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Characterization of plant growth promoting bacteria from crops in Bolivia

Charakterisierung pflanzenwachstumsfördernder Bakterien von Kulturpflanzen in Bolivien

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Abstract

The use of plant growth promoting bacteria (PGPB) is an economically and environmental friendly alternative to the application of chemical fertilizers resp. pesticides in agriculture. To obtain novel bacterial strains that could be used for plant growth promotion (PGP) in the agriculture of Bolivia, plant associated bacteria derived from horseradish tree (*Moringa oleifera*), sorghum (*Sorghum vulgare*), sunflower (*Helianthus annuus*), and safflower (*Carthamus tinctorius*) were screened for direct and indirect *in vitro* PGP traits. Subsequently, most promising strains were selected for *ad planta* studies. According to *in vitro* experiments, out of 59 tested isolates, 19% were declared as diazotrophs, 41% as solubilizers of phosphorus, 10% were able to reduce the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), and 17% exhibited phytohormone (IAA) synthesis. Only a small proportion of rhizobacterial strains (7% out of 276) showed *in vitro* antagonism against plant pathogenic *Colletotrichum* sp., whereas none of isolated stem and leaf endophytes inhibited growth of *Verticillium chlamydosporum* or *Bipolaris maydis* in dual culture. Based on results of *in vitro* screens, the rhizospheric strains *Pectobacterium cypripedii* M56, *Pantoea agglomerans* M72, and *P. agglomerans* M81 were selected for *ad planta* applications. Results revealed significant increases in number of beans per black bean plant resp. diameters of flower heads of sunflower plants compared to controls by *P. agglomerans* M72. We conclude that the assessment and selection of plant associated bacteria based on traits conferring theoretically PGP can provide the basis for the development of new microbial inoculants for agricultural purposes.

Key words: ACC, diazotrophs, endophytes, IAA, *Pantoea agglomerans*, PGPB, plant growth promoting bacteria

Zusammenfassung

Die Anwendung pflanzenwachstumsfördernder Bakterien in der Landwirtschaft kann eine profitable und umweltschonende Alternative zum Einsatz von chemischen Düngemitteln und Pestiziden darstellen. Um neue, bakterielle Pflanzenwachstumsförderer für die bolivianische Landwirtschaft zu gewinnen, wurden pflanzenassoziierte Bakterien von Meerrettichbaum (*Moringa oleifera*), Sorghumhirse (*Sorghum vulgare*), Sonnenblume (*Helianthus annuus*) und Färberdistel (*Carthamus tinctorius*) isoliert und *in vitro* auf direkte und indirekte Mechanismen der Pflanzenwachstumsförderung untersucht. Ausgehend von 59 unter Laborbedingungen getesteten Isolaten, waren 19% diazotroph, 41% wurden als Phosphor-Solubilisierer deklariert, 17% zeigten Phytohormonsynthese (IAA) und 10% wurden als Verwerter der Ethylen-Vorläufer-Substanz 1-Aminocyclopropan-1-Carbonsäure (ACC) erklärt. Nur ein geringer Anteil rhizobakterieller Stämme (7% von 276) zeigte antagonistische Aktivität *in vitro* gegen das Pflanzenpathogen *Colletotrichum* sp., während keiner der von Stängeln oder Blättern isolierten Endophyten das Wachstum von *Verticillium chlamydosporum* oder *Bipolaris maydis* in Dualkultur inhibieren konnte. Basierend auf den Resultaten der *in vitro* Tests, wurden die Rhizosphären-Stämme *Pectobacterium cypripedii* M56, *Pantoea agglomerans* M72 und *P. agglomerans* M81 für die Applikation in Freilandexperimenten selektiert, wobei *P. agglomerans* M72 zu signifikant höheren Bohnenzahlen bei schwarzer Bohne bzw. Blütenkorbdurchmessern bei Sonnenblume im Vergleich zu Kontroll-Behandlungen geführt hat. Die Bewertung und Selektion pflanzenassoziierter Bakterien, basierend auf deren pflanzenwachstumsfördernden Eigenschaften *in vitro*, stellt die Grundlage für die Entwicklung neuer biologischer Produkte für landwirtschaftliche Zwecke dar.

Stichwörter: ACC, bakterielle Pflanzenwachstumsförderer, diazotroph, Endophyten, IAA, *Pantoea agglomerans*

1 Introduction

Plant growth promoting bacteria (PGPB) and biological control agents (BCAs) (Bashan and Holguin 1998; Chernin and Chet 2002) perform beneficial effects on plants via direct resp. indirect mechanisms. PGPB directly serve the plants by supplying nutrients [e.g. via the fixation of atmospheric nitrogen (N_2), phosphorous (P) solubilization, segregation of iron (Fe) by siderophores], by means of phytohormone synthesis (e.g. indole-3-acetic acid, IAA), and by lowering the hosts ethylene level due to ACC deaminase activity (Dart 1986; Bar-Ness et al. 1991; Bloemberg and Lugtenberg 2001; Gull et al. 2004; Glick et al. 2005). BCAs support planth health via the suppression of plant pathogens, e.g. due to competition for nutrients and space, the synthesis of antimicrobial compounds, parasitism, or the induction of systemic resistance in host plants (O`Sullivan and O`Gara 1992; Van Loon 2007; Raaijmakers et al. 2008). Whereas it is sometimes difficult to screen biocontrol agents under *in vitro* conditions due to discrepancies between the antagonistic effects under *in vitro* conditions and the corresponding *in situ* efficacy (Weller and Cook 1983; Reddy et al. 1994), PGB traits can be assessed under laboratory conditions and allow the selection of strains that could lead to increased plant growth (Cattelan et al. 1999).

The inoculation of legumes with *Rhizobium* spp. is a prominent example for an effective, cheap, and environmental friendly method to ensure supply of nitrogen to agriculturally used areas (Kadam et al. 1977; Dobbelare et al. 2003). Also non-symbiotic bacterial strains have notable potentials to promote plant growth and health, and were even subjected to the development of ecosensitive products for the agricultural market (Kurze et al. 2001; Whipps 2001). However, plant associated microhabitats harbour a large number of bacterial strains with yet unknown abilities for direct and/or indirect PGP. The rhizosphere, as the soil compartment directly encompassing the roots, constitutes an attractive niche for bacterial colonization (Faure et al. 2008). Furthermore the interior of plant tissues termed as endosphere represents a niche

of close plant vicinity in that PGPB and BCAs may be selectively enriched (Berg and Hallmann 2006; Berg et al. 2008).

The aim of this study was to isolate and characterize new strains of plant associated bacteria that could serve in terms of PGP for a farm in Bolivia. This farm, which is named "San Rafael" and owned by the company DESA (Desarrollos Agrícolas), is located in the Bolivian department Santa Cruz, cultivated an area of 12.300 ha. On the farm different crop plants including soybean, sunflower and cotton are cultivated and, additionally, citrus fruits in plantations are grown. Interestingly, resident farmers produce its own biocontrol agents such as *Trichoderma harzianum* and *Beauveria bassiana*. To find PGPB with a broad host range for self production, bacteria derived from agricultural plants grown at the Bolivian farm were isolated and screened *in vitro* for N_2 fixation, IAA synthesis, ACC deaminase activity, P solubilization, and antagonistic activity against fungal plant pathogens. A screening scheme for the selection of most promising strains was designed and three chosen isolates were applied *ad planta*.

2 Materials and methods

2.1 *Sampling and isolation of plant associated bacteria*

For isolation of plant associated bacteria, horseradish tree (*Moringa oleifera* Lam.) and safflower (*Carthamus tinctorius* L.) plants were collected from fields of the farm San Rafael/DESA, whereas sorghum (*Sorghum vulgare* PERS.) and sunflower (*Helianthus annuus* L.) plants were collected from fields adjacent to this farm, located in the Bolivian department Santa Cruz. Samples of roots with adhering soils, stalks, and leaves were taken from six safflower- and four sorghum individuals from always four different sites of corresponding fields. From sunflower, samples of roots and shoots were taken from two individual plants from always four different field sites whereas in case of horseradish tree, roots from three individuals grown at different plantation sites were collected.

For preparation of bacterial rhizosphere suspensions, four to seven grams of roots with adhering soils were amended with 40 ml of sterile 0.85% NaCl in 50 ml tubes and shaken thoroughly by hand for 1.5 min. Suspensions of endophytic bacteria were obtained as follows: tap water washed roots as well as unwashed stalks and leaves were surface sterilized in 4% sodium hypochlorite (NaOCl) for five min, except for sorghum leaf samples, that were treated only for three min with NaOCl, and then washed three times with sterile water. Afterwards plant materials were imprinted on R2A medium as a sterility check. Only colonies that derived from samples showing negative sterility test results resp. exhibited different colony morphologies compared to bacteria grown on corresponding sterility test plates were used for subsequent analysis. Two to six grams of surface sterilized roots, stalks, and leaves were amended with five resp. 10 ml sterile 0.85% NaCl in a mortar and homogenized with a pestle. Resulting rhizosphere and endosphere bacterial suspensions were serially diluted with sterile 0.85% NaCl and plated onto R2A medium. Agar plates were incubated for five days at 24°C and afterwards colony forming units (CFUs) were counted and their means were assessed per g of sample fresh weight. Bacterial isolates selected from PGP screenings (as described below) were purified and stored in sterile nutrient broth containing 50% glycerol at −70°C.

2.2 In vitro *screenings for direct PGP abilities*

Overall, 54 bacterial isolates derived from the horseradish tree rhizosphere as well as selected bacterial *in vitro* antagonists, isolated from rhizospheres of sorghum (two), sunflower (two), and safflower (one), that exhibited growth inhibition of plant pathogenic *Colletotrichum* sp. that exceeded five mm in a dual culture assay (as described below), were subjected to the following screenings:

2.2.1 N_2 fixation and ACC deaminase activity. Bacteria were transferred to Brown & Dilworth (BD) minimal medium (Brown and Dilworth 1975) with and without the addition of 150 µl 5% ACC solution that was spread on BD medium as the unique nitrogen source for ACC deaminase active isolates. Bacteria were grown for five days at room temperature (RT) and their extent of growth was assessed after five days. Bacteria that exhibited heavily growth on BD plates were declared as diazotrophs and isolates showing more pronounced growth on BD + ACC solution in comparison to ACC free medium were declared as ACC utilizing strains.

2.2.2 IAA synthesis. Isolated strains were grown in five ml minimal growth medium (5 g glucose, 0.025 g yeast extract, 0.204 g L-tryptophan) in culture tubes in absence of light for 72 hours at 20°C under agitation (120 rpm). Afterwards 1 ml of bacterial suspensions were transferred to 1.5 ml Eppendorf tubes and centrifuged at 16.750 × g for 10 min. 90 µl of resulting supernatants were added to 60 µl Salkowski reagent (0.5 M $FeCl_3$ and 35% perchloric acid in a mixture 1:49) in the cavities of a 96 well plate and incubated for 30 min in the dark. Afterwards absorbance at 530 nm was measured with a microplate spectrophotometer (Infinite M200, Tecan). Absorbance arising from tested isolates was compared with that one of the IAA producing strain *Serratia plymuthica* HRO-C48. Strains whose corresponding absorbance values exceeded that of *S*. plymuthica HRO-C48 were declared as IAA synthesizers.

2.2.3 P solubilization. Bacterial isolates were transferred to National Botanical Research Institute`s phosphate growth agar (NBRIP) containing per liter: 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2 \times 6\ H_2O$, 0.25 g $MgSO_4 \times 7\ H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$, and 15 g agar to perform screening for P solubilization on plates. Isolates were grown for 5 days at RT and afterwards presence (P solubilizing positive) or absence (P solubilizing negative) of visible halo zones at the isolates growth sites on the plates was noted.

2.3 In vitro *screenings for indirect PGP qualities by detecting antagonistic activity against isolated fungal plant pathogens*

Root associated bacteria isolated from sorghum (50), sunflower (80), and safflower (81) as well as six selected rhizosphere strains derived from horseradish tree, positively tested in at least two of the four aforementioned *in vitro* screening criteria for direct PGP, were screened for their antagonistic capacity against the soilborne pathogen *Colletotrichum* sp. Moreover, bacterial endophytes from sorghum (11), sunflower (12), and safflower (15), isolated from stalks, were tested against stem-infesting *Verticillium chlamydosporum* whereas bacterial leaf endophytes harboured by sorghum (11) and safflower (10) were tested against the foliar pathogen *Bipolaris maydis*. Bacterial strains and respective pathogens were transferred on Waksman agar (per liter: 5 g bactopeptone, 10 g glucose, 5 g NaCl, 3 g meat extract, 20 g agar) and incubated at RT. When moulds were grown close enough to bacterial isolates, sizes of possible growth inhibition of the pathogens, outgoing from bacterial strains, were assessed.

Colletotrichum sp. was isolated from dry field stubbles of soybean. *V. chlamydosporum* was isolated from symptomatic sunflower stalks, and *B. maydis* was isolated from corn leaves exhibiting symptoms of Southern Corn Leaf Blight. Pathogenic fungi were isolated from plant material of the farm San Rafael/DESA, Santa Cruz, Bolivia, and identified by morpho-

logical observations. Identity of *Colletotrichum* sp. and *B. maydis* was confirmed by sequence analysis of the ribosomal internal transcribed spacer region as described below.

2.4 *Selection of screened isolates for* ad planta *experiments*

Based on direct PGP criteria a point scheme was generated to assess *in vitro* performance of tested strains and to select most promising candidates for *ad planta* studies. The point scheme was designed by the assignment of one point for each of the aforementioned positive fulfilled criteria for direct PGP (max. four points) to tested strains, points were summed up subsequently and strains to that highest sums of points were ascribed, were selected for *ad planta* experiments.

2.5 *Inoculation of black bean (*Phaseolus vulgaris*) and sunflower (*Helianthus annuus*)*

Two weeks old black bean plantlets and three weeks old sunflower plantlets (both at the four leaf stage) were inoculated with horseradish tree derived rhizobacterial strains *Pectobacterium cypripedii* M56, *Pantoea agglomerans* M72 or *Pantoea agglomerans* M81, selected by *in vitro* experiments (as described above). Chosen strains were grown in nutrient broth (NB: 5 g/l tryptone, 3 g/l meat extract) for 48 h at RT under agitation and plants were inoculated by 15 min root dipping in respective bacterial suspensions that contained 10^9 cells ml^{-1} for black bean inoculation and 2×10^9 cells ml^{-1} for sunflower inoculation. Plant roots dipped in sterile NB served as control. Inoculated and control plants were put in sterile water until they were transplanted. Black bean plants were transferred to pots containing loamy soil obtained from the superficial layer of a field of the farm San Rafael/DESA, Bolivia, mixed with sand in a proportion of 3:1. Sunflower plants were transplanted to a field with loamy soil located at farm San Rafael/DESA, Bolivia. 27 resp. 24 days after inoculation/transplantation 15 ml of 10^9 resp. 7×10^8 cells ml^{-1} containing suspensions prepared out of aforementioned strains, grown in NB for 44 hours at RT, were applied to each stem base (a control treatment, by applying 15 ml sterile NB, was performed as well) of black bean plants resp. sunflower plants. It is noteworthy that the used soil for the black bean experiment was taken from a boarder site of a field where no agricultural plants were grown and that was heavily exposed to solar radiation and may reflected approximated gnotobiotic conditions. Each treatment was performed with 40 black bean plants (8 plants per pot and 5 repetitions) and 84 sunflower plants (14 plants per plot with 6 repetitions) and both experiments were organized in a randomized complete block design (RCBD). Plants were irrigated by watering can and were grown without fertilization. Cultivation time of black bean plants was from May to August and from June to September 2008 for sunflower. As indicators for plant growth and harvest yields, numbers of beans per adult plant resp. diameters of flower heads of sunflowers were assessed.

2.6 *Isolation of DNA from bacterial strains possessing pronounced* in vitro *PGP qualities*

Bacterial cell material were transferred to 1.5 ml tubes, containing 300 µl extraction buffer [0.2 M Tris-HCl (pH 8), 0.25 M NaCl, 0.5% sodium dodecyl sulphate], and mixed well. Cell suspensions were transferred to 2 ml tubes containing 200 µl glass beads and were agitated two times for 30 s at level five with a Fast Prep™ machine (Qbiogene BIO 101® systems, Carlsbad, CA, USA). Then 150 µl of 3 M sodium acetate was added, tubes were vortexed and stored for 30 min at −20°C. Afterwards tubes were centrifuged for 10 min at 16,750 × g and supernatants were transferred to 1.5 ml tubes, DNA was purified by the phenol-chloroform extraction method and precipitated by the addition of isopropanol. DNA was dissolved in 50 µl of PCR grade water and stored at −20°C.

2.7 *Isolation of DNA from fungal plant pathogens*

100 – 250 mg of mycel of *Colletotrichum* sp. and *B. maydis*, grown on potato dextrose agar (PDA), were transferred to 2 ml tubes containing 400 µl salt homogenizing buffer (0.4 M NaCl, 10 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0) and agitated with a Fast Prep™ machine (Qbiogene BIO 101® systems, Karlsbad) for 15 s at level 6.5. Then, 40 µl of 20% sodium dodecyl sulphate were added and tubes were vortexed. Afterwards tubes were incubated at 60°C for one hour followed by the addition of 300 µl of 6 M NaCl. Then samples were centrifuged for 30 min at 10,000 × g, supernatants were transferred to fresh tubes and an equal volume of isopropanol was added before samples were incubated at −20°C for one hour. After tubes were centrifuged for 20 min at 16.750 × g, DNA was washed with 70% ethanol, dried, suspended in 50 µl PCR grade water, and stored at −20°C.

2.8 *Partial 16S rRNA gene sequence analysis of selected bacterial strains exhibiting pronounced* in vitro *PGP capabilities*

To get information of the identity of bacterial strains, exhibiting pronounced direct and/or indirect *in vitro* PGP activities, partial 16S rRNA genes from respective isolates were sequenced. For that, in a primary step, 16S rDNA fragments were amplified in a 60 µl master mix, containing 12 µl of 5 × Taq-&GO™ PCR Mastermix (Qbiogene), always 0.5 µM of primers Eubac1-forward (5'-GAG TTT GAT CCT GGC TCA G-3') and 1492r (5'-TAC GGY TAC CTT CGT TAC GCA CTT-3') as well as 3 µl of template DNA. PCR conditions were as follows: initial denaturation at 95°C for 5 min followed by nine amplification cycles consisting of 95°C for 30 s, 52°C for 30 s, and 72°C for 100 s followed by 19 amplification cycles consisting of 95°C for 30 s, 52°C for 30 s, and 72°C for 30 s (+ 10 s/cycle), and a final elongation step at 72°C for 5 min. PCR products were purified using the peqGOLD MicroSpin Cycle Pure Kit (PEQLAB, Erlangen, Germany). Purified PCR products were sequenced using the genetic analyzer AB3730 (Applied Biosystems) and the ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems). Obtained data was subjected to BLAST analysis (Altschul et al. 1997) with the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov).

2.9 *Internal transcribed spacer rDNA sequence analysis of fungal plant pathogens*

For confirmation of morphological identification of isolated fungal plant pathogens *Colletotrichum* sp. and *B. maydis*, their ribosomal internal transcribed spacer (ITS) rDNA regions were sequenced. The PCR master mix for amplification of fungal ITS genes for subsequent sequencing consisted of 8 µl of 5 × Phusion GC reaction buffer (Finnzymes), 0.2 mM dNTPs mix, 2 µl of 100% dimethyl sulfoxide, 2 units Phusion™ DNA Polymerase (Finnzymes), always 1 µM of primers ITS 1f (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS 4r (5'-TCC TCC GCT TAT TGA TAT GC-3'), 1.5 mM $MgCl_2$, and 2 µl template DNA in a final reaction volume of 40 µl. Touchdown PCR was performed as follows: initial denaturation at 95°C for 5 min followed by 37 amplification cycles consisting of 95°C for 30 s, 62°C (−0.32°C/cycle) for 35 s, 72°C for 40 s, and a final elongation step at 72°C for 10 min PCR products were purified using the peqGOLD MicroSpin Cycle Pure Kit (PEQLAB Erlangen, Germany) for the *B. maydis* derived PCR product whereas for *Colletotrichum* sp. the respective PCR product

was cut out from an agarose gel and purified using the GENECLEAN®Turbo Kit (Qbiogene). Purified PCR products were sequenced and subjected to phylogenetic analysis as described above.

2.10 Statistical analysis

Via Statistical Product and Service Solutions for Windows, Rel. 11.5.1 (SPSS Inc.) mean numbers of beans per black bean plant (DMS, P = 0.05) resp. diameters of flower heads of sunflowers, (Duncan`s multiple range test, P = 0.15) evaluated in *ad planta* studies, were compared.

2.11 Nucleotide sequence accession numbers

Obtained sequences were deposited in GenBank under accession numbers FJ669155 to FJ669165 (16S rRNA gene sequences) and FJ669166 to FJ669167 (ITS sequences).

3 Results

3.1 Isolation of plant associated bacteria

Bacterial counts (expressed as $\log_{10}$ CFU g^{-1} fresh weight of plant material) derived from microhabitats of horseradish tree (*M. oleifera*), sunflower (*H. annuus*), sorghum (*S. vulgare*), and safflower (*C. tinctorius*) were the highest for rhizosphere of *M .oleifera* (5.39×10^{10}, SD ± 4.97) and lowest for endosphere of shoot of *H. annuus* (5.48×10^3, SD ± 5.81) as declared in Table 1.

3.2 In vitro screenings for direct PGP abilities

To select strains that promote plant growth directly, 54 bacterial strains originated from the rhizosphere of horseradish tree as well as selected isolates from rhizospheres of sorghum (two), sunflower (two), and safflower (one) that exhibited pronounced *in vitro* antagonism against fungal plant pathogens (as shown below) were screened for direct PGP abilities. Among all 59 tested strains, 19% were declared as N_2 fixers, 41% were assigned to be P solubilizers, 17% synthesized IAA, and 10% were able to degrade ACC (data not shown). Altogether, 66% of tested strains were declared positive for at least one PGP criterion, 14% for at least two criteria and three strains (5%) for three criteria, whereas no "allround strain" was observed that fulfilled all four direct PGP criteria (data not shown). Organisms, declared positive for at least two screening criteria are listed in Table 2.

3.3 In vitro screenings for indirect PGP abilities

For selection of strains that potentially function as BCAs, bacterial strains isolated from plant derived microhabitats from sorghum (72), sunflower (92), and safflower (106) as well as six selected rhizosphere strains, derived from the horseradish tree rhizosphere, positively tested in at least two of the four aforementioned *in vitro* screening criteria for direct PGP, were analyzed for their capacity to inhibit growth of fungal pathogens *Colletotrichum* sp., *V. chlamydosporum* and *B. maydis*. The interaction of pathogens and their potential antagonists was analyzed according to their microhabitat origin (as described above). Conformable to that screen, highest amount of rhizobacterial antagonists that showed at least minimal antagonistic activity were harboured in the rhizosphere of sorghum (12%), followed by 9% and 8% of antagonists in rhizospheres of sunflower and safflower (Table 3). While 4% of sorghum associated bacteria from the endorhiza exhibited growth inhibition of *Colletotrichum* sp., in the corresponding microhabitat of sunflower and safflower none strain was tested positively (Table 3). Activity towards the soil-borne pathogen was found for 2% of all tested rhizobacterial strains (listed in Table 2). Conspicuously, none of the bacterial isolates obtained from endospheres of stems and leaves were tested positively for antagonistic activity against *V. chlamydosporum* resp. *B. maydis* (Table 3).

3.4 Selection of most promising strains for ad planta studies

To select most promising strains for *ad planta* experiments, a point scheme based on direct PGP criteria was generated by the assignment of one point for each of the aforementioned positive fulfilled PGP criteria (max. four points) (by even *in vitro* antagonists). Thus a selection of isolated bacteria that putatively serve for plant growth and health was achieved (Table 2).

The isolates *Pectobacterium cypripedii* M56, *Pantoea agglomerans* M72, and *Pantoea agglomerans* M81, declared in Table 2 to that most points (three) in point scheme were ascribed, were selected for *ad planta* studies.

Table 1: Bacterial CFUs per g fresh weight of plant material

Plant species	Habitat	CFU g^{-1} plant material
Horseradish tree (*Moringa oleifera*)	Rhizosphere	5.39×10^{10} SD ± 4.97
Sunflower (*Helianthus annuus*)	Rhizosphere	1.66×10^{8} SD ± 1.37
	Endorhiza	1.16×10^{5} SD ± 0.79
	Shoot endosphere	5.48×10^{3} SD ± 5.81
Sorghum (*Sorghum vulgare*)	Rhizosphere	1.14×10^{10} SD ± 1.38
	Endorhiza	1.92×10^{7} SD ± 1.82
	Shoot endosphere	1.57×10^{4} SD ± 0.99
	Leaf endosphere	2.22×10^{5} SD ± 1.18
Safflower (*Carthamus tinctorius*)	Rhizosphere	4.98×10^{8} SD ± 4.93
	Endorhiza	8.32×10^{5} SD ± 7.00
	Shoot endosphere	2.35×10^{5} SD ± 1.39
	Leaf endosphere	3.81×10^{5} SD ± 2.88

Table 2: Selected rhizobacterial strains exhibiting pronounced *in vitro* PGP traits by fulfilling at least two of the four tested direct PGP criteria and/or by representing halos of growth inhibition of *Colletotrichum* sp. ≥ 5 mm in a dual culture assay. Shaded lines indicate strains that were selected for *ad planta* experiments

Isolate	Host plant species	Closest NCBI match for partial 16S rRNA gene sequences/% similarity	IAA synthesis[a]	P solubilization[b]	ACC deaminase activity[c]	Growth on nitrogen-free medium[d]	Sum of points for direct PGP criteria[e]	*In vitro* growth inhibition of *Colletotrichum* sp.[f]
M41	*Moringa oleifera*	*Agrobacterium tumefaciens*, *Rhizobium* sp./100	+	–	–	+	2	–
M56	*Moringa oleifera*	*Pectobacterium cypripedii*/99	+	+	–	+	3	–
M71	*Moringa oleifera*	*Ochrobactrum* sp./100	+	+	–	–	2	–
M72	*Moringa oleifera*	*Pantoea agglomerans*/99	+	+	–	+	3	–
M80	*Moringa oleifera*	*Bacillus* sp./100	+	–	–	+	2	–
M81	*Moringa oleifera*	*Pantoea agglomerans*/100	+	+	–	+	3	–
S18	*Sorghum vulgare*	*Burkholderia* sp./100	–	+	–	+	2	+++
S36	*Sorghum vulgare*	*Burkholderia vietnamiensis*/99	–	+	–	–	1	++
H9	*Helianthus annuus*	*Streptomyces* sp./100	–	+	–	–	1	++
H112	*Helianthus annuus*	*Bacillus subtilis*/100	–	–	+	–	1	+++
C54	*Carthamus tinctorius*	*Burkholderia vietnamiensis*/99	–	+	+	–	2	+++

[a] IAA synthesis: – represents no IAA synthesis, + represents IAA synthesis.
[b] P solubilization: – represents no P solubilization activity, + represents P solubilization activity.
[c] ACC deaminase activity: – represents no ACC deaminase activity, + represents ACC deaminase activity.
[d] Growth on nitrogen-free medium: – represents no pronounced growth on nitrogen-free medium, + represents pronounced growth on nitrogen-free medium.
[e] Sum of points for direct PGP criteria: for each fulfilled criterion for direct PGP (as indicated by +), one point was assigned to respective isolates and points were summed up subsequently.
[f] Zones of growth inhibition of *Colletotrichum* sp. in a dual culture assay: – no growth inhibition (GI), ++ 5 – 10 mm GI, +++ > 10 mm GI.

Table 3: Amount of bacteria associated with different microhabitats and host plants showing at least minimal antagonistic activity *in vitro* against plant pathogenic *Colletotrichum* sp., *Verticillium chlamydosporum* or *Bipolaris maydis*

	Rhizosphere		Endorhiza		Stem endosphere		Leaf endosphere	
	∑ strains tested	% pos.	∑ strains tested	% pos.	∑ strains tested	% pos.	∑ strains tested	% pos
Sorghum vulgare								
Colletotrichum sp.	26	12	24	4				
Verticillium chlamydosporum					11	0		
Bipolaris maydis							11	0
Helianthus annuus								
Colletotrichum sp.	57	9	23	0				
Verticillium chlamydosporum					12	0		
Carthamus tinctorius								
Colletotrichum sp.	65	8	16	0				
Verticillium chlamydosporum					15	0		
Bipolaris maydis							10	0

3.5 Ad planta *experiments*

Effects of treatments with selected bacteria on number of beans per adult plant as an indicator for PGP and harvest yield were assessed and compared with the control treatment. Application of strain *P. agglomerans* M72 resulted in a maximum mean number of beans per plant (8), that was significantly (DMS, P = 0.05) more than in case of fewest beans per plant (6) harbouring control plants (Fig. 1).

As an indicator for PGP and harvest yield for sunflower plants, diameters of flower heads were measured and compared with control plants. Application of strain *P. agglomerans* M72 resulted in the highest mean for flower head diameters (10.13 cm) that was significantly different (Duncan`s multiple range test, P = 0.15) to control plants (9.72 cm) (Fig. 2). Lowest mean diameter of flower heads resulted from application of strain *P. cypripedii* M56 (9.66 cm) (Fig. 2).

3.6 *Partial 16S rRNA gene sequence analysis of selected bacterial strains exhibiting pronounced* in vitro *PGP capabilities*

Potential PGPB were subjected to 16S rRNA gene sequence and BLAST analysis to obtain information about their taxonomic affiliation. The 11 strains could be assigned to eight different bacterial genera: *Agrobacterium/Rhizobium*, *Pectobac-*

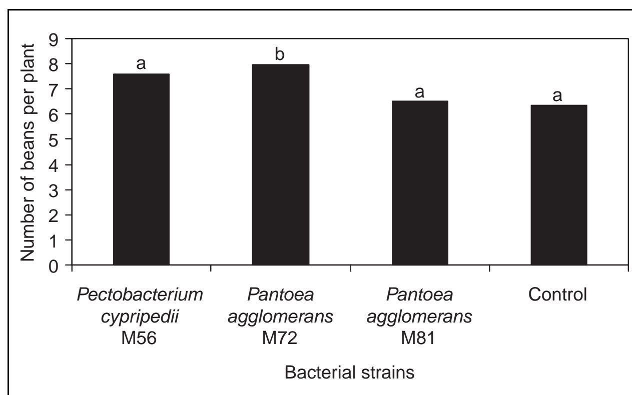

Fig. 1: Mean number of beans per black bean plant after treatment with bacterial strains selected from *in vitro* studies. Different letters signify significant differences between mean values (DMS, $P = 0.05$).

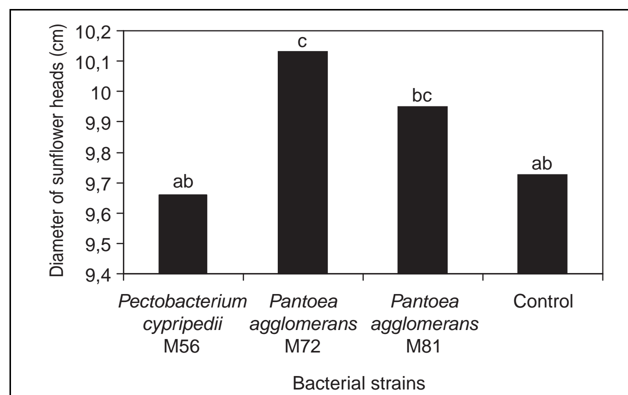

Fig. 2: Mean diameters of flower heads of sunflower plants treated with bacterial strains selected from *in vitro* studies. Different letters signify significant differences between mean values (Duncan`s multiple range test, $P = 0.15$).

terium, *Ochrobactrum*, *Pantoea*, *Bacillus*, *Burkholderia*, and *Streptomyces*. The genera *Bacillus* and *Pantoea* could always be assigned to two isolates, whereas the genus *Burkholderia* could be affiliated to three isolates. Partial 16S rDNA sequences showed at least 99% similarities to database entries.

3.7 ITS rDNA sequence analysis of fungal plant pathogens

To confirm identification of plant pathogenic *Colletotrichum* sp. and *B. maydis* based on morphological observations, their respective ITS rDNA sequences were subjected to BLAST analysis that revealed 100% similarities to *Colletotrichum* sp. resp. *Cochliobolusheterostrophus* (sexual stage of *B. maydis*).

4 Discussion

As microbial ecosystem, each plant harbours microorganisms which confer plant growth and health to their hosts (Berg et al. 2005; Compant et al. 2005; Raajmakers et al. 2008). Results obtained by this study suggest that 66% of tested bacterial isolates, obtained from rhizospheres of horseradish tree, sorghum, sunflower, and safflower in Bolivia, possess at least one out of four tested properties (N_2 fixation, ACC deaminase activity, P solubilization, IAA synthesis) that are involved in PGP. This is in agreement with a comparative study investigating bacteria derived from the rhizosphere of soy bean and bulk soil, which revealed that 68% of screened isolates exhibited positive results for IAA synthesis, ACC deaminase activity or P solubilization (Cattelan et al. 1999). Based on the fact that especially in the rhizosphere of the investigated plants extraordinary high abundances up to 10^{10} g^{-1} were found, a high indigenous plant growth promoting potential of microbial populations was determined. In comparison to the antagonistic potential in the rhizosphere, which is approx. 1/3 of the microbial populations (Berg et al. 2006), the potential to promote directly growth of observed host plants was higher.

By the use of a scheme to assess PGP traits *in vitro* of tested strains, a selection of promising candidates to support plant growth and health was obtained. Taxonomic analysis revealed that the majority of selected strains belong to *Pantoea*, *Bacillus*, *Burkholderia*, *Streptomyces*; bacterial genera that are well known as PGPB resp. BCAs (El-Shanshoury 1989; Krebs et al. 1998; Sessitsch et al. 2005; Sergeeva et al. 2007). In contrast, *Pectobacterium cypripedii* is a novel discovered PGPB. It is the only non-phytopathogenic and non-pectinolytic species in the genus *Pectobacterium*. However, isolate M41 is phylogenetically highly related to *Agrobacterium tumefaciens*, which is a notorious phytopathogenic organism. This underlines the importance of an early taxonomic analysis of presumable BCAs or PGPB.

According to the results obtained *in vitro*, *Pectobacterium cypripedii* M56, *Pantoea agglomerans* M72, and *Pantoea agglomerans* M81, isolated from the rhizosphere of horseradish tree, were selected for *ad planta* studies. As the unique isolates they fulfilled three out of four assessed criteria for direct PGP. Most positive responses regarding number of beans per black bean plant and flower head diameters of sunflower plants, parameters that indicate plant growth and harvest yield as well, were noted from the application of strain *P. agglomerans* M72, as mean values for aforementioned parameters determined from plants inoculated with respective strain were always highest. The effect of *P. agglomerans* M72 on plant growth and harvest yield was more pronounced for the black bean experiment (DMS, $P = 0.05$) than for the sunflower inoculation study (Duncan`s multiple range test, $P = 0.15$) when compared to respective control treatments. Although the effect on PGP resp. harvest yield by *P. agglomerans* M72 was less intensive in case of the sunflower field trial, *P. agglomerans* M72 may has the capability to serve as a PGPB for different agricultural plants by exploiting its full potential that could be assessed by dose-response assays (Bonaterra et al. 2003). The PGP effect of *P. agglomerans* M72 is not surprising as *P. agglomerans* strains are known for serving as PGPB (Sergeeva et al. 2007).

By our knowledge this is the first report that deals with the analysis of bacteria for PGP that were associated with *M. oleifera*. The Moringa tree (that originates from India) belongs to the plant family of Moringaceae and is mainly used for oil production (technical purposes and cosmetic industry) and as vegetable. The synthesis of antimicrobial compounds like glucosinolates and isothiocyanates by horseradish tree is remarkable (Fahey et al. 2001; Kjaer et al. 1979). In other plant species with this defence system such as canola, a significant impact on its associated microbial community was shown (Rumberger and Marschner 2003).

Due to the phenomenon of plant-driven selection of plant associated microbes (Hartmann et al. 2009) it is not self-evident that a horseradish tree derived bacterial strain is able to establish itself in rhizospheres shaped by other host plants like black bean or sunflower. Although the rhizosphere competence, as a prerequisite for plant growth promoting rhizobacteria (Bloemberg and Lugtenberg 2001), was not assessed for *P. agglomerans* M72, results obtained by this study suggest at least the effective colonization of the black bean rhizosphere by *P. agglomerans* M72 due to its significant reaction on that host plant. The soil used for the black bean inoculation survey, that was heavily exposed to solar radiation and might corresponded to approximated gnotobiotic conditions (as described above), may also be a reason for the stronger benefi-

cial effect on black bean plants in comparison to sunflower plants by *P. agglomerans* M72.

Results obtained by *in vitro* screenings for the assessment of bacterial PGP cannot hundred percent reflect the reality e.g. at the field or in the greenhouse. Not all bacterial traits that confer plant growth can be determined *in vitro* as e.g. for induced systemic resistance. However, *in vitro* screenings for bacterial PGP can provide a tool to select strains out of the vast amount of bacteria living in plant associated habitats that "fulfil *in situ* what they promise *in vitro*".

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References

ALTSCHUL, S.F., T.L. MADDEN, A.A. SCHAFFER, J.H. ZHANG, Z. ZHANG, W. MILLER, D.J. LIPMAN, 1997: Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**, 3389-3402.

BASHAN, Y., G. HOLGUIN, 1998: Proposal for the division of plant growth-promoting rhizobacteria into two classifications: Biocontrol-PGPB (plant growth-promoting bacteria) and PBPB. Soil. Biol. Biochem. **30**, 1225-1228.

BERG, G., A. KRECHEL, M. DITZ, R.A. SIKORA, A. ULRICH, J. HALLMANN, 2005: Endophytic and ectophytic potato-associated bacterial communities differ in structure and antagonistic function against plant pathogenic fungi. FEMS Microbiol. Ecol. **51**, 215-229.

BERG, G., J. HALLMANN, 2006: Control of plant pathogenic fungi with bacterial endophytes. In: B. SCHULZ, C. BOYLE, T. SIEBER (eds.): Microbial Root Endophytes, pp. 53-70. Springer, Berlin, Heidelberg, New York.

BERG, G., K. OPELT, C. ZACHOW, J. LOTTMANN, M. GÖTZ, R. COSTA, K. SMALLA, 2006: The rhizosphere effect on bacteria antagonistic towards the pathogenic fungus *Verticillium* differs depending on plant species and site. FEMS Microbiol. Ecol. **56**, 250-261.

BERG, G., H. MÜLLER, C. ZACHOW, K. OPELT, K. SCHERWINSKI, R. TILCHER, A. ULLRICH, J. HALLMANN, R. GROSCH, A. SESSITSCH, 2008: Endophytes: structural and functional diversity and biotechnological applications in control of plant pathogens. Ecol. Gen. **6**, 17-26.

BLOEMBERG, G.V., B.J.J. LUGTENBERG, 2001: Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Curr. Opin. Plant Biol. **4**, 343-350.

BONATERRA, A., L. RUZ, E. BADOSA, J. PINOCHET, E. MONTESINOS, 2003: Growth promotion of *Prunus* rootstocks by root treatment with specific bacterial strains. Plant Soil **255**, 555-569.

BROWN, C.M., M.J. DILWORTH, 1975: Ammonia assimilation by *Rhizobium* cultures and bacteroids. J. Gen. Microbiol. **86**, 39-48.

CATTELAN, A.J., P.G. HARTEL, J.J. FUHRMANN, 1999: Screening for plant growth-promoting rhizobacteria to promote soybean growth. Soil Sci. Soc. Am. J. **63**, 1670-1680.

CHERNIN, L., I. CHET, 2002: Microbial enzymes in the biocontrol of plant pathogens and pests. In: R.G. BURNS, R.P. DICK (eds.): Enzymes in the Environment – Activity, Ecology and Applications, pp. 171-226. Marcel Dekker, New York.

COMPANT, S., B. DUFFY, J. NOWAK, C. CLÉMENT, E.A. BARKA, 2005: Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Appl. Environ. Microbiol. **71**, 4951-4959.

DART, P.J., 1986: Nitrogen fixation associated with non-legumes in agriculture. Plant Soil **90**, 303-334.

DOBBELARE, S., J. VANDERLEYDERN, Y. OKON, 2003: Plant-growth promoting effects of diazotrophs in the rhizosphere. Crit. Rev. Plant Sci. **22**, 107-149.

EL-SHANSHOURY, A.R., 1989: Growth promotion of wheat seedlings by *Streptomyces atroolivaceus*. J. Agron. Crop Sci. **163**, 109-114.

FAHEY, J.W., A.T. ZALCMANN, P. TALALAY, 2001: The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry **56**, 5-51.

FAURE, D., D. VEREECKE, J.H.J. LEVEAU, 2008: Molecular communication in the rhizosphere. Plant Soil, DOI 10.1007/s11104-008-9839-2.

GLICK, B.R. 2005: Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. FEMS Microbiol. Lett. **252**, 1-7.

GULL, M., F.Y. HAFEEZ, M. SALEEM, K.A. MALIK, 2004: Phosphorus uptake and growth promotion of chickpea by co-inoculation of mineral phosphate solubilising bacteria and a mixed rhizobial culture. Austral. J. Exp. Agric. **44**, 623-628.

HARTMANN, A., D. VAN TUINEN, G. BERG, 2009: Plant-driven selection of microbes. In: P. LEMANCEAU (ed.), Rhizosphere, a Major Place of Interactions. Springer, Berlin, Heidelberg, New York, in press.

KADAM, S.S., K.G. KACHHAVE, J.K. CHAVAN, D.K. SALUNKHE, 1977: Effect of nitrogen, *Rhizobium* inoculation and simazine on yield and quality of Bengal gram (*Cicer arietinum* L.). Plant Soil **47**, 279-281.

KJAER, A., O. MALVER, B. EL-MENSHAWI, J. REISCH, 1979: Isothiocyanates in myrosinase-treated seed extracts of *Moringa peregrina*. Phytochemistry **18**, 1485-1487.

KREBS, B., B. HÖDING, S. KÜBART, M.A. WORKIE, H. JUNGE, G. SCHMIEDEKNECHT, R. GROSCH, H. BOCHOW, M. HEVESI, 1998: Use of *Bacillus subtilis* as biocontrol agent. I. Activities and characterization of *Bacillus subtilis* strains. J. Plant Dis. Protect. **105**, 181-197.

KURZE, S., H. BAHL, R. DAHL, G. BERG, 2001: Biological control of fungal strawberry diseases by *Serratia plymuthica* HRO-C48. Plant Dis. **85**, 529-534.

O`SULLIVAN, D.J., F. O`GARA, 1992: Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. Microbiol. Rev. **56**, 662-676.

RAAIJMAKERS, J.M., T.C. PAULITZ, C. STEINBERG, C. ALABOUVETTE, Y. MOENNE-LOCCOZ, 2008: The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. Plant Soil, DOI 10.1007/s11104-008-9568-6.

REDDY, M.S., R.K. HYNES, G. LAZAROVITS, 1994: Relationship between *in vitro* growth inhibition of pathogens and suppression of preemergence damping-off and postemergence root rot of white bean seedlings in the greenhouse by bacteria. Can. J. Microbiol. **40**, 113-119.

RUMBERGER, A., P. MARSCHNER, 2003: 2-Phenylethylisothiocyanate concentration and microbial community composition in the rhizosphere of canola. Soil. Biol. Biochem. **35**, 445-452.

SERGEEVA, E., L. DANIELLE, M. HIRKALA, L.M. NELSON, 2007: Production of indole-3-acetic acid, aromatic amino acid aminotransferase activities and plant growth promotion by *Pantoea agglomerans* rhizosphere isolates. Plant Soil **297**, 1-13.

SESSITSCH, A., T. COENYE, A.V. STURZ, P. VANDAMME, E.A. BARKA, J.F. SALLES, J.D. VAN ELSAS, D. FAURE, B. REITER, B.R. GLICK, G. WANG-PRUSKI, J. NOWAK, 2005: *Burkholderia phytofirmans* sp. nov., a novel plant-associated bacterium with plant-beneficial properties. Int. J. Syst. Evol. Microbiol. **55**, 1187-1192.

VAN LOON, L.C., 2007: Plant responses to plant growth promoting bacteria. Eur. J. Plant Pathol. **119**, 243-254.

WELLER, D.M., R.J. COOK, 1983: Suppression of take-all of wheat by seed treatments with fluorescent pseudomonads. Phytopathology **73**, 463-469.

WHIPPS, J.M., 2001: Microbial interactions and biocontrol in the rhizosphere. J. Exp. Bot. **52**, 487-511.