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Plant Growth Promoting Properties of Rhizobacteria Isolated from Wheat and Pea Grown in Loamy Sand Soil

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Abstract: Microbes are important catalysts to regulate functional properties of terrestrial ecosystems. In this study, rhizosphere and phyllosphere bacteria were isolated from wheat and peas and examined for their plant growth promoting properties. The effects of bacterial inoculants on the growth of peas and wheat were studied in a series of pot experiments using loamy sand soil. The results showed that the colonisation of bacteria was higher in the rhizosphere as compared to the phyllosphere of both plants. Bacterial strains were identified as *Pseudomonas, Bacillus, Kocuria,* and *Microbacterium,* and *Cellulomonas* species. The response of wheat and peas when inoculated with bacteria was significantly positive over the control. After inoculation with effective bacterial strains, the root and shoot growth, and nodulation of peas were increased. However, the strains stimulated only the roots of wheat. Independent of the origin (rhizosphere vs. phyllosphere), bacterial strains produced indole-3 acetic acid (IAA), which most probably accounted for the overall synergistic effect on growth of peas and wheat.

Key Words: Rhizosphere, phyllosphere, auxin, wheat, peas

Tınlı Topraklarda Yetişen Buğday ve Bezelye Bitkisinden İzole Edilen Rhizobakterilerin Bitki Büyüme Uyarıcı Özellikleri

Özet: Karasal ekosistemlerin fonksiyonel özelliklerini düzenlemede mikroplar önemli katalistlerdir. Bu çalışmada buğday ve bezelyeden izole edilen rhizosfer ve phyllosfer bakterileri bitki büyümesini uyarıcı özellikleri çalışılmıştır. Buğday ve bezleyelere bakteri ınokulasyonlarının etkileri bir seri saksı denemesinde tınlı kumlu topraklarda çalışılmıştır. Sonuçlara gore her bitki için rhizosphere'lerin kolanizasyonu phyllosphere'ye göre daha fazla olmuştur. Bakteri suşları *Pseudomonas, Bacillus, Kocuria* ve *Microbacterium* ve *Cellumonas* türleri olarak tanımlanmıştır. Bakteri ile inoküle edilen buğday ve bezelyenin cevabı control üzerine pozitif etki yapmıştır. Bakteri suşlarının inokülasyonundan sonra kök, gövde ve bezelye nodülasyonunda artış gözlenmiştir. Fakat, suşlar yalnız buğday köklerinde etkili olmuştur. Bakteri suşlarının menşeyinden bağımsız, bakteri suşları indol-3-asetik asit üretmesi buğday ve bezelye büyümesi üzerine sinerjik etkisini gösterir.

Anahtar Sözcükler: Rhizosfer, phyllosfer, oksin, buğday, bezelye

Introduction

Microbes are important regulators of terrestrial ecosystems. The challenges for the next decades include understanding the behaviour of microbes in their natural and often complex habitats, such as the rhizosphere (1). Analysis of the genotypic and phenotypic characteristics of indigenous rhizobacteria can help us to understand the mechanism of interactions between them and plant roots (2). Plant growth promoting bacteria are a group of organisms that have close association with plants and can help plants to establish in degraded ecosystems, protect plants from diseases, and promote plant growth (3).

Numerous plant growth promoting rhizobacteria (PGPR) of the genera *Pseudomonas, Bacillus, Arthrobacter, Azospirillum, Klebsiella*, and *Enterobacter* have been isolated from the rhizosphere of various crops and noted for their synergistic effects on plant growth (4-6). Understanding the diversity and beneficial activity of the plant-bacterial association is important to sustain agroecosystems for sustainable crop production (3). Beneficial effects of micro-organisms have often been evaluated on the basis of faster seed germination, better seedling emergence, and increased plant growth. Other than rhizobacteria, some phyllosphere bacterial strains

such as *Pseudomonas* spp., *Pantoea* spp., and *Agrobacterium* spp. have been reported to increase plant growth and nutrient uptake of grasses, cereals, and legumes (7). The mechanisms of plant growth stimulation by associative bacteria are mobilisation of nutrients, stimulation of root growth by production of phytohormones, and antagonism against soil-borne plant pathogens (8,9). However, the nitrogen-fixing ability did not correlate with the growth-promoting properties of the PGPR bacteria (10,11). The production of phytohormones (e.g., auxins) has been suggested to be one of the mechanisms by which PGPR bacteria stimulate plant growth (4). A diverse group of soil micro-organisms are capable of producing physiologically active auxins that may have pronounced beneficial effects on plant growth and development (12). Barea et al. (13) reported that about 86% of the bacterial strains isolated from the rhizosphere of various plants produced auxins.

The objectives of the study were to isolate plant growth promoting rhizosphere and phyllosphere bacteria from peas and wheat, and evaluate their beneficial effects on plant growth properties.

Materials and Methods

Soil and plants

Pot experiments were conducted with a neutral (pH 6.9) loamy sand soil that contained 7 g organic C, 600 mg N, 62 mg P, and 74 mg K kg^{-1}. The soil was collected from fields under agricultural production at Muncheberg, Germany. Total soil organic C content was determined by elementary analysis while total N content was determined by the Kjeldahl method. The molybdenum blue method was used to determine the total P content of soil. Potassium content was determined by using the flame photometric method (14). Soil pH was measured by means of an electrometer. Wheat (*Triticum aestivum* cv. Naxo) and peas (*Pisum sativum* cv. Grapis) were used as experimental plants.

Isolation and characterisation of soil micro-organisms

Wheat and peas were grown in pots containing 0.3 kg of soil. Both plants were harvested 28 days after the emergence of the seeds. After harvesting, the roots and shoots of wheat were separated. For determination of rhizosphere colonisation, 1 g of fresh root was chopped into pieces and subsequently shaken with 9 ml of sterile water containing 1 ml of 40% tetramethylthiuramdisulphide solution for 30 min. To isolate phyllosphere colonisation, a 1-g leaf sample was macerated and subsequently shaken with 9 ml of sterile water. From the suspension, a serial dilution (1:10) was prepared and the resulting suspensions were spread over the surface of a glycerol-peptone agar (peptone 10 g, glycerol 10 ml, NaCl 5 g, KH_2PO_4 0.1 g, and agar 15 g l^{-1} of sterile water) (15). Four inoculated plates per dilution were incubated at 28 °C and the bacterial colony forming units (CFUs) were counted after 4 days of incubation. Twenty bacterial colonies from each plate were randomly selected for further studies. The isolates were streaked twice on the original medium, checked for purity. The isolates were identified by using conventional tests (e.g., morphological, physiological, and chemotaxonomic characterisation and commercial identification systems, like Biolog (GN, GP) from the Biolog Inc. and API 20E or API 20NE (bioMérieux) (16).

Auxin production

The production of indole-3-acetic acid (IAA) was determined by following Bric et al. (17). Selected bacterial strains were grown in glycerol-peptone broth with and without tryptophan (500 mg ml^{-1}) and incubated at 28 °C for 3 days. A 2-ml culture was taken from each tube and centrifuged at 10,000 rpm for 15 min. One millilitre of the supernatant fluid was taken to a clean dry tube to which 100 ml of 10 mM orthophosphoric acid and 2 ml of reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$) were added. After 25 min, the absorbance of the pink colour was measured spectrophotometrically at 530 nm. The IAA concentration in the culture was determined by using a calibration curve of pure IAA as a standard (18).

Antagonistic activity

The isolates were tested in vitro to select bacteria capable of inhibitory effects against *Fusarium culmorum* by using a plate bioassay with potato dextrose agar (PDA). Fungal isolates were grown in a PDA plate at 30 °C for 7 days. Discs of fresh culture of the fungus (5 mm diameter) were cut out and placed in the centre of 9-cm petri plates with PDA. Bacteria (grown in peptone agar plates) were streaked on the test plate perpendicular to the fungi. Plates were incubated at 30 °C for 7 days until the fungi had grown over control plates without any bacterial isolates. Antifungal activity was recorded as the width of the zone of growth inhibition between the

fungus and the test bacterium. The experiment was conducted by using a completely randomised design with 3 replications per treatment. The experiment was performed twice.

Plant growth promotion

A second set of pot experiments with loamy sand was used to evaluate the effects of bacterial strains isolated from the rhizosphere and phyllosphere of wheat and pea on shoot and root growth of both plants. Prior to sowing, the pots were filled with 350 g of fresh soil. The soil was moistened with water and maintained at 60% of its moisture holding capacity. Four seeds of wheat and 3 seeds of peas were sown per pot. After germination, the plants were thinned to 2 seedlings per pot. Seedlings of each plant were inoculated with 1 ml of the appropriate bacterial suspensions, resulting in inoculum density of ca. 10^6 cfu ml^{-1}. The control was considered un-inoculated plants. Plants were placed in a temperature regulated growth chamber at a light intensity 20 kLux for 16 h at 16 °C during the day and 12 °C at night. Four weeks after germination, plant shoots and roots were separated and oven dried at 105 °C. The inoculation treatments were set-up in a completely randomised design with 6 replicates. The data were analysed for significant differences ($P < 0.05$) of main effects using 2-way ANOVA and Student-Newman-Keuls tests.

Results

Averaged across treatments, the pea rhizosphere colonisation by bacteria was significantly higher (log10[cfu/g of root] value 7.64) compared to that of wheat rhizosphere (log10[cfu/g of root] value 6.51). However, the phyllosphere colonisation of both plants with bacteria was significantly lower than that of rhizosphere colonisation. The pea phyllosphere colonised by bacteria (log10[cfu/g of shoot] value 3.84) was significantly higher than in the wheat phyllosphere (log10[(cfu/g of shoot] value 2.77).

There were 20 strains identified for further studies. Among the isolates, the representative bacterial strains in the rhizosphere were identified as *Bacillus lentus* 10/1, *Bacillus* sp. 41/1, *Cellulomonas* sp. 32, *Bacillus subtilis* 1, *Bacillus lentus* 28, *Bacillus* sp. 31 and phyllosphere *Cellulomonas* sp. 20/2, *Microbacterium* sp. 44, *Bacillus lentus* 17, *Bacillus cohnii* 19, *Pseudomonas fluorescens* 45, *Bacillus* sp. 39/1, *Kocuria varians* 13, and *Bacillus halodurans* 12.

The experimental results showed that the bacterial strains independent from the origin increased the root, shoot, and dry weight of peas by more than 26% compared to the control (Table 1). The strains increased the main root nodulation of peas by 35% to 75%. Moreover, the nodulation of lateral roots significantly increased by 200% after inoculation with bacterial strains.

The growth promotion of winter wheat after inoculation showed that most of bacterial strains were responsible for increasing only the root dry weights. There were no significant effects on shoot growth (Figure). All bacterial strains produce IAA regardless of their origin, rhizosphere, or phyllosphere (Table 2). The highest concentration of IAA (2.0 µg to 2.70 IAA ml^{-1} filtrate) were produced by bacterial strain *P. fluorescens* 45 and *Kocuria varians* 13 isolated from the phyllosphere of peas. Among 20 strains, 7 bacterial strains were found to be antagonistic to *Fusarium culmorum* fungi.

Discussion

The importance of rhizosphere and phyllosphere bacteria in promoting plant growth is well recognised (3,9). Our results have demonstrated that, independent of the origin, selected growth-stimulating rhizosphere and phyllosphere bacterial isolates were able to increase the growth of wheat and peas. Höflich et al. (19) reported that rhizosphere bacterial strains (*P. fluorescens* PsIA12 and *Agrobacterium rhizogenes* A1A4) increased the growth of legume and wheat under field conditions. Similar results were reported by Defreitas and Germida (20), that *Pseudomonas* stimulated wheat growth. The shoot and root growth, nodule numbers, and nutrient uptake of peas and soybean were increased when seeds were inoculated by rhizobacteria (21,22). The mechanisms of plant growth stimulation by associative bacteria are most probably related to greater mobilisation of nutrients, antagonism against soil-borne plant pathogens, and phytohormone production (9,23). Rhizosphere bacteria colonised in the root surface, which is relatively rich in organic substrates, and synthesised phytohormone auxin as secondary metabolites. Phytohormones are known to play a key role in plant growth regulation. They promote seed germination, root elongation, and stimulation of leaf expansion (24). Lindberg et al. (10) suggested that greater hormone

Table 1. Influence of selected bacterial strains on relative growth of root and shoot biomass of peas, and nodulation of peas.

Bacterial strains	Dry matter (g pot^{-1})		Nodulation (# plant^{-1})	
	Shoot	Root	Main root	Lateral root
Control	100	100	100	100
	(0.1896)	(0.2356)	(20)	(6)
Bacillus lentus 10/1	110	116	155*	150
Bacillus sp. 41/1	115*	118	135*	217
Cellulomonas sp. 32	122*	112	175*	267*
Bacillus subtilis 1	105	102	145*	117
Bacillus lentus 28	105	123*	145*	167
Bacillus sp. 31	105	110	145*	267*
ni 23	102	104	145*	133
ni 8	119*	121	160*	217
ni 16	107	121	125	283*
ni 7	119*	126*	140*	233*
Cellulomoas sp. 20/2	120*	123	130	233*
Bacillus cohnii 19	110	117	135*	217*
Microbacterium sp. 44	109	110	135*	83
Kocuria varians 13	109	106	140*	150
ni 51	101	116	130*	183
Bacillus lentus 17	103	100	140	200
ni 48	112	107	160*	233*
Bacillus halodurans 12	106	112	145*	217
Pseudomonas fluorescens 45	107	120	125	183
ni 39/1	106	111	155*	250*
LSD < 0.05	14	23	31	133

* Significantly different from the control for P < 0.05

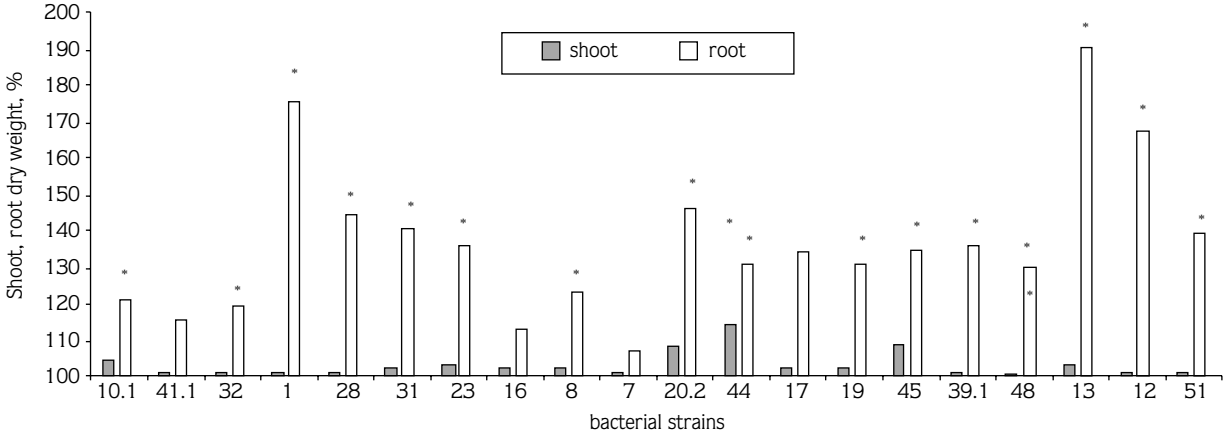

Figure. The effect of bacterial strains on the shoot and root dry matter of winter wheat (pot experiments, loamy sand, 4 weeks, control = 100% shoot-0.1119 g/plant; root- 0.2170 g/plant).

Table 2. Indole-3-acetic acid (IAA) production by bacterial strains (µg IAA ml^{-1} filtrate).

Origin	Bacterial strains	IAA (µg ml^{-1} filtrate)	Antagonistic activity against *F. culmorum*
Rhizosphere			
Pea	*Bacillus lentus* 10/1	1.75	-
Pea	*Bacillus* sp. 41/1	1.35	-
Winter wheat	*Cellulomonas* sp. 32	0.3	-
Winter wheat	*Bacillus subtilis* 1	1.7	A
Winter wheat	*Bacillus lentus* 28	1.25	A
Winter wheat	*Bacillus* sp. 31	0.9	-
Winter wheat	ni 23	0.9	-
Winter wheat	ni 16	0.25	-
Winter wheat	ni 8	2.7	-
Winter wheat	ni 7	1.6	A
Phyllosphere			
Pea	*Cellulomonas* sp. 20/2	1.45	-
Pea	*Microbacterium* sp. 44	1.65	-
Pea	*Bacillus lentus* 17	1.65	A
Pea	*Bacillus cohnii* 19	1.1	A
Pea	*P.s fluorescens* 45	2.0	A
Pea	*Bacillus* sp. 39/1	1.3	-
Pea	ni 48	1.95	-
Pea	*Kocuria varians* 13	2.70	-
Winter wheat	*Bacillus halodurans* 12	1.8	A
Winter wheat	ni 51	0.8	-

ni - not identified, and A-antagonist

production is an important part of the overall effect of bacteria on plant growth. They regulate plant growth by modifying physiological and morphological processes at very low concentrations. A diverse group including pseudomonads has been found to synthesise IAA, which influences root length due the hormonal effects (25). In our experiments, most of the bacterial strains produced IAA. Similarly, Leinhos and Vacek (26) reported a production of 1.6 to 3.3 mg auxin ml^{-1} filtrate by rhizosphere bacteria isolated from wheat. Furthermore, Prikryl et al. (27) have reported production of IAA and some other auxins in liquid culture of *Pseudomonas cepacia* and *P. fluorescens* isolated from maize and bean rhizosphere. Production of growth substances such auxin (indole-3-acetic acid) by bacteria has also been confirmed in other studies (12,28). The production of growth promoting compounds indole-3-acetic acid by *P. polymyxa* has been suggested to be growth stimulants of crested wheatgrass (29). Some of our isolated bacterial strains were antagonistic against *Fusarium culmorum* pathogens. In addition, greater root development and proliferation of plants in response to bacterial activities enhanced water and nutrient uptake (24).

In conclusion, the bacteria associated plant growth properties shown in our experiments suggested that, independent of origin (rhizosphere, phyllosphere), PGPR bacteria were able to produce phytohormone auxin (IAA). However, the bacterial strains were not plant specific; they stimulated the plant growth of wheat and pea regardless of their origin.

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