Appl Microbiol Biotechnol (2013) 97:4639–4649
DOI 10.1007/s00253-012-4249-z

ENVIRONMENTAL BIOTECHNOLOGY

Comparison of prominent *Azospirillum* strains in *Azospirillum–Pseudomonas–Glomus* consortia for promotion of maize growth

Olivier Couillerot · Augusto Ramírez-Trujillo · Vincent Walker · Andreas von Felten · Jan Jansa · Monika Maurhofer · Geneviève Défago · Claire Prigent-Combaret · Gilles Comte · Jesus Caballero-Mellado · Yvan Moënne-Loccoz

Received: 21 March 2012 / Revised: 7 June 2012 / Accepted: 14 June 2012 / Published online: 18 July 2012

Abstract *Azospirillum* are prominent plant growth-promoting rhizobacteria (PGPR) extensively used as phytostimulatory crop inoculants, but only few studies are dealing with *Azospirillum*-containing mixed inocula involving more than two microorganisms. We compared here three prominent *Azospirillum* strains as part of three-component consortia including also the PGPR *Pseudomonas fluorescens* F113 and a mycorrhizal inoculant mix composed of three *Glomus* strains. Inoculant colonization of maize was assessed by quantitative PCR, transcription of auxin synthesis gene *ipdC* (involved in phytostimulation) in *Azospirillum* by RT-PCR, and effects on maize by secondary metabolic profiling and shoot biomass measurements. Results showed that phytostimulation by all the three-component consortia was comparable, despite contrasted survival of the *Azospirillum* strains and different secondary metabolic responses of maize to inoculation. Unexpectedly, the presence of *Azospirillum* in the inoculum resulted in lower phytostimulation in comparison with the *Pseudomonas–Glomus* two-component consortium, but this effect was transient. *Azospirillum*'s *ipdC* gene was transcribed in all treatments, especially with three-component consortia, but not with all plants and samplings. Inoculation had no negative impact on the prevalence of mycorrhizal taxa in roots. In conclusion, this study brought new insights in the functioning of microbial consortia and showed that *Azospirillum–Pseudomonas–Glomus* three-component inoculants may be useful in environmental biotechnology for maize growth promotion.

Keywords PGPR · *Azospirillum* · *Pseudomonas* · *Glomus* · Microbial consortia · Inoculum survival · Maize

This paper is dedicated to the late Jesus Caballero-Mellado.

Electronic supplementary material The online version of this article (doi:10.1007/s00253-012-4249-z) contains supplementary material, which is available to authorized users.

O. Couillerot · V. Walker · C. Prigent-Combaret · G. Comte · Y. Moënne-Loccoz (✉)
Université de Lyon,
69622 Lyon, France
e-mail: yvan.moenne-loccoz@univ-lyon1.fr

O. Couillerot · V. Walker · C. Prigent-Combaret · G. Comte · Y. Moënne-Loccoz
Université Lyon 1,
Villeurbanne, France

O. Couillerot · V. Walker · C. Prigent-Combaret · G. Comte · Y. Moënne-Loccoz
Ecologie Microbienne, CNRS, UMR5557,
Villeurbanne, France

A. Ramírez-Trujillo · J. Caballero-Mellado
Centro de Ciencias Genómicas (CCG),
Universidad Nacional Autónoma de México,
Cuernavaca, Mor., México

A. von Felten · M. Maurhofer · G. Défago
Institute of Integrative Biology, ETH Zurich,
Universitätstrasse 2,
CH-8092 Zürich, Switzerland

J. Jansa
Institute of Agricultural Sciences, ETH Zurich,
Eschikon 33,
CH-8315 Lindau, Switzerland

Introduction

Azospirillum are prominent plant growth-promoting rhizobacteria (PGPR) used as inoculants for phytostimulation of several types of crops (mainly cereals) under different climatic conditions, and they may lead to improved crop yields (Charyulu et al. 1985; Okon and Labandera-Gonzalez 1994; Dobbelaere et al. 2001; Pedraza et al. 2009). This environmental biotechnology is also receiving attention as a mean to reduce chemical fertilizer doses without affecting crop yield, and can thus be evaluated as a component of integrated management strategies in agriculture (El Zemrany et al. 2006; Fuentes-Ramirez and Caballero-Mellado 2006; Adesemoye et al. 2009). Several modes of action have been documented in *Azospirillum* PGPR, noticeably nitrogen fixation (James 2000), nitric oxide production (Creus et al. 2005), and 1-aminocyclopropane-1-decarboxylate deaminase activity (Prigent-Combaret et al. 2008), but production of phytohormones such as auxins is often proposed as the main phytobeneficial mechanism (Dobbelaere et al. 2003).

Interactions of *Azospirillum* PGPR inoculants with other rhizosphere microorganisms have been considered by studying the ecological impact of inoculation on non-target, resident microorganisms and the effect on plant of mixed inocula involving *Azospirillum* strains. On one hand, studies on the ecological impact of *Azospirillum* inoculation did not evidence any positive interaction with indigenous microorganisms (Basaglia et al. 2003; Russo et al. 2005; Herschkovitz et al. 2005a, b; Lerner et al. 2006; Naiman et al. 2009; Baudoin et al. 2009b). On the one hand, the potential use of *Azospirillum* PGPR strains in mixed inocula is promising (Bashan 1998; Cassan et al. 2009; Singh et al. 2010; Combes-Meynet et al. 2011), but it remains poorly documented. By combining microorganisms with different metabolic capacities (N_2 fixation, P mobilization, production of phytohormones, and antimicrobials, etc.), we could expect additive or synergistic effects resulting from the combination of different phytobeneficial capacities, which may be important to enhance performance consistency.

Several studies have focused on *Azospirillum* dual-inoculation with (1) other *Azospirillum* strains (Han and New 1998; Bashan et al. 2000), (2) *Bacillus* (El-Komy 2005), (3) *Bradyrhizobium* (Steinberg et al. 1989; Cassan et al. 2009), (4) phosphate-solubilizing bacteria (*Arthrobacter* or *Agrobacterium*; Belimov et al. 1995), (5) *Rhizobium* (Raverkar and Konde 1988; Er et al. 2004; Remans et al. 2008), (6) *Pseudomonas* (Joe and Sivakumar 2010; Combes-Meynet et al. 2011; Couillerot et al. 2011, Khorshidi et al. 2011,) and (7) *Glomus* (Mar Vázquez et al. 2000; Pulido et al. 2003), but only some of the combinations have shown enhanced plant growth stimulation compared to single inoculation (Belimov et al. 1995; El-Komy 2005; Remans et al. 2008; Combes-Meynet et al. 2011). To our knowledge, five studies have focused on interactions established in complex, *Azospirillum*-based mixed-inocula involving more than two other microorganisms, i.e., *Rhizobium* and arbuscular mycorrhizal fungi (AMF; Biró et al. 2000), *Azotobacter* and AMF (Singh et al. 2010), *Burkholderia, Gluconacetobacter* and *Herbaspirillum* (Oliveira et al. 2009), *Pseudomonas* and *Rhizobium* (Khan et al. 2009), or *Pseudomonas* and AMF (Walker et al. 2012). Depending on the strain combination, microbial interactions within these consortia had positive or negative effects on inoculant establishment on roots and resulted or not in enhanced plant growth in comparison with single inoculation, highlighting the importance of identifying synergistic strain combinations.

In the case of maize, the use of *Azospirillum-Pseudomonas-Glomus* consortia under low-fertilizer input conditions resulted in enhanced root system development compared with the non-inoculated control (Walker et al. 2012), but these positive effects might be enhanced if selection of the microbial partners was optimized. In this study, three prominent *Azospirillum* strains were compared when inoculated as part of a three-component consortium also including another PGPR inoculant (*Pseudomonas fluorescens* F113) and an AMF inoculant mix composed of three *Glomus* species. *P. fluorescens* F113 is another PGPR extensively studied as crop inoculant, and this strain has been shown to be a mycorrhiza helper bacteria (MHB; Garbaye 1994; Barea et al. 1998). In the case of maize, *Azospirillum lipoferum* CRT1 is one of the important PGPR strains used in Europe (Jacoud et al. 1998; Lucy et al. 2004; El Zemrany et al. 2006), whereas *Azospirillum brasilense* UAP-154 and CFN-535 inoculants are extensively used under agronomic conditions in Mexico, which is one of the leading countries in field inoculation (Dobbelaere et al. 2001; Fuentes-Ramirez and Caballero-Mellado 2006). Phytostimulation by *Azospirillum* strains has been shown to be cell-density dependant (Jacoud et al. 1999), and effective early root colonization is often required for effective stimulation (Dobbelaere et al. 2002).

Thus, the objective of this study was to compare *A. lipoferum* CRT1, *A. brasilense* UAP-154 and CFN-535 in *Azospirillum–Pseudomonas–Glomus* consortia for early promotion of maize growth. The experiment was performed under greenhouse conditions. Monitoring focused on rhizosphere survival of inoculated PGPR, root mycorrhization (noticeably to check lack of negative impact on indigenous AMF taxa), as well as on the effects on plant growth and on plant metabolic markers indicative of established plant–microbe interactions.

Materials and methods

Microorganisms

Bacteria used in this study are deposited at the MIAE strain collection in Dijon, France (http://www2.dijon.inra.fr/

umrmse/spip.php?rubrique47), except for commercial strains *A. brasilense* UAP-154 and CFN-535 available at UNAM's Centro de Ciencias Genómicas in Cuernavaca, Mexico (http://www.ccg.unam.mx/en). *A. brasilense* strains UAP-154 and CFN-535 were both isolated from maize in Mexico (Dobbelaere et al. 2001), *A. lipoferum* CRT1 (MIAE 00337) was recovered from maize in France (Fages and Mulard 1988) and *P. fluorescens* F113 (MIAE 00794) was isolated from sugarbeet in Ireland (Fenton et al. 1992). To obtain *Azospirillum* inocula, strains were grown in NFb liquid medium (Döbereiner et al. 1976) supplemented with NH_4Cl (0.2 gL^{-1}) for 2 days at 30 °C with shaking (200 rpm). The cells were then washed two times with $MgSO_4$ 10 mM and the suspension adjusted to an optical density (OD_{600}) of 0.6, giving 5×10^7 (for *A. lipoferum* CRT1), 2×10^9 (for *A. brasilense* UAP-154) and 7×10^9 (for *A. brasilense* CFN-535) CFU/mL. *P. fluorescens* F113 was grown in Luria Bertani medium supplemented with 0.25 gL^{-1} $MgSO_4{\cdot}7H_2O$ (LB-Mg) for 8 h at 30 °C and 200 rpm. The cells were washed two times with $MgSO_4$ 10 mM and adjusted to an OD_{600} of 0.2, giving 2×10^7 CFU/mL. Colony counts of inocula were obtained on RC agar (Rodriguez Caceres 1982) for *Azospirillum* strains and LB-Mg agar for *P. fluorescens* F113, after a 72-h incubation of plates at 30 °C.

The mycorrhizal inoculum consisted of a mixture of the Swiss isolates *Glomus intraradices* JJ291 (BEG accession 158 at the International Bank for the Glomeromycota; www.kent.ac.uk/bio/beg/), *Glomus claroideum* JJ360 (BEG 155), and *Glomus mosseae* JJ964 (BEG 161) (Jansa et al. 2005). Each was produced in plant cultures following commercial procedures by Symbio-M (Lanškroun, Czech Republic). Mycorrhized roots were chopped, mixed together and with zeolite carrier. The inoculum product contained 5.3×10^7 (*G. intraradices* JJ291), 2.9×10^7 (*G. claroideum* JJ360), and 2.5×10^6 (*G. mosseae* JJ964) gene copies of the nuclear Large ribosomal Sub-Unit (nLSU) of the respective AMF species per gram, as determined using the method of Thonar et al. (2011).

Greenhouse experiment

A greenhouse experiment was performed with sieved (4 mm) non-sterile soil taken from the loamy-sandy surface horizon of a Mexican oxisol from a field at Zacatepec near Cuernavaca, Morelos (clay 4.8 %, silt 7.9 %, sand 87.3 %, organic matter 4.3 %, pH 7.5). Seeds of maize (*Zea mays*) var. Costeño Mejorado (PROSASOL, Huitchila Morelos, Mexico) were surface-disinfected by stirring in sodium dichloroisocyanurate-containing Bayrochlor Mini solution (Bayrol, Dardilly, France) for 15 min, and washed several times with sterile distilled water (Couillerot et al. 2010a). The seeds were then germinated on water agar (8.5 gL^{-1}) for 24 h in the dark at 30 °C.

Treatments included (1) a non-inoculated control, (2) inoculation with a two-component consortium composed of *P. fluorescens* F113 and *Glomus* mix, and (3) inoculation with a three-component consortium containing the two-component consortium and either *A. lipoferum* CRT1, *A. brasilense* UAP-154 or CFN-535. For each bacterial strain, inoculation was done by adding 1 mL of cell suspension (described above) to each germinated seed. In addition, 65 g of zeolite-formulated *Glomus* inoculum was placed approximately 3 cm below each germinating seed. Sterile water (2 mL) and non-inoculated zeolite (65 g) were used in the non-inoculated control and 1 mL sterile water in the *Pseudomonas–Glomus* treatment.

For the 10-day sampling, four maize plants were grown in 1-dm^3 pots containing 1.5 kg soil previously supplemented with 270 mL sterile nutrient solution (described in Rodriguez-Salazar et al. 2009). For the later samplings (i.e., at 21 and 35 days), two maize plants were grown in 2-dm^3 pots containing 2.3 kg soil previously supplemented with 340 mL of sterile nutrient solution. Five pots were used per treatment at each sampling, and the 75 pots were placed in a greenhouse (randomized block design) located at Cuernavaca (Mexico), with controlled temperature (26±4 °C) and natural light. Watering was done by adding 270 and 340 mL of nutrient solution each day in 1 and 2 dm^3 pots, respectively.

Sampling

Watering of the pots was reduced 48 h before the second and third samplings and stopped 24 h before each sampling. At each sampling, all shoots were cut off and dried for 2–4 days at 70 °C for biomass determination; one root system per pot was used for *ipdC* RT PCR analysis, and another root system per pot for real-time PCR quantification of PGPR inoculants (and AMF genotypes at the 35-day sampling, after splitting the root system in two parts). In addition, two other root systems per pot were used for plant metabolomic analysis at the first sampling (10 days).

DNA preparation

For PGPR inoculant monitoring, each root system was shaken vigorously to discard soil loosely adhering to the roots. Roots and tightly adhering soil were transferred in a 50-mL Falcon tube and flash-frozen in liquid nitrogen. Samples were then lyophilized for 48 h in Falcon tubes and homogenized by crushing in the tubes using a spatula, and 250–300 mg of lyophilized sample (rhizosphere soil+roots) were transferred in Lysing Matrix E tubes from the FastDNA® SPIN® kit (BIO 101 Inc., Carlsbad, CA). DNA was then extracted and eluted in 50 μL of sterile ultra-pure

water, according to the manufacturer's instructions. DNA concentrations were assessed by OD measurements at 260 nm using NanoDrop (Nanodrop technologies, Wilmington, DE).

For AMF monitoring, roots from the third sampling were cut in 5-cm pieces. They were washed in ice-cold tap water, flash-frozen in liquid nitrogen, and lyophilized for 48 h in Eppendorf tubes. Lyophilized roots samples (25–35 mg) were homogenized by dry bead-beating three times 45 s with glass balls (1 mm diameter) in Biospec Beadbeater-8 (Fisher Scientific AG, Wohlen, Switzerland). DNA was then extracted with Plant DNeasy kit (Qiagen, Courtaboeuf, France) following manufacturer's recommendations.

Real-time PCR assessments

Bacterial root colonization was assessed by real-time PCR, as described in Couillerot et al. (2010a; for *A. brasilense* inoculants), Couillerot et al. (2010b; for *A. lipoferum* inoculant) and von Felten et al. (2010; for *P. fluorescens* inoculant). Briefly, real-time PCR for *Azospirillum* strains was done using the LightCycler® FastStart DNA Master SYBR® Green I kit and a LC-480 LightCycler (Roche Applied Science, Indianapolis, IN), and that for *P. fluorescens* F113 using the Fast SYBR® Green I kit and a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Primers are described in Table S1.

AMF root colonization was assessed by real-time PCR targeting the nLSU RNA genes of *G. claroideum, G. mosseae, G. intraradices, Gigaspora, Scutellospora* (as described by Thonar et al. 2011) and *Diversispora* (as described by Wagg et al. 2011). Primers are described in Table S1. Additionally, two TaqMan markers targeting the mitochondrial Large ribosomal Sub-Unit (mtLSU) RNA gene of the *G. intraradices* species (i.e., mt5 marker) or the strain *G. intraradices* JJ291 (i.e., mt4 marker) were designed and validated (Table S2). Briefly, the inoculant strain *G. intraradices* JJ291, as well as the species *G. intraradices, G. claroideum,* and *G. mosseae* (to which the inoculants belonged) and the AMF genera *Gigaspora, Scutellospora*, and *Diversispora* were assessed using LightCycler® TaqMan® chemistry and a LightCycler 2.0 (Roche Applied Science), as described previously (Thonar et al. 2011; Wagg et al. 2011) or in the current supplementary information.

Melting curve calculation and determination of Tm values were performed using the polynomial algorithm function of LightCycler Software v.1 (Roche Applied Science) or of the Sequence detection Software v.1.4 (Applied Biosystems).

Normalization of the data

Plasmid APA9 (i.e., pUC19 with cassava virus insert; Genbank accession number AJ427910) was used as internal standard to normalize C_T values. Real-time analyzes for *Azospirillum, Pseudomonas,* and *Glomus* AMF were carried out in different laboratories with different LightCyclers, so normalization was done separately. The protocol for *A. brasilense* inoculants is described in Couillerot et al. (2010a), for *A. lipoferum* inoculant in Couillerot et al. (2010b), for *P. fluorescens* inoculant in von Felten et al. (2010), and for AMF in Thonar et al. (2011). Briefly, known quantities of purified plasmid APA9 were added at the first step of each DNA extraction protocol, and real-time PCR analyzes with specific markers targeting the internal standard sequence were then performed on each DNA extract to estimate recovery rates of the internal standard. C_T values thus obtained for the internal standard were used to normalize DNA extraction efficiency (Park and Crowley 2005).

Generation of standard curves for real-time PCR assessments

Real-time PCR quantification of PGPR inoculants in the rhizosphere required development of standard curves. Briefly, Lysing-Matrix E tubes (BIO 101 Inc.) containing 250–300 mg lyophilized sample (i.e., rhizosphere soil+roots) from the non-inoculated control (obtained as described above) were inoculated with one of the four PGPR strains. DNA extraction was performed using the FastDNA® SPIN® kit (BIO 101 Inc.) and real-time PCR was done as described above. A standard curve for each strain was generated by plotting the C_T number against the logarithm of CFU added per g of soil, for the three independent replicates. Amplification efficiency was calculated from the slope of the standard curve using the formula $E = 10^{-1/\text{slope}} - 1$ and standard curves were then used to estimate inoculant cell number in the rhizosphere of seed-inoculated maize plants. Real-time PCR quantification data were expressed as log cell equivalents per g of lyophilized rhizosphere sample (i.e., roots+tightly adhering soil).

Real-time PCR assays of the different AMF phylotypes was calibrated by using serially diluted cloned fragments (pGEM-T Easy vector, Promega) of the corresponding mtLSU, as described by Jansa et al. (2008) and Thonar et al. (2011). Real-time PCR quantification data were converted to gene copy number per gram of lyophilized root.

ipdC reverse transcriptase PCR analysis

Primers ipdCF3 (5′-CTTGCCCTTCTTCAAGGTGG-3′) and ipdCR3 (5′-GGGGGATTTCCAGATAGACC-3′) were designed for transcription analysis of auxin synthesis gene *ipdC* in *Azospirillum* spp., after alignment of all published *ipdC* sequences. Primer ipdCF3 displays 0 or 1 mismatch with *Azospirillum ipdC* sequences (n=8) and at least 4

mismatches with the other *ipdC* sequences (n=11), whereas primer ipdCR3 displays 0 mismatch with *Azospirillum ipdC* sequences and at least two mismatches with the other *ipdC* sequences (Fig. S2). Primer validation was performed by PCR (as described below) using genomic DNA from 33 *Azospirillum* strains, which showed that primers amplified *ipdC* in all 15 *A. brasilense* strains (including the two inoculants), in 2 of 11 *A. lipoferum* strains (including strain CRT1) and in *Azospirillum doebereinerae* GSF71, but not in six strains from *Azospirillum canadense, Azospirillum halopraeferens, Azospirillum irakense, Azospirillum oryzae*, or *Azospirillum zeae* (confirmed by sequencing).

Transcription of *ipdC* by *Azospirillum* spp. was measured by reverse-transcriptase (RT) PCR. Each studied root system (with tightly adhering soil) was placed in a 50-mL Falcon tube and flash frozen in liquid nitrogen. Samples were then washed with 35 mL solution of 100 mM $CaCl_2$ and 50 mM Tris–HCl pH 7.0 (prepared with 0.5 %*v*/*v* DEPC treated water) supplemented with β-mercaptoethanol (5 %). This solution was centrifuged for 5 min at 6×*g*. Soil particles and root debris were discarded and the supernatant was centrifuged for 5 min at 2,250×*g*. The resulting cell pellet was resuspended in 1 mL of TRIZOL reagent (Invitrogen, Carlsbad, CA). DNAse treatment was then performed and cDNA synthesis was done with RevertAid™ H minus cDNA synthesis kit (Fermentas, Ontario, Canada). Low amounts of RNA were recovered, so only 600 ng of RNA were used per reaction. Amplification was done with 1.0 U of *Taq* DNA polymerase (Invitrogen), 2 μL of synthesized cDNA as template, 1× reaction buffer, 10 % DMSO, 1.5 mM $MgCl_2$, 200 μM deoxynucleosides triphosphates, and 0.4 pmol of primers ipdCF3/ipdCR3. Because amplification was not observed directly in PCR assays, it was necessary to repeat it. For the second amplification, 4 μL of PCR product were diluted in 100 μL of water and 2 μL were used. Both PCR amplifications consisted of an initial denaturation for 5 min at 95 °C, 35 PCR cycles (30 s denaturation at 95 °C, 30 s annealing at 58 °C, and 30 s elongation at 72 °C), a final elongation for 10 min at 72 °C, and a cooling step at 5 °C.

Metabolomic analysis

At 10 days, the root systems from two plants per pot were washed with ice-cold distilled water and placed in aluminum envelops before being flash-frozen in liquid nitrogen. Samples were then lyophilized for 72 h and stored at −80 °C until analysis. Freeze-dried roots were introduced in Eppendorf tubes, to which liquid nitrogen was added. Roots were crushed using a ball mill (TissueLyser II, Qiagen), and extraction was performed using 2 mL methanol for 10 mg of dry sample, as described by Walker et al. (2011). Extraction was done twice and extracts were dried using Speedvac-assisted evaporation. Each sample was then resuspended in methanol to reach 10 mg dry extract per milliliter.

Chromatographic analysis of the extracts was achieved with an Agilent 1200 series HPLC (Agilent Technologies, Santa Clara, CA) equipped with a degasser (G132A), a quaternary pump module (G1311A), an automatic sampler (G1329A) and a Diode Array Detector (DAD G1315B), as described by Walker et al. (2011). The separation was carried out at room temperature using a NUCLEODUR sphinx C18 column (250×4.6 mm; 5 μm-Macherey-Nagel®, Düren, Germany). For each sample, 20 μL of extract was injected and the column was eluted at 1 mL min^{-1}, with an optimized gradient established using solvents A (acetic acid 4‰ (*v*/*v*) in water) and B (acetic acid 4‰ (*v*/*v*) in acetonitrile) (Carloerba ® reagents, Val de Reuil, France). A step by step gradient was used with an increase of proportion of solvent B until 15 % during 5 min, then an isocratic level from 30 min, with a flux of 1 mL per minute. Chromatograms were recorded and processed at 254, 280, 310, and 366 nm. The Chemstation Agilent software was used for integration and comparison of chromatograms. Each chromatogram was integrated after standardization of integration parameters. Background peaks present on chromatograms were not integrated. Individual compounds were identified based on the results of Walker et al. (2011).

Statistics

Statistical analyses of real-time quantification data, *ipdC* expression data, and shoot biomass were performed at $P<0.05$, using S plus software (TIBCO Software Inc., Palo Alto, CA). Chromatographic data obtained from root extracts, i.e., retention time and relative area of each integrated peak, were compiled in a matrix for discriminant principal component analysis (PCA), as described by Walker et al. (2011). Treatments were studied by ANOVA followed with Fisher's LSD tests ($P<0.05$).

Results

Effect of microbial consortia on maize growth

By comparison with the non-inoculated control, inoculation of maize with the *Pseudomonas–Glomus* two-component consortium (i.e., *P. fluorescens* F113 and a mix of three *Glomus* isolates) resulted in higher shoot biomass at the first two samplings (Table 1). Shoot biomass was also higher in all three-component consortia than in the non-inoculated control at the first two samplings, but results were not influenced by the identity of the *Azospirillum* strain (Table 1). In addition, *Azospirillum* addition did not give any positive effect by comparison with the two-component

Table 1 Effect[a] of consortium[b] inoculation on shoot dry weight (gram per plant) of maize (mean±standard error; $n=5$)

Sampling	Non-inoculated control	Two-component control	Three-component consortia		
			CRT1	UAP-154	CFN-535
10 days	0.134±0.002[c]	0.174±0.004[a]	0.159±0.004[b]	0.159±0.002[b]	0.160±0.005[b]
21 days	1.421±0.043[b]	1.591±0.048[a]	1.568±0.050[a]	1.588±0.057[a]	1.711±0.074[a]
35 days	1.601±0.083	1.601±0.116	1.526±0.100	1.498±0.066	1.484±0.092

[a] Statistical differences between treatments are indicated with letters (ANOVA and Fisher's LSD tests; $P<0.05$)

[b] The two-component control entailed inoculation with *P. fluorescens* F113 and an AMF inoculant mix composed of three *Glomus* species, whereas the three-component consortia included also an *Azospirillum* strain

consortium at the first sampling, and indeed shoot biomass in the two-component control was higher than those in the three-component consortia. The effect of inoculation on shoot biomass was not statistically significant at the third sampling, but this result was of limited significance. Indeed, roots had entirely colonized the whole soil volume in the pots by then, regardless of whether seeds were inoculated or not, and they could not expand further, thereby limiting the possibility of phytostimulation effects.

Inoculant colonization of maize roots

When the *Pseudomonas–Glomus* two-component consortium was used, *P. fluorescens* F113 was enumerated by real-time PCR at about 10^8 cell equivalents per g of rhizosphere at the first sampling and at 2 log units lower by the third sampling (Fig. 1a). The presence of an *Azospirillum* inoculant resulted in higher F113 population levels at one of the three samplings (with *A. lipoferum* CRT1 or *A. brasilense* UAP-154) or had no effect on the pseudomonad (with *A. brasilense* CFN-535). As expected, strain F113 was not found in the non-inoculated treatment.

The population size of *A. brasilense* CFN-535 dropped from 1.5×10^7 to 2.7×10^5 cell equivalents per gram of rhizosphere from the first to the third sampling based on by real-time PCR analysis (Fig. 1b). In comparison, *A. brasilense* UAP-154 and especially *A. lipoferum* CRT1 were recovered at lower levels, which fell below detection limit (4×10^3 cell equivalents per g of rhizosphere) by the second (for strain CRT1) or third sampling (for strain UAP-154). None of the *Azospirillum* inoculants (i.e., even the two Mexican isolates) was found in the non-inoculated treatment.

Among AMF inoculants, a real-time PCR quantification method was only available for *G. intraradices* JJ291. At the third sampling, this strain was not found in the non-inoculated treatment, but was detected in two of five replicate samples (at 41 and 128 mtLSU gene copies per

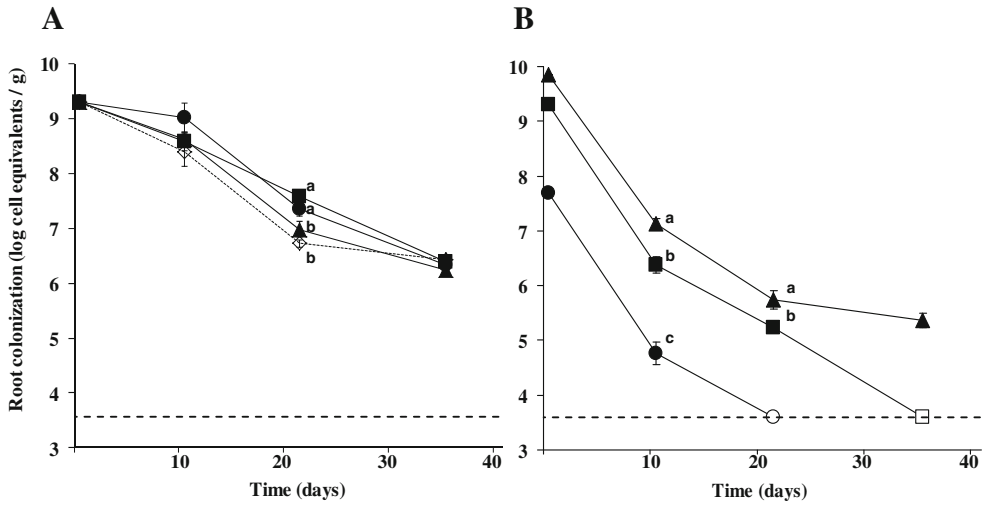

Fig. 1 Root colonization of the PGPR strains *P. fluorescens* F113 (**a**) and *Azospirillum* (**b**) used as part of two-component (F113+AMF mix; *empty diamond*) or three-component consortia (with *A. lipoferum* CRT1 (*filled circle*), *A. brasilense* UAP-154 (*filled square*), or CFN-535 (*filled triangle*)). Data represent means±standard error ($n=5$) of log cell equivalents per gram of rhizosphere. The detection limit (4×10^3 cell equivalents per gram of rhizosphere) is shown by *dotted lines* and *symbols* appear in white for *Azospirillum* inoculants below detection limit. Statistical differences between treatments at each sampling time are indicated with letters (ANOVA and Fisher's LSD tests; $P<0.05$)

Table 2 Effect[a] of consortium inoculation on AMF root colonization[b] (expressed as thousand gene copies per mg of dry root) at 35 days after inoculation (mean±standard error; $n=5$)

AMF group	Non-inoculated control	Two-component control	Three-component consortia		
			CRT1	UAP-154	CFN-535
G. intraradices (nLSU)	183±35[bc]	377±27[a]	172±23[bc]	198±33[b]	121±12[c]
G. intraradices (mtLSU; mt5)	196±83[b]	556±89[a]	325±39[b]	251±57[b]	246±55[b]
G. claroideum (nLSU)	38.4±5.3	42.8±4.3	31.0±3.4	32.6±4.8	26.2±4.0
Diversispora (nLSU)	0.54±0.10	0.57±0.17	1.19±0.24	1.20±0.28	1.09±0.35

[a] Statistical differences between treatments are indicated with letters (ANOVA and Fisher's LSD tests; $P<0.05$)

[b] Using the mt4 marker, the inoculant strain *G. intraradices* JJ291 was detected (but with weak amplification) in 3 of the 25 samples analyzed (i.e., 2 in the two-component control, and 1 with the three-component consortium involving *A. brasilense* CFN-535). Similarly, only 3 weak amplifications were obtained for species *G. mosseae* and 2 for the genus *Gigaspora*. *Scutellospora* was not detected in any sample

milligram of dry root) when the *Pseudomonas–Glomus* two-component consortium was used. With the three-component consortia, *G. intraradices* JJ291 was only found in one of five replicates (at 60 mtLSU gene copies per milligram of dry root) in the treatment where *A. brasilense* CFN-535 was included.

Effect of inoculation on root-associated AMF populations

In the non-inoculated treatment, at the third sampling, the *G. intraradices* species was enumerated at 1.8×10^5 (nLSU method) and 2.0×10^5 (mtLSU method) gene copies, the *G. claroideum* species at 3.8×10^4 nLSU gene copies, and the *Diversispora* genus at 5.4×10^2 nLSU gene copies per mg of dry root (Table 2). When the two-component consortium was used, *G. intraradices* was recovered at higher level in comparison with the control, regardless of the method. With the three-component consortia, the population size of *G. intraradices* was comparable to that in the non-inoculated control and lower than that in the two-component control was used. Inoculation had no effect on the size of the *G. claroideum* species or the *Diversispora* genus (Table 2). The *G. mosseae* species and the AMF genera *Scutellospora* and *Gigaspora* were not found in any of the treatments. Thus, there was no negative effect of inoculation on root mycorrhization by indigenous AMF taxa.

ipdC transcription in *Azospirillum*

The RT-PCR approach developed in this work targets *ipdC* from *Azospirillum* strains (mostly *A. brasilense*), but not in the other auxin-producing bacteria tested, including pseudomonads (not shown). Successful RT-PCR amplification of *Azospirillum*'s *ipdC* mRNAs from rhizosphere samples was observed even in the absence of *Azospirillum* inoculation, i.e., in the non-inoculated control (at the first two samplings) and when the two-component consortium was used (at the first sampling) (Table 3). When three-component consortia were applied, transcription of *Azospirillum ipdC* genes (1) was found in 3–4 of 5 replicates at the first two samplings (versus only two replicates or less in the other treatments) and (2) was also detected at the third sampling.

Effect of inoculation on secondary metabolite profiles of maize roots

Chromatograms at 280 nm for root methanolic extract gave 18 major integrated peaks, 11 of them corresponding to benzoxazinoid derivatives based on UV spectra (Walker et al. 2011). Polar compounds (based on water elution) were cyclic hydroxamic acids, whereas two more apolar compounds were benzoxazinone derivatives.

Discriminant PCA indicated that all inoculation treatments resulted in changes in the secondary metabolite

Table 3 RT-PCR detection of *Azospirillum ipdC* gene, as indicated by the number of plants (out of 5) for which amplification was successful

Treatments	First sampling (10 days)	Second sampling (21 days)	Third sampling (35 days)
Non-inoculated control	1	2	0
Two-component control	2	0	0
Three-component consortium (CRT1)	4	3	1
Three-component consortium (UAP-154)	3	4	2
Three-component consortium (CFN-535)	3	3	2

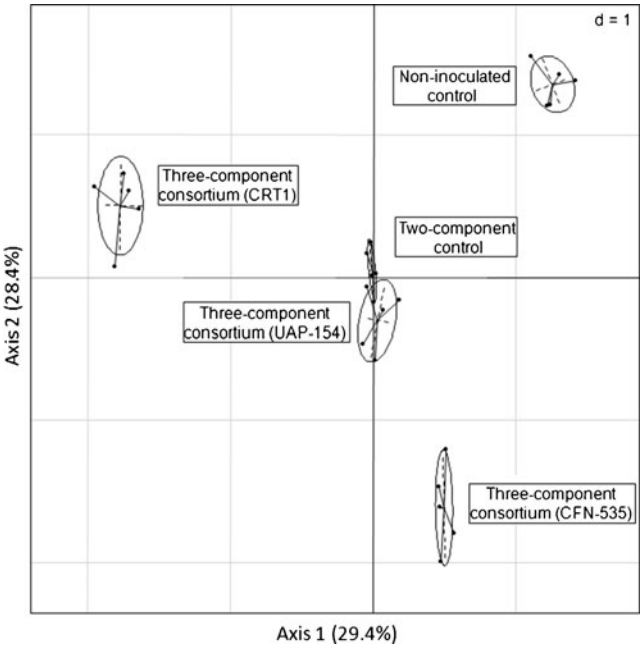

Fig. 2 Discriminant PCA performed on chromatographic data obtained for each methanolic extract of maize. Analyses were based on peak areas and retention times. Each point represents two pooled extracts (i.e., two plants) of the same treatment

profile of maize (Fig. 2). The inoculation impact varied according to the consortium, except that presence of *A. brasilense* UAP-154 within the *Pseudomonas–Glomus* two-component consortium had no effect. When assessing individual compounds responsible for treatment discrimination, it appeared that the prevalence of five PCA-discriminant secondary metabolites (including three benzoxazinoid derivatives and one cinnamic acid) differed significantly between treatments based on ANOVA and Fisher's test (Fig. 3).

Discussion

Azospirillum PGPR strains have been extensively studied as phytostimulatory inoculants of cereal crops (Okon and Labandera-Gonzalez 1994; Dobbelaere et al. 2001), and to a much lesser extent in mixed inocula combining phytobeneficial microorganisms with different metabolic capacities (Bashan 1998). Indeed, most studies on mixed inocula containing diazotrophic bacteria have been performed with bacteria other than *Azospirillum* (Biró et al. 2000; Remans et al. 2008; Cassan et al. 2009; Oliveira et al. 2009). Several attempts have been made to combine *Azospirillum* with a microorganism such as *Pseudomonas* (Corich et al. 1995; Joe and Sivakumar 2010; Combes-Meynet et al. 2011; Couillerot et al. 2011; Khorshidi et al. 2011), which may function also as a biocontrol agent. Combining *Pseudomonas* antagonistic biocontrol agents and *Azospirillum* requires special attention regarding potential inhibitory effects of *Pseudomonas* antimicrobial metabolites, such as 2,4-diacetylphloroglucinol (DAPG), against *Azospirillum* (Couillerot et al. 2011). We verified that the three *Azospirillum* strains used in this study were rather resistant to DAPG, as growth inhibition required as much as 500 μM of synthetic DAPG. In fact, synergistic effects might even be expected since DAPG can act a signal enhancing the expression of phytostimulation-relevant genes in *Azospirillum* (Combes-Meynet et al. 2011).

In all inoculation treatments, *P. fluorescens* F113 colonized maize roots extensively. Its population level was

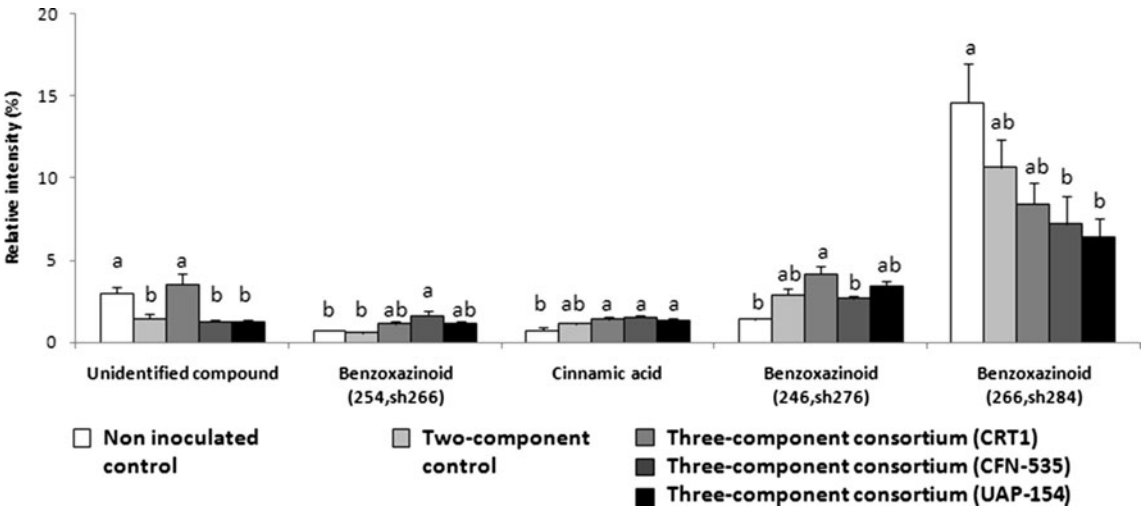

Fig. 3 Effect of maize seed inoculation on root content in individual secondary metabolites that distinguished treatments in discriminant PCA. Compound identification was based on UV spectra. For each compound, statistical differences between treatments are indicated with letters (ANOVA and Fisher's tests; $P<0.05$; $n=5$)

significantly enhanced when the pseudomonad was in presence of certain *Azospirillum* inoculants, which here was only found at one sampling time but confirms a previous observation under field conditions (Walker et al. 2012). The three *Azospirillum* strains showed very different root colonization abilities. Only *A. brasilense* CFN-535 managed to colonize roots significantly (i.e., at levels above 10^5 cell equivalents per gram of rhizosphere) and durably (i.e., till the last sampling). *A. lipoferum* CRT1 declined as fast (Walker et al. 2012) or faster (El Zemrany et al. 2006; Baudoin et al. 2009a) than in maize trials done in Europe, and perhaps soil type and/or maize variety play an important part in this phenomenon. Auxin synthesis is often considered the main mode of action in *Azospirillum* PGPR (Dobbelaere et al. 2003), but here the *ipdC* gene proved too conserved within the *Azospirillum* genus to enable strain-specific PCR monitoring of *ipdC* expression. In this work, the RT-PCR approach developed for *A. brasilense* and related strains proved operational with rhizosphere samples, even though amplification was not successful with all plants studied. The fact that plants not inoculated with *Azospirillum* yielded *ipdC* RT-PCR signals points to the contribution of resident *Azospirillum* strains present in the soil used. Further work will be necessary to understand why, at the third sampling, *Azospirillum ipdC* transcription was found in one third of *Azospirillum*-inoculated plants (including in treatments where the *Azospirillum* inoculant was below detection limit) versus none of the plants not inoculated with *Azospirillum*.

Little is known about the genetic diversity of root-associated AMF communities, despite their importance for plant growth. Here, recent PCR methods for analysis of AMF community composition were used to probe AMF taxa colonizing maize roots in Mexican soils, and results showed that several AMF taxa were well established. The prevalence of *G. intraradices* was twice as high when the two-component consortium was used, regardless of whether a mtLSU or nLSU approach was used (Table 2), in accordance with the good correspondence between both approaches (Fig. S1). It is tempting to speculate that this was due to the inoculation of *G. intraradices* JJ291, but PCR monitoring of the latter fell below expectations. Indeed, *G. intraradices* JJ291 was not present in the non-inoculated control (as expected), but its detection was poorly effective in this and other inoculation treatments, thereby limiting the usefulness of this assessment in the current experiment. Strong competition with indigenous AMF can be expected (Biró et al. 2000). Results indicate also that presence of DAPG-producing *P. fluorescens* F113 had no apparent deleterious impact on root-associated AMF, despite antifungal properties of DAPG (Barea et al. 1998; Mar Vázquez et al. 2000; Gaur et al. 2004).

AMF establishment in the three-component consortia was comparable to that in the non-inoculated control. When compared to the two-component consortium, this suggests that presence of *Azospirillum* prevented enhanced establishment of *G. intraradices*. Previous analyses failed to evidence any effect of *Azospirillum* on mycorrhization (Mar Vázquez et al. 2000; Russo et al. 2005), but methodology differed.

Stimulation of maize shoot growth was significant when seeds were inoculated with any of the three-component consortia, i.e., whatever the *Azospirillum* strain involved. It is interesting to note that this took place despite (1) contrasted survival dynamics for different *Azospirillum* inoculants, and (2) maize secondary metabolite profiles that varied between most treatments. Maize elaborated specific metabolic patterns according to the *Azospirillum* strain present, showing thus a specific impact of *Azospirillum* presence within the three-component consortia. The variation induced by microbial inoculation concerned several types of secondary compounds, including some already identified (Walker et al. 2011, 2012). In addition, it was rather unexpected that presence of *Azospirillum* in the inoculum resulted in lower maize stimulation in comparison with the *Pseudomonas–Glomus* two-component consortium, but this effect was transient. Its molecular basis remains unknown.

In conclusion, this study indicated that *Azospirillum–Pseudomonas–Glomus* three-component consortia may be useful for early stimulation of maize growth. Despite evidence for distinct interaction functioning according to the *Azospirillum* strain included, the identity of the *Azospirillum* strain was not a significant factor determining phytostimulation efficiency. This is an important finding in biotechnological terms, as it will facilitate development of microbial consortia for crop inoculation.

Acknowledgements OC and JART contributed equally to this work. This work was supported in part by the European Union (FW6 STREP project MicroMaize 036314). We are grateful to F. Bellvert, P. Mavingui (UMR CNRS 5557 Ecologie Microbienne), N. Gómez-Hernández (UNAM), and C. Schwer, A. Steffen, T. Flura (ETH Zürich) for technical help and/or discussion. We thank A. Látr (Symbio-M, Lanškroun, Czech Republic) for supplying AMF inoculum. This work made use of FR41's DTAMB platform in Université Lyon 1.

References

Adesemoye A, Torbert H, Kloepper J (2009) Plant growth-promoting rhizobacteria allow reduced application rates of chemical fertilizers. Microbial Ecol 58:921–929

Barea JM, Andrade G, Bianciotto VV, Dowling D, Lohrke S, Bonfante P, O'Gara F, Azcon-Aguilar C (1998) Impact on arbuscular mycorrhiza formation of *Pseudomonas* strains used as inoculants for biocontrol of soil-borne fungal plant pathogens. Appl Environ Microbiol 64:2304–2307

Basaglia M, Casella S, Peruch U, Poggiolini S, Vamerali T, Mosca G, Vanderleyden J, De Troch P, Nuti MP (2003) Field release of genetically marked *Azospirillum brasilense* in association with *Sorghum bicolor* L. Plant Soil 256:281–290

Bashan Y (1998) Inoculants of plant growth promoting bacteria for use in agriculture. Biotech Adv 16:729–770

Bashan Y, Moreno M, Troyo E (2000) Growth promotion of the seawater-irrigated oilseed halophyte *Salicornia bigelovii* inoculated with mangrove rhizosphere bacteria and halotolerant *Azospirillum* spp. Biol Fertil Soils 32:265–272

Baudoin E, Couillerot O, Spaepen S, Moënne-Loccoz Y, Nazaret S (2009a) Applicability of the 16S–23S rDNA internal spacer for PCR detection of the phytostimulatory PGPR inoculant *Azospirillum lipoferum* CRT1 in field soil. J Appl Microbiol 108:25–38

Baudoin E, Nazaret S, Mougel C, Ranjard L, Moënne-Loccoz Y (2009b) Impact of inoculation with the phytostimulatory PGPR *Azospirillum lipoferum* CRT1 on the genetic structure of the rhizobacterial community of field-grown maize. Soil Biol Biochem 41:409–413

Belimov AA, Kojemiakov AP, Chuvarliyeva CV (1995) Interaction between barley and mixed cultures of nitrogen fixing and phosphate-solubilizing bacteria. Plant Soil 173:29–37

Biró B, Köves-Péchy K, Vörös I, Takács T, Eggenberger P, Strasser RJ (2000) Interrelations between *Azospirillum* and *Rhizobium* nitrogen-fixers and arbuscular mycorrhizal fungi in the rhizosphere of alfalfa in sterile, AMF-free or normal soil conditions. Appl Soil Ecol 15:159–168

Cassan F, Perrig D, Sgroy V, Masciarelli O, Penna C, Luna V (2009) *Azospirillum brasilense* Az39 and *Bradyrhizobium japonicum* E109, inoculated singly or in combination, promote seed germination and early seedling growth in corn (*Zea mays* L.) and soybean (*Glycine max* L.). Eur J Plant Pathol 45:28–35

Charyulu PBBN, Foucassie F, Barbouche AK, Rondro Harisoa L, Omar AMN, Marie R, Balandreau J (1985) Field inoculation of rice using *in vitro* selected bacterial and plant genotypes. In: Klingmüller W (ed) *Azospirillum* III: genetics, physiology, ecology. Springer-Verlag, Heidelberg, pp 163–179

Combes-Meynet E, Pothier JF, Moënne-Loccoz Y, Prigent-Combaret C (2011) The *Pseudomonas* secondary metabolite 2,4-diacetylphloroglucinol is a signal inducing rhizoplane expression of *Azospirillum* genes involved in plant-growth promotion. Mol Plant Microbe Interact 24:271–284

Corich V, Giacomini A, Concheri G, Ritzerfeld B, Vendramin P, Struffi P, Basaglia M, Squartini A, Casella S, Nuti MP, Peruch U, Poggiolini S, de Troch P, Vanderleyden J, Fedi S, Fenton A, Moënne-Loccoz Y, Dowling DN, O'Gara F (1995) Environmental impact of genetically modified *Azospirillum brasilense, Pseudomonas fluorescens* and *Rhizobium leguminosarum* released as soil/seed inoculants. In: Jones DD (ed) Proceedings of the third international symposium on the biosafety results of field tests of genetically-modified plants and microorganisms. University of California, Monterey, pp 371–388

Couillerot O, Poirier MA, Prigent-Combaret C, Mavingui P, Caballero-Mellado J, Moënne-Loccoz Y (2010a) Assessment of SCAR markers to design real-time PCR primers for rhizosphere quantification of *Azospirillum brasilense* phytostimulatory inoculants of maize. J Appl Microbiol 109:528–538

Couillerot O, Bouffaud ML, Muller D, Caballero-Mellado J, Moënne-Loccoz Y (2010b) Development of a real-time PCR method to quantify the PGPR strain *Azospirillum lipoferum* CRT1 on maize seedlings. Soil Biol Biochem 42:2298–2305

Couillerot O, Combes-Meynet E, Pothier JF, Bellvert F, Challita E, Poirier MA, Rohr R, Comte G, Moënne-Loccoz Y, Prigent-Combaret Y (2011) The role of the antimicrobial compound 2,4-diacetylphloroglucinol in the impact of biocontrol *Pseudomonas fluorescens* F113 on *Azospirillum brasilense* phytostimulators. Microbiology 157:1694–1705

Creus CM, Graziano M, Casanovas EM, Pereyra MA, Simontacchi M, Puntarulo S, Barassi CA, Lamattina L (2005) Nitric oxide is involved in the *Azospirillum brasilense*-induced lateral root formation in tomato. Planta 221:297–303

Dobbelaere S, Croonenborghs A, Amber T, Ptacek D, Vanderleyden J, Dutto P, Labandera-Gonzalez C, Caballero-Mellado J, Aguirre JF, Kapulnik Y, Shimon B, Burdman S, Kadouri D, Sarig S, Okon Y (2001) Responses of agronomically important crops to inoculation with *Azospirillum*. Austral J Plant Physiol 28:1–9

Dobbelaere S, Croonenborghs A, Thys A, Ptacek D, Okon Y, Vanderleyden J (2002) Effect of inoculation with wild type *Azospirillum brasilense* and *A. irakense* strains on development and nitrogen uptake of spring wheat and grain maize. Biol Fertil Soils 36:284–297

Dobbelaere S, Vanderleyden J, Okon Y (2003) Plant growth-promoting effects of diazotrophs in the rhizosphere. Crit Rev Plant Sci 22:107–149

Döbereiner J, Marriel IE, Nery M (1976) Ecological distribution of *Spirillum lipoferum* Beijerinck. Can J Microbiol 22:1464–1472

El Zemrany H, Cortet J, Peter Lutz M, Chabert A, Baudoin E, Haurat J, Maughan N, Felix D, Défago G, Bally R, Moënne-Loccoz Y (2006) Field survival of the phytostimulator *Azospirillum lipoferum* CRT1 and functional impact on maize crop, biodegradation of crop residues, and soil faunal indicators in a context of decreasing nitrogen fertilisation. Soil Biol Biochem 38:1712–1726

El-Komy HMA (2005) Coimmobilization of *Azospirillum lipoferum* and *Bacillus megaterium* for successful phosphorus and nitrogen nutrition of wheat plants. Food Technol Biotech 43:19–27

Er F, Kilic M, Brohi AR, Ogut M (2004) Nodulation and growth of beans [*Phaseolus vulgaris* L.] inoculated with genetically modified *Rhizobium etli* strains and *Azospirillum brasilense*. Agrochimica 48:124–131

Fages J, Mulard D (1988) Isolement de bactéries rhizosphériques et effet de leur inoculation en pots chez *Zea mays*. Agronomie 8:309–314

Fenton AM, Stephens PM, Crowley J, O'Callaghan M, O'Gara F (1992) Exploitation of gene(s) involved in 2,4-diacetylphloroglucinol biosynthesis to confer a new biocontrol capability to a *Pseudomonas* strain. Appl Environ Microbiol 58:3873–3878

Fuentes-Ramirez L, Caballero-Mellado J (2006) Bacterial biofertilizers. In: Siddiqui ZA (ed) PGPR: biocontrol and biofertilization. Springer-Verlag, Heidelberg, pp 143–172

Garbaye J (1994) Helper bacteria: a new dimension to the mycorrhizal symbiosis. New Phytol 128:197–210

Gaur R, Shani N, Kawaljeet, Johri BN, Rossi P, Aragno M (2004) Diacetylphloroglucinol-producing pseudomonads do not influence AM fungi in wheat rhizosphere. Curr Sci 88:453–457

Han SO, New PB (1998) Variation in nitrogen fixing ability among natural isolates of *Azospirillum*. Microbial Ecol 36:193–201

Herschkovitz Y, Lerner A, Davidov Y, Okon Y, Jurkevitch E (2005a) *Azospirillum brasilense* does not affect population structure of specific rhizobacterial communities of inoculated maize (*Zea mays*). Environ Microbiol 7:1847–1852

Herschkovitz Y, Lerner A, Davidov Y, Rothballer M, Hartmann A, Okon Y, Jurkevitch E (2005b) Inoculation with the plant-growth-promoting rhizobacterium *Azospirillum brasilense* causes little disturbance in the rhizosphere and rhizoplane of maize (*Zea mays*). Microbial Ecol 50:277–288

Jacoud C, Faure D, Wadoux P, Bally R (1998) Development of a strain-specific probe to follow inoculated *Azospirillum lipoferum* CRT1 under field conditions and enhancement of maize root development by inoculation. FEMS Microbiol Ecol 27:43–51

Jacoud C, Job D, Wadoux P, Bally R (1999) Initiation of root growth stimulation by *Azospirillum lipoferum* CRT1 during maize seed germination. Can J Microbiol 45:339–342

James EK (2000) Nitrogen fixation in endophytic and associative symbiosis. Field Crops Res 65:197–209

Jansa J, Mozafar A, Frossard E (2005) Phosphorus acquisition strategies within arbuscular mycorrhizal fungal community of a single field site. Plant Soil 276:163–176

Jansa J, Smith FA, Smith SE (2008) Are there benefits of simultaneous root colonization by different arbuscular mycorrhizal fungi? New Phytol 177:779–789

Joe MM, Sivakumar PK (2010) Seed priming with co-flocs of *Azospirillum* and *Pseudomonas* for effective management of rice blast. Arch Phytopathol Plant Protect 43:1551–1563

Khan MA, Khokhar SN, Ahmed R, Afzal A (2009) Wheat growth and yield in response to coinoculation of *Rhizobium, Azospirillum* and *Pseudomonas* under rainfed conditions. Int J Biol Biotechnol 6:257–263

Khorshidi YR, Ardakani MR, Ramezanpour MR, Khavazi K, Zargari K (2011) Response of yield and yield components of rice (*Oryza sativa* L.) to *Pseudomonas fluorescens* and *Azospirillum lipoferum* under different nitrogen levels. American-Eurasian J Agric & Environ Sci 10:387–395

Lerner A, Herschkovitz Y, Baudoin E, Nazaret S, Moënne-Loccoz Y, Okon Y, Jurkevitch E (2006) Effect of *Azospirillum brasilense* inoculation on rhizobacterial communities analyzed by denaturing gradient gel electrophoresis and automated ribosomal intergenic spacer analysis. Soil Biol Biochem 38:1212–1218

Lucy M, Reed E, Glick BR (2004) Applications of free living plant growth-promoting rhizobacteria. Anton Leeuw Int J G 86:1–25

Mar Vázquez M, César S, Azcón R, Barea JM (2000) Interactions between arbuscular mycorrhizal fungi and other microbial inoculants (*Azospirillum, Pseudomonas, Trichoderma*) and their effects on microbial population and enzyme activities in the rhizosphere of maize plants. Appl Soil Ecol 15:261–272

Naiman AD, Latronico A, Garcia de Salamone IEG (2009) Inoculation of wheat with *Azospirillum brasilense* and *Pseudomonas fluorescens*: impact on the production and culturable rhizosphere microflora. Eur J Plant Pathol 45:44–51

Okon Y, Labandera-Gonzalez CA (1994) Agronomic applications of *Azospirillum*: an evaluation of 20 years worldwide field inoculation. Soil Biol Biochem 26:1591–1601

Oliveira ALM, Stoffels M, Schmid M, Reis VM, Baldani JI, Hartmann A (2009) Colonization of sugarcane plantlets by mixed inoculations with diazotrophic bacteria. Eur J Soil Biol 45:106–113

Park JW, Crowley DE (2005) Normalization of soil DNA extraction for accurate quantification of target genes by real-time PCR and DGGE. Biotechniques 38:579–586

Pedraza RO, Bellone CH, Carrizo de Bellone S, Boa Sorte PMF, Teixeira KRdS (2009) *Azospirillum* inoculation and nitrogen fertilization effect on grain yield and on the diversity of endophytic bacteria in the phyllosphere of rice rainfed crop. Eur J Plant Pathol 45:36–43

Prigent-Combaret C, Blaha D, Pothier JF, Vial L, Poirier MA, Wisniewski-Dyé F, Moënne-Loccoz Y (2008) Physical organization and phylogenetic analysis of *acdR* as leucine-responsive regulator of the 1-aminocyclopropane-1-carboxylate deaminase gene *acdS* in phytobeneficial *Azospirillum lipoferum* 4B and other Proteobacteria. FEMS Microbiol Ecol 65:202–219

Pulido LE, Cabrera A, Medina N (2003) Biofertilization using rhizobacteria and AMF in the production of tomato (*Lycopersicon esculentum* Mill.) and onion (*Allium cepa* L.) seedlings: root colonization and nutritional status. Cultivos Tropicales 24:5–13

Raverkar K, Konde B (1988) Effect of *Rhizobium* and *Azospirillum lipoferum* inoculation on the nodulation, yield and nitrogen uptake of peanut cultivars. Plant Soil 106:249–252

Remans R, Ramaekers L, Schelkens S, Hernandez G, Garcia A, Reyes J, Mendez N, Toscano V, Mulling M, Galvez L, Vanderleyden J (2008) Effect of *Rhizobium–Azospirillum* coinoculation on nitrogen fixation and yield of two contrasting *Phaseolus vulgaris* L. genotypes cultivated across different environments in Cuba. Plant Soil 312:25–37

Rodriguez Caceres EA (1982) Improved medium for isolation of *Azospirilllum* spp. Appl Environ Microbiol 44:990–991

Rodriguez-Salazar J, Suarez R, Caballero-Mellado J, Iturriaga G (2009) Trehalose accumulation in *Azospirillum brasilense* improves drought tolerance and biomass in maize plants. FEMS Microbiol Lett 296:52–59

Russo A, Felici C, Toffanin A, Götz M, Collados C, Barea J, Moënne-Loccoz Y, Smalla K, Vanderleyden J, Nuti M (2005) Effect of *Azospirillum* inoculants on arbuscular mycorrhiza establishment in wheat and maize plants. Biol Fertil Soils 41:301–309

Singh SR, Zargar MY, Singh U, Ishaq M (2010) Influence of bioinoculants and inorganic fertilizers on yield, nutrient balance, microbial dynamics and quality of strawberry (*Fragariax ananassa*) under rainfed conditions of Kashmir valley. Indian J Agric Sci 80:275–281

Steinberg C, Gamard P, Faurie G, Lensi R (1989) Survival and potential denitrifying activity of *Azospirillum lipoferum* and *Bradyrhizobium japonicum* inoculated into sterilized soil. Biol Fertil Soils 7:101–107

Thonar C, Erb A, Jansa J (2011) Real-time PCR to quantify composition of arbuscular mycorrhizal fungal communities—marker design, verification, calibration, and field validation. Mol Ecol Resour 12:219–232

von Felten A, Défago G, Maurhofer M (2010) Quantification of *Pseudomonas fluorescens* strains F113, CHA0 and Pf153 in the rhizosphere of maize by strain-specific real-time PCR unaffected by the variability of DNA extraction efficiency. J Microbiol Meth 81:108–115

Wagg C, Jansa J, Stadler M, Schmid B, van der Heijden MGA (2011) Mycorrhizal fungal identity and diversity relaxes plant–plant competition. Ecology 92:1303–1313

Walker V, Bertrand C, Bellvert F, Moënne-Loccoz Y, Bally R, Comte G (2011) Host plant secondary metabolite profiling shows complex, strain-dependent response of maize to plant growth-promoting rhizobacteria of the genus *Azospirillum*. New Phytol 189:494–506

Walker V, Couillerot O, Von Felten A, Bellvert F, Jansa J, Maurhofer M, Bally R, Moënne-Loccoz Y, Comte G (2012) Variation of secondary metabolite levels in maize seedling roots induced by inoculation with *Azospirillum, Pseudomonas* and *Glomus* consortium under field conditions. Plant Soil 356:151–163