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Diversity of Endophytic Actinomycetes from Wheat and its Potential as Plant Growth Promoting and Biocontrol Agents

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Abstract: A total of 35 endophytic actinomycetes strains was isolated from the roots, stems and leaves tissues of healthy wheat plants and identified as *Streptomyces* sp. (24), *Actinopolyspora* sp. (3), *Nocardia* sp. (4), *Saccharopolyspora* sp. (2) *Pseudonocardia* (1) and *Micromonospora* sp. (1). Seventeen endophytic actinomycetes isolate showed abilities to solubilize phosphate and produce IAA in the range of 5 to 42mg/100ml and 18-42µg/ml respectively. Nineteen isolates produced catechol-type of siderophore ranging between 1.3-20.32µg/ml. Also, hydroxamate-type siderophore produced by 9 isolates in the range of 13.33-50.66µg/ml. Maximum catechol-type of siderophore production was observed in *Streptomyces roseosporus* W9 (20.32µg/ml) which was also displaying maximum antagonistic activity against ten different pathogenic fungi. The results indicated that internal tissues of healthy wheat plants exhibited endophytic actinomycetes diversity not only in terms of different types of isolates but also in terms of functional diversity.

Keywords: Endophytic actinomycetes, IAA, Siderophores, Antagonistic activity, Pathogenic fungi.

1. Introduction

Actinomycetes are bacteria known to constitute a large part of the rhizosphere microbiota. Their isolation is an important step for screening of new bioactive compounds. The actinomycete that resides in the tissue of living plants and does not visibly harm the plants are known as endophytic actinomycetes (Stone *et al*., 2000). Frankia strains, symbionts of actinorhizal plants, can induce Nitrogen-fixing root nodules on certain non-leguminous plants and be identified as actinomycetes (Benson and Silvester, 1993). Endophytic streptomycetes were isolated from surface-sterilized roots of 28 plant species in northwestern Italy (Sardi *et al*., 1992), from roots and leaves of maize in north-east Brazil (de Araujo *et al*., 2000) and from roots of wheat (Coombs and Franco, 2003). It has been observed that endophytic actinomycetes promote growth by various ways through secretion of plant growth regulators like IAA, Pteridic acid A and B which has auxin-like activity (Igarashi *et al.*, 2002) and promote plant establishment under adverse conditions (Hasegawa *et al*., 2006).

These endophytic actinomycetes can be used as biological control agents of soil borne-root disease due to their ability to colonize healthy plant tissue and produce antibiotic *in situ* (Kunoh, 2002). Around sixty endophytic actinomycetes isolated from wheat root, have shown ability to reduce the impact of "take all" disease on wheat by up to 70% in a glass house trial using naturally infested soil (Coombs *et al.*, 2004). A number of endophytic actinomycetes exhibited a suppression of different soil-borne plant pathogens including *Rhizoctonia solani*, *Pythium* spp. *Fusarium oxysporum*, and *Colletotrichum orbiculare* indicating their potential use as biocontrol agents (Krechel, 2002; Cao *et al.*, 2005; El-Tarabily *et al.*, 2009; Shimizu *et al.*, 2009). The present study is the first report from India on isolation and identification of endophytic actinomycetes from healthy wheat plants and their role

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in pathogen defense and growth regulation of their hosts.

2. Materials and Methods

2.1 Sample collection

Wheat samples were collected from different locations of Ludhiana. Twenty healthy plants were uprooted and brought to the laboratory in polythene bags and screened for endophytic actinomycetes on the same day.

2.2 Isolation of Endophytic Actinomycetes

The plants were washed in running tap water to remove soil particles and each sample was dissected into leaves, stems and roots. Tissue pieces were sterilized by segmental immersion in 70% (v/v) ethanol for 5 minutes and sodium hypochlorite solution (0.9% available chlorine) for 20 minutes and then surface-sterilized samples were washed in sterile water three times to remove surface sterilization agents. The samples were soaked in 10% (w/v) sodium bicarbonate solution for 10 minutes to retard the growth of endophytic fungi. Each sample was divided into small fragments. These fragments were mashed using sterile pestle and mortar. An aliquot of 1ml of root, stem and leaves suspension was poured on to Petri plates containing tryptic soy agar (TSA) media and spread with the help of glass spreader. Petri plates were incubated at 28^0C for 7-10 days. Isolated colonies were picked and streaked on TSA medium and then subcultured on slants and stored at 4^0C. Effectiveness of surface sterilization was tested by the method of Schulz *et al*., (1993).

2.3 Identification of Actinomycetes

Cultural and morphological characteristics, including the presence of aerial mycelia, spore mass color, distinctive reverse colony color, color of diffusible pigments and spore chain morphology were used as identification characters (Goodfellow and Cross, 1984). Visual observation of both morphological and microscopic characteristics using light microscopy and Gram stain properties were also performed. All morphological characters were observed on TSA and the criteria used for classification and differentiations were as follows:

(1) **Aerial mass color**: The mass color of mature sporulating aerial mycelium was observed following growth on TSA plates. The aerial mass was classified according to Bergey's manual of systematic bacteriology.
(2) **Substrate mycelium**: Distinctive colors of the substrate mycelium were recorded.
(3) **Diffusible pigments**: The production of diffusible pigment was also considered.
(4) **Spore chain morphology**: The shape of the spore chains observed under light microscope was also used as an important step in the identification.
(5) **Biochemical screening**: Physiological criteria such as ability to degrade casein, starch, esculin, tween 80, tyrosine, xanthine and hypoxanthine as substrates by the various actinomycete strains were also used for genus confirmation.

2.4.1 Phosphate Solubilization

A phosphate solubilization test was conducted qualitatively by inoculating the endophytic actinomycetes isolates on National Botanical Research Institute's phosphate agar medium (NBRIP) containing $Ca_3(PO_4)^2$ according to Nautiyal (1999). The presence of a halo clearing zone around growing colony after incubating at 28^0C for 7 days was used as an indicator for positive P-solubilization. The ability of isolates to solubilize insoluble phosphate was also tested quantitatively in liquid culture. The tested isolates were inoculated into NBRIP medium containing $Ca_3(PO_4)^2$ and incubated at 28^0C for 7 days. The cultures suspensions were centrifuged at 3000 rpm for 30 min. soluble phosphate in supernatants was determined according to Jackson (1973).

2.4.2 Indole acetic acid (IAA) production

The production of IAA by 35 actinomycetes isolates was determined by colorimetric assay (Gordon and Weber, 1951). The actinomycetes isolates were grown on yeast malt extract agar at 28^0C for 5 days. Eight millimeter-diameter agar discs were inoculated into 100ml of yeast malt extract broth containing 0.2% L-tryptophan and incubated at 28^0C with shaking at 125 rpm for seven days. Cultures were centrifuged at 11,000 rpm for 15 min. One milliliter of the supernatant was mixed with 2ml of the Salkowski reagent. Development of pink color indicated IAA production. Optical density (OD) was read at 530nm using a spectrophotometer. The level of IAA produced was estimated by comparison with an IAA standard.

2.4.3 Siderophore production

The endophytic actinomycetes isolates were inoculated on Chrome azurol S (CAS) agar medium and incubated at 28^0C for 5 days (Schwyn and Neilands, 1987). The colonies with orange zones were considered as siderophore producing isolates. The active isolates (width of the orange zone on CAS plate > 20mm) were cultured on glycerol yeast broth and incubated at 28^0C with shaking at 125 rpm for 10 days. Catechol-type siderophores were estimated by using an Arnow's method (Arnow, 1937) and hydroxamate-type siderophores were estimated by the Csaky test (Csaky, 1948).

2.5 *In vitro* antagonistic bioassay

The endophytic actinomycetes isolates were evaluated for their antagonistic activity against ten pathogenic fungi: *Aspergillus niger*, *A. versicolor*, *A. flavus*, *Alternaria brassicicola*, *Botrytis cinerea*, *Chaetomium globosum*, *Fusarium oxysporum*, *Penicillium pinophilum*, *Phytophthora dresclea* and *Rhizoctonia solani* by dual-culture *in vitro* assay. Fungal discs (8mm in diameter), 5 days old on PDA at 28^0C were placed at the center of PDA plates. Two actinomycete discs (8mm) 5 days old, grown on yeast malt extract agar were incubated at 28^0C placed on opposite sides of the plates, 3cm away from fungal disc. Plates without the actinomycete disc served as controls. All the plates were incubated at 28^0C for 14 days. The width of inhibition zone between the pathogen and the actinomycete isolates was measured and evaluated as follows: +++, 20mm <; ++, 11-19mm; +, 2-10mm; ±, ≤mm; -, 0mm.

3. Results and Discussion

3.1 Isolation and characterization of endophytic actinomycetes diversity

The present study revealed that healthy living tissues of wheat plants harbour a variety of endophytic actinomycetes (Table 1).

A total of 35 isolates of endophytic actinomycetes were obtained from the roots, leaves and stems. Out of 35 isolates, the majority (n=21) was recorded from roots, followed by stems (n=9) and leaves (n=5). Since roots are the site of water and nutrient uptake for plants, they are an ideal substrate for actinomycete colonization. Also, endophytic actinomycetes may easily gain access to root through the damaged epidermal layer created when lateral roots grow out from the existing root structure. Such is the case for the well-studied endophytic actinomycete, *Frankia*, which forms root nodules in non-leguminous woody plants but also exists in soil in substantial numbers (Hahn *et al*., 1999). Once inside the roots, it is conceivable that endophytic actinomycetes other than *Frankia* could benefit plants by fixing nitrogen without forming nodules (Valdes *et al*., 2005) and/or producing antibiotics or siderophores to protect against infection by soil-borne pathogens are consistent with the reports of other investigators (Taechowisan *et al*., 2003; Verma *et al*., 2009). *Streptomyces* sp. was the most frequently isolated species (68.6%) followed by *Nocardia* sp. (11.4%) *Actinopolyspora* sp. (8.6%), *Saccharopolyspora* sp. (5.8%), *Micromonospora* sp. and *Pseudonocardia* sp. (2.8% each) respectively. The predominance of *Streptomyces* sp. among the endophytic actinomycetes of wheat that was observed in the present study was in conformity with the other reports from different plants (Sardi *et al*., 1992; Coombs and Franco, 2003; Taechowisan *et al*., 2003; Tan *et al*., 2006). Maximum *Streptomyces* strains were recovered from roots than leaves. Based on colony and cultural characteristics, various subgroups were identified among *Streptomyces* sp., the subgroup *S. albosporus* (n=6) was most frequently isolated followed by *S. roseosporus* (n=5) and *S. viridis* (n=4), *S. aureus* (n=2), *S. lavendulae*, *S. globisporus*, *S. cinereus*, *S. flavus*, *S. griseofuscus*, *S. griseorubruviolaceus*, *S. hygroscopicus* (n=1 for each) respectively. Maximum *Streptomyces* sp. was recovered from roots and minimum from a leaf. The results also revealed that the surface treatment was adequate for the isolation of endophytic actinomycetes, as surface sterilized imprinted Petri plate (control) did not produce any growth. Thus, all the actinomycetes recorded in this experiment must have been endophytic and not the epiphytic.

Table 1. Distribution of endophytic actinomycetes isolates from wheat plants.

Type	No. of Endophytic actinomycetes isolated from		
	Root	Stem	Leaf
Streptomyces albosporus	4	1	1
S. aureus	1	1	0
S. cinereus	1	0	0
S. flavus	0	1	0
S. griseofuscus	0	0	1
S. globisporus	1	0	0
S. roseosporus	3	1	1
S. hygroscopicus	1	0	0
S. lavendulae	1	0	0
S. viridis	2	2	0
S. griseorubruviolaceus	0	1	0
***Actinopolyspora* sp.**	2	0	1
***Micromonospora* sp.**	0	1	0
***Nocardia* sp.**	2	1	1
***Saccharopolyspora* sp.**	2	0	0
***Pseudonocardia* sp.**	1	0	0
Total	21	9	5

3.2 Characterization of Actinomycetes Isolates for PGP Traits

3.2.1 Phosphate solubilization

Seventeen isolates were able to solubilize phosphate on NBRI-BPB medium were further evaluated for amount of phosphate solubilized. The amount of phosphate solubilized by the isolates ranged from 5 to 42mg/100ml (Table 2). The maximum amount of phosphate solubilized by *S. roseosporus* W24 (42mg/100ml). These results are in accordance with some earlier reports (Hamdali *et al*., 2008), where high amount of phosphate solubilizing activity by *Streptomyces cavourensis* (83.3mg/100ml) followed by *Streptomyces griseus* (58.9mg/100ml) and *Micromonospora aurantiaca* (39mg/100ml) is reported. Microbial solubilization of mineral phosphate might be either due to the acidification of external medium or the production of chelating substances that increase phosphate solubilization (Whitelow, 1999). Hence, P-solubilizing actinomycetes play an important role in the improvement of plant growth.

3.2.2 Indole acetic acid (IAA) production

A total of seventeen endophytic actinomycetes isolates produced an amount of IAA in the range of 18-42µg/ml. The maximum IAA was produced by *saccharopolyspora* W3 while *Strptomyces albosporus* W2, *Streptomyces roseosporus* W9 and *Actinopolyspora* W20 produced the minimum yield of IAA (Table 3). Igrarashi *et al*., (2002) isolated *Streptomyces hygroscopicus* from *Pteridium aquilinum* and found that *S. hygroscopicus* produced novel pteridic acids A and B as plant growth promoters with auxin-like activity. Nimnoi and Pongslip (2009) reported that isolates of IAA synthetic bacteria enhanced root and shoot development of *Raphanus sativus* and *Brassica oleracea* more than fivefold when compared with control. Our findings are in accordance with Nimnoi *et al*., (2010) who isolated *Nocardia alba* from surface-sterilized roots of *Aquilaria crassna* that showed high ability to produce IAA (14.53µg/ml). The presence of endophytic actinomycetes mainly inside root tissues that produce IAA may have an important role in plant growth.

3.2.3 Siderophore production

Siderophore is iron-containing chelating compound impart antimicrobial capacity to the producing microbe. Out of 35 isolates, 19 were observed to produce catechol type siderophore in range of 1.3-20.32µg/ml while hydroxamate-type siderophore was produced by only nine isolates in the range of 13.33-50.66µg/ml (Table 4). The isolate *Streptomyces roseosporus* W9 produced maximum Catechol type (20.32µg/ml) and isolate *Streptomyces globisporus* W26 produced maximum hydroxamate-type (50.66µg/ml) siderophores on glycerol yeast broth (Table 4). This finding is in agreement with Nimnoi *et al*., (2010) who reported that *Pseudonocardia halophobica* isolated from roots of *Aquilaria crassna* exhibited high ability for siderophore production and produced 39.30µg/ml Khamna *et al*., (2009) also reported that *Streptomyces* CMU-SK 126 isolated from *Curcuma mangga* rhizospheric soil exhibited high ability for siderophore production and produced catechol type (16.19µg/ml) as well as hydroxamate-type (54.9µg/ml) siderophores. Usually, siderophores are produced by various soil microbes to bind Fe^{3+} from the environment, transport it back to the microbial cell and make it available for growth (Leong, 1996; Neilands and Leong, 1986). Plants also utilize microbial siderophores as an iron source (Bar-Ness *et al*., 1991; Wang *et al*., 1993). Even though siderophores do not promote plant growth directly; they deliver iron to plants and provide nutrition to stimulate plant growth.

Table 2. Phosphate solubilization by endophytic actinomycetes isolates.

Actinomycetes isolates	Phosphate solubilization (mg/100ml)
***Streptomyces albosporus* W1**	13
***Saccharopolyspora* W3**	5
***Streptomyces viridis* W4**	5
***Nocardia*W6**	5
***Streptomyces lavendulae* W7**	5.5
***S. viridis* W8**	5
***S. roseosporus* W9**	8.5
***Micromonospora* W11**	5.5
***Streptomyces albosporus* W13**	5
***S. griseofuscus* W18**	6.5
***Actinopolyspora* W20**	6.5
***Streptomyces roseosporus* W24**	42
***S. flavus* W25**	5.5
***Nocardia* W29**	6.5
***Streptomyces roseosporus* W31**	10
***S. cinereus* W32**	5.5
***Saccharopolyspora* W33**	10
CD@5%	1.02

Table 3. IAA production by endophytic actinomycetes isolates after 7 days incubation.

Actinomycetes isolates	IAA production (µl/ml)
***Streptomyces albosporus* W1**	19.2
***S. albosporus* W2**	18
***Saccharopolyspora* W3**	42
***Streptomyces viridis* W4**	32.2
***S. lavendulae* W7**	21.5
***S. viridis* W8**	31.5
***S. roseosporus* W9**	18
***S. albosporus* W13**	24.2
***S. griseofuscus* W18**	34
***Actinopolyspora* W20**	18
***Nocardia* W21**	34
***Streptomyces roseosporus* W24**	24
***S. globisporus* W26**	20.5
***S. viridis* W28**	36
***Pseudonocardia* W34**	19
***Streptomyces griseorubruviolaceus* W35**	20
CD@5%	1.96

Table 4. Siderophore production by endophytic actinomycetes after 10 days incubation.

Isolates	Siderophore (μg/ml)	
	Catechols	Hydroxamate
***Streptomyces albosporus* W1**	16.46	43.99
***S. albosporus* W2**	1.4	-
***Saccharopolyspora* W3**	8.66	28.0
***Streptomyces viridis* W4**	2.9	-
***S. viridis* W8**	1.3	-
***S.roseosporus* W9**	20.32	13.33
***S. aureus* W10**	1.2	-
***Micromonospora* W11**	10.70	45.33
***Streptomyces albosporus* W13**	1.3	-
***Nocardia* W15**	11.51	40.0
***Streptomyces hygroscopicus* W17**	2.8	-
***S. viridis* W19**	16.37	44.0
***Actinopolyspora* W20**	22.19	47.99
***Streptomyces roseosporus* W24**	13.42	39.95
***S. flavus* W25**	1.6	-
***S. globisporus* W26**	12.56	50.66
***S. cinereus* W32**	2.6	-
***Actinopolyspora* W33**	1.8	-
***Pseudonocardia* W34**	2.1	-
CD@5%	0.247	0.50

3.2.4 *In vitro* antagonistic bioassay

Eighteen endophytic actinomycetes isolates (51.4%) have strong antagonistic activity against *Fusarium oxysporum* and *Phytophthora drechsleri* (Table 5). Different isolates of *Streptomyces* sp. displayed an array of activity against pathogenic fungi. *Streptomyces roseosporus* W9 strongly inhibited all of the pathogenic fungi. *Streptomyces hygroscopicus* W17 was showing antagonism towards all the test fungi. *Pseudonocardia* W34 displayed antagonistic activity against all except *Penicillium pinophillum*. *Streptomyces viridis* W19 antagonized all tested fungi except *A. niger, C. globosum, R. solani* and *B. cinerea*. *Streptomyces roseosporus* W24 was displaying antagonistic activity against all fungi except *R. solani*. *Actinopolyspora* W20 was able to antagonize all the test fungi except *C. globosum* and *P. pinophillum*. This is in conformity with the results of several studies carried out by other investigators (Crawford *et al*., 1993; Taechowisan *et al*., 2003; Tian *et al*., 2004; Verma *et al*., 2009). *Streptomyces* species have also been implicated in the biological control of a number of other pathogens. *Streptomyces lydicus* WYEC108 inhibited *Pythium ultimum* and *R. solani in vitro* by the production of antifungal metabolites (Yuan and Crawford, 1995). Similarly, 23 *Streptomyces* isolates showed activity against five phytopathogenic fungi *Alternaria brassicicola, Penicillium digitatum, Fusarium oxysporum, Sclerotium rolfsii* and *Penicillium* spp. (Khamna *et al*., 2009).

Table 5. Antagonistic activities of wheat isolate against plant pathogenic fungi.

Actinomycetes isolates	1	2	3	4	5	6	7	8	9	10
***Saccharopolyspora* W3**	+	-	+	-	+	+	-	++	-	++
***Streptomyces albosporus* W5**	+	-	+	+	-	++	-	+	-	++
***S. lavendulae* W7**	-	-	-	-	-	++	-	+	-	-
***S. roseosporus* W9**	++	+++	++	+++	+++	+++	+++	+++	+++	+++
***S. aureus* W10**	+	-	++	+	-	+	-	-	++	+
***Micromonospora* W11**	-	-	-	+	-	-	-	++	-	+
***Streptomyces albosporus* W13**	-	-	-	+	-	+	-	++	+	++
***S. hygroscopius* W17**	++	+	+	++	++	+++	+	++	+++	++
***S. griseofuscus* W18**	++	++	++	++	-	++	+++	++	++	+++
***S. viridis* W19**	-	+	++	++	-	+++	++	+	-	-
***Actinopolyspora* W20**	+	+	+	++	-	+	-	++	+	++
***Streptomyces albosporus* W22**	-	-	-	-	-	++	-	+	-	-
***S. roseosporus* W24**	+	+	+	++	+	+++	+	+	-	++
***S. globisporus* W26**	+	-	-	-	-	+	-	+	+	-
***S. viridis* W28**	+	-	+	+	-	+	-	++	+	+
***S. albosporus* W30**	-	-	-	+	-	+	-	++	+	+
***S. cinereus* W32**	++	+	++	++	+	+	-	++	++	++
***Pseudonocardia* W34**	+	+	+	++	+	++	-	+	++	++

Biocontrol effects of endophytic actinomycetes both *in vitro* and planta have been reported. In another study, thirty-eight strains of endophytic actinomycetes isolated from surface sterilized wheat and barley roots were tested for their antagonistic activity to wheat root pathogens *Gaeumannomyces graminis*, *Rhizoctonia solani* and *Pythium* sp. (Coombs *et al*., 2004). It was observed that 17 of 38 isolates displayed statistically significant activity in planta against *G. graminis*. Tian *et al*., (2004) isolated 274 actinomycete strains from surface-sterilized roots and leaves of field-grown rice plants and reported that about 50% of the isolates showed antagonism to some fungal pathogens. The ability of isolates to inhibit the growth of fungal pathogens is implication of the volatile secondary metabolites. Those endophytic actinomycetes may play an important role in protecting the plant host against pathogenic microorganisms.

The isolation of microorganisms from within the tissue of healthy wheat suggests that the host derives some benefit from harboring the endophytes. In this case, the advantage may take the form of a secondary metabolite produced by the endophytic actinomycetes, since actinomycetes are well known for their ability to produce a broad range of antibacterial, antifungal and plant growth-regulatory metabolites. This study reveals a novel plant-microbe interaction with implications for biotechnological application of these endophytic actinomycetes.

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