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RESEARCH ARTICLE

ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF ENDOPHYTIC BACTERIA-AZOSPIRILLUM SP. ANDPSEUDOMONAS SP. FROM BHENDI (ABELMOSCHUS ESCULENTUS L. MOENCH)

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ABSTRACT

Thirty five isolates of endophytic bacteria (Azospirillum sp. and Pseudomonas sp.) were isolated from bhendi from three different localities. All the bacterial isolates were evaluated for its biochemical characterization and nitrogen fixation. The results showed, among them, six isolates were effective in nitrogen fixation. Among the six, two strains were selected as efficient to other four, they were molecularly characterized by sequencing their 16SrDNA and the results confirmed their sequences are Azospirillum brasilense and Pseudomonas fluorescens. This study indicates that there is huge number of endophytic microbes occupy a relatively privileged niche within plant and usually contribute to plant health.

INTRODUCTION

Background

Among the immature fruit vegetables, brinjal and okra are occupying major area in cultivation and major vegetables by consumption in Tamilnadu. Bhendi *Abelmoschus esculentus* L. (Moench). is an economically important vegetable crop. It is grown commercially in India, India ranks first in the world with 3.5 million tonnes (70% of the total world production) of bhendi produced from over 0.35 million ha land (FAOSTAT 2008). Bhendi is said to be very useful against genito-urinary disorders, spermatorrhoea and chronic dysentery (Nadkarni, 1927). Its medicinal value has also been reported in curing ulcers and relief from hemorrhoids (Adams, 1975). Beneficial plant–microbe interactions that promote plant health and development have been the subject of considerable study. At the most basic level, endophyte simply means the location of an organism, with "endo" means "inside" and "phyte" means "plants". Therefore, endophyte refers to organisms that live within plants (Wilson, 1995).

Objective

This study was undertaken to isolate and identify endophytic bacteria (*Azospirillum sp.* and *Pseudomonas sp*). associated with Okra from three localities.

Beneficial plant–microbe interactions that promote plant health and development have been the subject of considerable study. Fungi and bacteria are the most common organisms associated with the term endophyte. Endophytic organisms associated with plants are varied and complex. Endophytic microbes occupy a relatively privileged niche within plant and usually contribute to plant health. Some groups of endophytic microorganisms have been believed to be mutualists that protect plants against biotic stresses. Co-evolution may exist between endophytes and their host in resist to environmental stresses. During the last two decades endophytes have been targeted as valuable sources of new bioactive compounds (Tadych, & White, 2009). Some of the endophytes are proved to be able to enhance plant growth by nitrogen fixation(Chelius *et al*.,2000). Endophytic microorganisms depend on the nutrient supplied by host plants, so parameters affect plant nutrient supplies will consequently influent endophytic communities. Endophytic bacteria are defined as bacteria that are detected "from inside surface-disinfested plants or extracted from inside plants and have no visibly harmful effects on the plants"extracted from inside plants and have no visibly harmful effects on the plants"(Hallmann, *et al*., 1997). Endophytes enter plant tissue primarily through the root zone; however, aerial portions of plants, such as flowers, stems, and cotyledons, may also be used for entry (**Kobayashi, D. Y., and J. D. Palumbo.** 2000). Specifically, the bacteria enter tissues via germinating radicles (**Gagne *et al.*,** 1987), secondary roots (**Agarwal, S., and S. T. Shende.** 1987), stomates (**Roos, I. M. M., and M. J. Hattingh.** 1983), or as a result of foliar damage (**Leben *et al.*,** 1968). Endophytes inside a plant may either become localized at the point of entry or spread throughout the plant (**Hallmann *et al.*,** 1997). These microorganisms can reside within cells **(Jacobs *et al.*,**1985), in the intercellular spaces, (**Patriquin, D. G., and J. Dobereiner.** 1978**)** or in the vascular system (**Bell *et al.*,** 1995). Generally, bacterial populations are larger in roots and decrease in the stems and leaves **(Lamb *et al.*,**1996).

Every plant found so far associate with at least one kind of endophytic microbes (Guo *et al*., 2008). Variety of interactions is going on ranging from microbe-host interaction to microbe-microbe interaction. Endophytes colonizing inside plant tissues contribute to the fitness of host and in return, they gain nutrient and protection from the host. Endophytic bacteria living within plant tissue as biotrophic symbionts and these bacteria can be either obligate or facultative. Some endophytic bacteria are able to colonize thousands of different plant species, while some are restricted to plant families. Endophytic

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bacteria produce a wide range of phytohormones, such as auxins, cytokinins, and the gibberellins. In addition, endophytic bacteria help to enhance the nutrient ability and fix nitrogen for plants.The bioactive natural products from endophytes are promising resources for medicine, agriculture and industry (Guo *et al*., 2008).

MATERIALS AND METHODS

Collection of plant material

Bhendi variety *Arka anamika* was collected from Agriculture field of Annamalai nagar, Puthur, Tamilnadu and Karaikal, Pondicherry, India. The plant samples were transported to refrigerated box at 4°C to the laboratory.

Sterilization

The plant parts such as root, stem and leaves were thoroughly washed in tap water followed by distilled water to get rid of adhering soil particles and cut into small pieces. They were soaked in 0.1% mercuric chloride solution for 5 minutes to sterilize the surface and thoroughly washed in tap water followed by sterile distilled water and 1gm of each sample was taken for extraction by macerating in pestle and mortar.

Isolation of endophytes

The samples extracts volume was made up to10ml using sterile distilled water. From that, 1ml was taken and serially diluted using test tubes containing 9ml of sterilized distilled water. 0.1ml from the dilutions10^{-4}, 10^{-5} and 10^{-6}were transferred to Petri-plates containing nitrogen free malate medium (NFB- Dobereiner,1992) and King's B (Kaare Johnsen and Preben Nielsen, 2006) medium for the isolation of *Azospirillum sp.* and *Pseudomonas sp.* respectively. The plates were rotated in clockwise and anticlockwise direction for uniform distribution of medium and incubated at 37ºc for 48hrs. Meanwhile, 1ml of extracts was dissolved in semisolid NFB to observe the sub-surface pellicle formation by *Azospirillum sp.* and kept at room temperature. The isolated colonies in petri dishes were brought to pure culture by several subcultures. Pellicle forming *Azospirillum sp.* in test tubes containing semisolid NFB was streaked in petri plates containing solid NFB. The purified strains were maintained in nutrient agar slants and stored at 4ºC for future use.

Purification of *Azospirillum sp.* and *Pseudomonas sp.*

Individual colonies which become scarlet upon storage were picked out and streaked on Potato infusion agar (BMS) plates (Baldiani and Dobereiner, 1980) and incubated at 32˚C for 7days. Typical pink colour often wrinkled colonies were then transferred to nutrient agar slants for further study of *Azospirillum species.*

The isolates of *Pseudomonas fluorescens* were purified using King's B medium and examined after various periods of incubation under ultraviolet light at 360 mμ. for confirmation of fluorescens.

Colony and Cell Morphological Characterization

Colony and cell morphological features of bacterial isolates were observed by culturing the isolates after 48 hours of spreading on nitrogen free maleic medium and King's B medium for *Azospirillum sp.* and *Pseudomonas sp.* respectively in petri plates. A loop full of bacterial culture was transferred on glass slide with a drop of sterile water and observed under light microscope for recording cell morphology.

Further the isolates were subjected to various biochemical tests for their species level identification. The representative strains of *Azospirillum sp*. and *Pseudomonas sp*. were identified by the following identification tests.

Preliminary identification tests (Bergey's Manual of Determinative bacteriology. 9thEdition)

The Gram Stain

This is a differential stain. It requires a primary stain and counter stain. It divides most of the Eubacteria into two large groups: Gram positive and Gram negative. The heat fixed bacterial smear was flood with crystal violet (primary stain) for 1min., then smeared with Iodine (Mordant) 1 min., then with Ethanol to decolorize, then with saffranin for 1min. After the application of every stain the smear was washed thoroughly in water. Finally the smear was blotted, air dried and observed under microscope.

Catalase test

This test is used to identify organisms that produce the enzyme, catalase. This enzyme detoxifies hydrogen peroxide by breaking it down into water and oxygen gas. The bubbles resulting from production of oxygen gas clearly indicate a catalase positive result. The sample on the right below is catalase positive.

$$2H_2O \longrightarrow 2H_2o+O_2$$

Slant method

Three to four drops of the 3% hydrogen peroxide was allowed to flow over the entire surface of the inoculated slant containing culture and examine the absence or the formation of bubbling or foaming.

Oxidase test

This test is used to identify microorganisms containing the enzyme cytochrome oxidase (important in the electron transport chain). cytochrome oxidase transfers electrons from the electron transport chain to oxygen (the final electron acceptor) and reduces it to water. in the oxidase test, artificial electron donors and acceptors are provided. when the electron donor is oxidized by cytochrome oxidase it turns a dark purple. this is considered a positive result. a few drops of electron donar phenylenediamine a redox dye was added to what man filter paper containing culture and the colour changes to blue-purple was observed.

Carbohydrate fermentation test

This test is used to determine which fermentation pathway is used to utilize glucose. In the mixed acid fermentation pathway, glucose is fermented and produces several organic acids (lactic, acetic, succinic, and formic acids). The stable production of enough acid to overcome the phosphate buffer will result in a pH of below 4.4. If the pH indicator (methyl red) is added to an aliquot of the culture broth and the pH is below 4.4, a red color will appear. If the MR turns yellow, the pH is above 6.0 and the mixed acid fermentation pathway has not been utilized. Trypticase, sodium chloride and phenol red were dissolved in 100ml distilled water then 0.5% to 1%

Table 1 List of endophytic bacterial isolates of *Azospirillum* and *Pseudomonas* straine from Okra at three different localities

S.No	Bacterial isolates	Bacterial name	Isolated from	Host plant	Locality
1.	AORU1	Azospirillum sp.	Root	Okra	Annamalai nagar
2.	AORU2	Azospirillum sp.	Root	Okra	Annamalai nagar
3.	AORU3	Azospirillum sp.	Root	Okra	Annamalai nagar
4.	AORU4	Azospirillum sp.	Root	Okra	Annamalai nagar
5.	AORU5	Azospirillum sp.	Root	Okra	Annamalai nagar
6.	AORU6	Azospirillum sp.	Root	Okra	Annamalai nagar
7.	AOSU7	Azospirillum sp	Stem	Okra	Annamalai nagar
8.	AOSU8	Azospirillum sp	Stem	Okra	Annamalai nagar
9	AOSU9	Azospirillum sp	Stem	Okra	Annamalai nagar
10.	AOLU10	Azospirillum sp	Leaf	Okra	Annamalai nagar
11.	AORK11	Azospirillum sp	Root	Okra	Karaikal
12.	AORK12	Azospirillum sp	Root	Okra	Karaikal
13.	AORK13	Azospirillum sp	Root	Okra	Karaikal
14.	AORK14	Azospirillum sp	Root	Okra	Karaikal
15.	AOSK15	Azospirillum sp	Stem	Okra	Karaikal
16.	AOSK16	Azospirillum sp	Stem	Okra	Karaikal
17.	AORP17	Azospirillum sp	Root	Okra	Putthur
18.	AORP18	Azospirillum sp	Root	Okra	Putthur
19.	AORP19	Azospirillum sp	Root	Okra	Putthur
20.	AOSP20	Azospirillum sp	Stem	Okra	Putthur
21.	AOLP21	Azospirillum sp	Leaf	Okra	Putthur
22.	PORU1	Pseudomonas sp.	Root	Okra	Annamalai nagar
23.	PORU2	Pseudomonas sp.	Root	Okra	Annamalai nagar
24.	PORU3	Pseudomonas sp.	Root	Okra	Annamalai nagar
25.	POSU4	Pseudomonas sp.	Stem	Okra	Annamalai nagar
26.	POSU5	Pseudomonas sp.	Stem	Okra	Annamalai nagar
27.	POLU6	Pseudomonas sp.	Leaf	Okra	Annamalai nagar
28.	PORK7	Pseudomonas sp.	Root	Okra	Karaikal
29.	PORK8	Pseudomonas sp.	Root	Okra	Karaikal
30.	POSK9	Pseudomonas sp.	Stem	Okra	Karaikal
31.	POSK10	Pseudomonas sp.	Stem	Okra	Karaikal
32.	POLK11	Pseudomonas sp.	Leaf	Okra	Karaikal
33.	PORP12	Pseudomonas sp.	Root	Okra	Putthur
34.	PORP13	Pseudomonas sp.	Root	Okra	Putthur
35.	POSP14	Pseudomonas sp.	Stem	Okra	Putthur

desired carbohydrate was added into each conical flasks containing the above said chemicals. Then durham was inserted invertly into each conical flasks which should be fully filled with broth containing culture. They were sterilized and incubated at 37^0cfor 18 to 24 hrs and the results in each flasks were noticed.

Motility test (Hanging drop method)

This test is used to determine whether an organism is equipped with flagella or not. A drop of bacterial culture was placed in the centre of cavity slide and a thin film of petroleum jelly was placed at the edges of the cover slip and the cavity slide was turned upside down in the glass slide and observed under oil immersion in microscope.

Biotin requirement (Allen, 1953)

The biotin requirements of the bacterial isolates are tested using semisolid nitrogen free malic acid medium prepared in two sets of tubes, one set of medium prepared with the addition of biotin (100 µg l-1) and other without biotin. The growth was observed by the change in colour from yellowish green to blue.

Nitrate reductase (Yordi and Rouff, 1981)

The cultures were grown in 10ml of malate broth supplemented with 10mM of sodium nitrate and incubated at 32ºC for 5 days in shake culture conditions. Then it was centrifuged and the supernatant was collected. To 10ml of the supernatant 0.3ml of 1% sulphanilamide in 1.5N Hcl and 0.2 ml of 0.002% N (α Napthyl) ethylene diamine and dilute Hcl were added the appearance of pink colour indicated the presence of Nitrate activity.

Nitrite Reductase

A loopful of malate grown cultures was transformed to 5ml of malate medium containing 5Mm of sodium nitrate as the source of nitrogen and incubated at 32ºC for 5 days. The broth was centrifuged and the supernatant was collected. To 1ml of supernatant 0.3ml of 1% sulphanilamide in 1.5N Hcl and 0.2 ml of 0.002% N (α Napthyl) ethylene diamine and dilute Hcl were added the disappearance of pink colour indicated the presence of Nitrite activity.

Dinitrogen fixation

The nitrogen fixation of the isolates was determined by Microkjeldahl Assay described by Humphris, 1956.

IAA production (Garden and Paleg, 1957)

The isolates were grown in Erlenmeyer flasks contained 100ml of N free malate broth, supplemented with tryptophan at $25\mu gL^{-1}$ for 5hrs and 7 days at30ºC and the flasks were wrapped with black paper during incubation to avoid photoinactivation of the biologically active compounds.

Extraction and estimation of IAA produced

After the incubation period, the broth cultures were centrifuged at 5000rpm for 15min and the supernatant was filtered through filter paper. The P^H was adjusted to 2.8.

50ml filtrate was taken in a separating funnel and the equal volume of peroxide free cold diethyl was added and mixed

Table 2 Biochemical test for species characterization of Azospirillum and Pseudomonas isolates from Okra

Isolate Number	Acid production from Glucos	Utilization of different Carbon source				Biotin needs	Nitrate Reductase Activity	Nitrite Reductase Activity	Spore formation	Gram's stain	Catalase activity	Oxidase activity	Motility
		Malate	Succinate	Mannitol	Fructose								
AORU1	-	+	-	+	+	+	+	+	-	-	-	+	+
AORU2	-	-	-	+	-	+	+	+	-	-	+	-	+
AORU3	+	+	-	-	+	-	+	+	-	+	+	+	+
AORU4	+	-	-	+	+	+	+	+	-	+	+	-	+
AORU5	+	-	+	+	-	-	+	+	-	-	+	+	+
AORU6	+	-	-	+	+	+	+	+	-	-	+	+	+
AOSU7	-	-	+	-	-	-	+	+	-	-	+	+	+
AOSU8	-	+	-	+	+	+	+	+	-	-	-	+	+
AOSU9	-	-	-	+	-	+	+	+	-	-	+	-	+
AOLU10	+	-	+	+	-	-	+	+	-	-	+	+	+
AORK11	+	-	-	+	+	+	+	+	-	-	+	+	+
AORK12	-	-	+	-	-	-	+	+	-	-	+	+	+
AORK13	-	+	-	+	+	+	+	+	-	-	-	+	+
AORK14	+	-	+	+	-	-	+	+	-	-	+	+	+
AOSK15	+	-	-	+	+	+	+	+	-	+	+	-	+
AOSK16	+	-	+	+	-	-	+	+	-	-	+	+	+
AORP17	+	-	+	+	-	-	+	+	-	-	+	+	+
AORP18	-	-	+	-	-	-	+	+	-	-	+	+	+
AORP19	-	+	-	+	+	+	+	+	-	-	-	+	+
AOSP20	+	-	-	+	+	+	+	+	-	+	+	-	+
AOLP21	+	-	+	+	-	-	+	+	-	-	+	+	+
PORU1	+	+	+	-	-	+	+	+	-	-	+	+	+
PORU2	+	-	-	+	+	+	+	+	-	-	+	+	+
PORU3	-	-	+	-	-	-	+	+	-	-	+	+	+
POSU4	+	-	+	+	-	-	+	+	-	-	+	+	+
POSU5	-	-	+	-	-	-	+	+	-	-	+	+	+
POLU6	-	+	-	+	+	+	+	+	-	-	-	+	+
PORK7	+	-	+	+	-	-	+	+	-	-	+	+	+
PORK8	+	+	+	-	-	+	+	+	-	-	+	+	+
POSK9	-	+	-	+	+	+	+	+	-	-	-	+	+
POSK10	+	-	+	+	-	-	+	+	-	-	+	+	+
POLK11	+	-	+	+	-	-	+	+	-	-	+	+	+
PORP13	+	+	+	-	-	+	+	+	-	-	+	+	+
PORP14	-	+	-	+	+	+	+	+	-	-	-	+	+
POSP15	+	-	+	+	-	-	+	+	-	-	+	+	+

thoroughly .the contents of the funnel was allowed to stand for 4hrs for 4ºC with intermittent shaking . theaquoes phase was subjected to more extraction and organic phase was pooled together. The ether phase was evaporatedto dryness and the residue was dissolved in 2ml isopropyl alcohol. From the suspension, 0.5 ml was taken in test tubes and added with1.5ml of distilled water followed by 4ml of salper's reagent (10ml of 0.5M ferric chloride in 50ml of 35% perchloric acid). The tubes were incubated in complete darkness for 1hr at 28ºC. the intensity of colour developed was measured at 535 in spectronic. The standard prepared with known qualities of IAA used to calculate the quantity of IAA produced in test tubes.

Molecular characterzation

Isolation of DNA

Around 0.5 g of sample was placed in a mortar and homogenized with 2 ml of extraction buffer. The extraction buffer (pH 8.0) consisted of 100 mMTris, 20 mM EDTA, 0.5 M NaCl, 7 M Urea, 0.1% β-mercaptoethanol and 2% SDS. An equal volume of phenol:chloroform :Isoamlyalcohol (25:24:1) was added to the tubes and mixed well by gently shaking the tubes. The tubes were centrifuged at room temperature for 15 min at 15,000 rpm. The upper aqueous phase was collected in a new tube and an equal volume of chloroform: Isoamlyalcohol (24:1) was added and mixed. The upper aqueous phase obtained after centrifuging at room temperature for 10 min at 15,000 rpm was transferred to a new tube. The DNA was precipitated from the solution by adding 0.1 volumes of 3 M Sodium acetate pH 7.0 and 0.7 volume of Isopropanol. After 15 min of incubation at room temperature the tubes were centrifuged at 4ºC for 15 min at 15,000 rpm. The DNA pellet was washed twice with 70% ethanol and then very briefly with 100% ethanol and airdried. The DNA was dissolved in TE (Tris-Cl 10 mM pH 8.0, EDTA 1 mM). To remove RNA 5 µl of DNAse free RNAseA (10 mg/ml) was added to the DNA.

PCR analysis

AP-PCR ANALYSIS: AP-PCR amplification was carried out in a volume of 25 µl containing 100 ng of template DNA, 2 mM MgCl2, 5µm 27f primer, 2.5 µl of 10X assay buffer which includes (10 mMTris (pH 9.0), 50 mMKCl, 1.5 mM MgCl2, and 0.01% Gelatin), 10 mM each of dNTPs and 5units/µl of of Taq DNA Polymerase. 1 cycle of 5 min at 94ºC for denaturation, 5 min at 45ºC for annealing, and 5 min at 72ºC extension and 35 cycles of 1 min at 94ºC, 1 min at 45ºC and 1 min at 72ºC, followed by a final 10 min extension at 72ºC.

16SrDNA Sequence Determination

The amplified products of approximately 1,461 bp of *Azospirillum sp*. and 1.341 bp of *Pseudomonas sp*. were sequenced by 16s primer containing 27f (forward primer) and 1492 (reverse primer) sequencing products wee purified and the results were recorded. The sequence data of 16SrDNA was compared with sequences in the National centre for

Table3 Nitrogen fixation and IAA production potentiality *Azospirillum* and *pseudomonas* isolates from bhendi

Isolate number	N Fixation (µg of $Ng^{-1}$of malate)	IAA production µgml^{-1}
AORU1	6.52±0.036	1.15±0.035
AORU2	4.18±0.045	1.13±0.045
AORU3	15.5 ±0.045	1.64 ±0.035
AORU4	10.24 ±0.036	1.28 ±0.040
AORU5	16.17 ±0.025	1.84 ±0.032
AORU6	9.02±0.015	1.15±0.040
AOSU7	3.11 ±0.043	0.52 ±0.047
AOSU8	2.02 ±0.043	1.05 ±5.557
AOSU9	7.50 ±0.025	0.48 ±0.032
AOLU10	1.09±0.032	0.52±0.041
AORK11	10.6±0.035	1.23±0.045
AORK12	1.82±0.035	0.19±0.035
AORK13	7.52±0.041	1.20±0.612
AORK14	8.14±0.030	1.80±0.050
AOSK15	1.44±0.035	0.82±0.041
AOSK16	2.05±0.026	0.65±0.025
AORP17	9.05±0.025	1.60±0.035
AORP18	12.08 ±0.037	1.42 ±0.041
AORP19	5.12 ±0.041	0.86 ±0.035
AOSP20	9.05 ±0.035	0.77 ±0.040
AOLP21	1.24±0.030	1.39±0.047
PORU1	10.19±0.028	1.52±0.063
PORU2	6.52±0.063	1.16±0.049
PORU3	4.18±0.049	1.19±0.014
POSU4	2.04±0.028	0.84±0.049
POSU5	1.16±0.049	12.4±0.028
POLU6	8.5 ±0.4242	0.12±0.063
PORK7	5.85±0.049	12.4±0.042
PORK8	11.23±0.063	1.77±0.007
POSK9	2.28±0.042	0.64±0.049
POSK10	2.12±0.014	0.40±0.042
POLK11	1.12±0.056	0.18±0.007
PORP13	8.02±0.021	1.72±0.049
PORP14	6.12±0.056	1.47±0.014
POSP15	2.18±0.021	1.04±0.0282

Biotechnology Information data bank using the BLAST program.

RESULTS

A total number of thirty five strains were isolated from okra at three localities (Table 1). Among the isolates, 10 Azospirillum sp. were isolated sample collected from Annamalai nagar, includes 6 from root, 2 from stem and 1 from leaves. From sample collected at karaikal, 4 from root and 2 from stem was isolated. Sample from puthur includes 3 from root, 1 from stem and 1 from leaf. There were 13 strains of Pseudomonas sp. including 6 from Annamalai nagar, 5 from karaikal and 2 from puthur. They were biochemically characterized for acid production from glucose, utilization of different carbon sources, biotin needs, nitrate and nitrite reductase, spore formation, gram's stain, catalase, oxidase and motility (Table 2). Among them nitrogen fixing ability was found maximum in AORU5 and PORU1 isolated from the sample of Annamalai nagar (Table 3). Molecular characterization confirmed the efficient isolates AORU5 and PORU1 are *Azospirillum brasilense* and *Pseudomonas fluorescens* respectively.

CONCLUSION

On the basis of the results obtained, it could be concluded that bhendi plant harboring rich population of both *Azospirillum* and *Pseudomonas* bacterial endophytes. This study indicates that there is huge number of endophytic microbes occupy a relatively privileged niche within plant and usually contribute to plant health. Colonization of endophytic bacteria is a challenging field of research, from fundamental as well as an applied focus. The first attempts to use endophytic bacteria for the improvement of pest control or phytoremediation processes have been promising, but considerable research efforts are required to optimize the practical applications. More knowledge of the population dynamics and activity of endophytic bacteria in their host plants are required.

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