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Research Article

Evaluation of streptomyces and non-streptomyces actinomycetes isolates for growth promotion in *Vigna radiata* and their use as biocontrol agent against *Sclerotium rolfsii*

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Abstract

Two streptomyces (ARHS/PO26 and ARHS/PO27)and two non streptomyces (ARHS/Mn3 and ARHS/Mn7) actinomycetes isolates obtained from the rhizosphere soil of *Solanum tuberosum* and *Mangifera indica* were found to be phosphate solubilizers and showed antagonistic activity against *Sclerotium rolfsii*. Isolates ARHS/PO26 and ARHS/PO27 were identified morphologically and confirmed by the National Centre for Fungal Taxonomy, as *Streptomyces griseus* (NCFT 2578.08; NAIMCC-B-00916) and *Streptomyces griseolus* (NCFT 2579.08). ARHS/Mn 3 and *Streptomyces griseolus* (ARHS/PO27) could inhibit 68% and 59.7% growth of *Sclerotium rolfsii in vitro. In vivo* evaluation of the isolates ARHS/Mn 3, *Streptomyces griseolus* (ARHS/PO27) and *Streptomyces griseus* (ARHS/PO26) showed maximum growth promotion on *Vigna radiata* by enhancing key defense enzymes like chitinase, β-1,3-glucanase, phenylalanine ammonia lyase and peroxidase. The results emphasize the fact that soil actinomycetes could be used as potential biocontrol agents.

Keywords: Non-streptomyces Actinomycetes, *Streptomyces griseus, Streptomyces griseolus, Vigna radiata*, Growth promotion, Defense enzymes, *Sclerotium rolfsii*

Introduction

Mung bean or Green gram *Vigna radiata* (L.) Wilczek (syn: *Phaseolus aureus* Roxb.) constitutes the important group of grain legumes which form a major source of dietary proteins of high biological value, energy, minerals and vitamins (Taylor *et al.*, 2005). Those who can not eat animal protein this plant belonging to the family Fabaceae or leguminosae is a good source of protein. However, the yield of mung bean is greatly reduced due to various factors of which diseases caused by fungi and viruses are of major concern (Satya *et al.*, 2011). Now it has become necessary to find out ways of increasing yield and decreasing disease incidence in *Vigna*.

Streptomycetes are a group of actinobacteria which are part of the microbial flora of most natural substrates (Moustafa *et al.*, 1963) and mainly found in the rhizosphere of plants in association with other microorganisms like rhizobacteria and fungi. They utilize humic acid and other organic matter in soil. In their natural habitat, such as forests, the actinomycetes interact in various ways with the higher plants (Lo *et al.*, 2002). These organisms are part of PGPM or plant growth promoting microorganisms. Streptomycetes affect plant health in various ways like by producing plant growth promoting hormones like IAA (Manulis *et al.*, 1994), production of siderophores (Tokala *et al.*, 2002) which influence plant growth or by protecting the plant against plant pathogenic microorganisms. It has been reported that secondary metabolites produced by some *Streptomyces* spp. inhibit growth of phytopathogenic fungi like *Colletotrichum musae* and *Fusarium oxysporum* (Taechowisan *et al.*, 2005).Many of non-streptomycete actinomycetes (NSA) taxa are therefore rarely reported in literature dealing with routine isolations of biocontrol agents and plant growth promoters from plant and soil. Seed-coating with powder formulation of the biocontrol agent was as effective as drench application of the fungicide, oxine benzoate (No-Damp), in controlling *Rhizoctonia* damping-off and superior to the commercial biocontrol agent, *Streptomyces*

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griseoviridis (Mycostop), applied to tomato seeds as seed-coating. Ensign *et al.* (1993) reviewed the physiology of some NSA as a component of soil microflora. Although Lechevalier (1988) and Doumbou *et al.* (2001) reviewed the literature on the biological control of soil-borne fungal plant pathogens and plant growth promotion by actinomycetes, they covered activities mainly of *Streptomyces* spp.

The present study reflects the role of non-streptomyces strains- ARHS/Mn 3 and 7 and streptomyces strains (*Streptomyces griseus* and *S. griseolus*) as plant growth promoters and biocontrol agents in reducing sclerotial disease in *Vigna radiata*.

Material and Methods

Isolation of actinomycetes

Actinomycetes were isolated by the standard serial dilution plate technique by Warcup (1955) using starch casein nitrate agar (SCN) medium.

Biochemical Characterization

Biochemical characterization of actinomycetes isolates were performed including starch hydrolysis, catalase and indole tests.

Screening for phosphate solubilizing activity

Isolates were screened in Pikovskaya medium for phosphate solubilization activity (Pikovskaya, 1948). Isolates were inoculated in Pikovskaya media and incubated for 7 days. A halo zone around the growth indicates positive result for phosphate solubilization activity.

Selection of two non-Streptomyces and two Streptomyces actinomycetes strains

On the basis of *in vitro* plant growth promoting activities out of the isolates of actinomycetes, two non-streptomyces strains (ARHS/Mn 3 and ARHS/Mn 7) and two streptomyces strains- ARHS/PO26 and ARHS/PO27 were selected. ARHS/PO26 and ARHS/PO27 were morphologically identified and confirmed by the National Centre for Fungal Taxonomy, Delhi as *Streptomyces griseus* (NCFT 2578.08; NAIMCC-B-00916) and *Streptomyces griseolus* (NCFT 2579.08).

In vitro antagonistic effect on Sclerotium rolfsii

Streptomycetes and non-streptomycetes isolates were tested for antagonistic effect against *Sclerotium rolfsii* by dual culture method (Skidmore and Dickinson, 1976). Inhibitions of the radial growth of fungal pathogen by the isolates confirm antagonism.

Inoculation technique and disease assessment

15 days old plant (*Vigna radiata*) was used for artificial inoculation with fungal pathogen. Sand maize meal media containing fungal inoculum were added carefully in the rhizosphere and ensured that inocula were attached to healthy roots. Disease assessment was done 15 days after inoculation. In order to determine the effects of two non-streptomyces and two streptomyces strains on disease reduction, four treatments were taken in each case: untreated control; inoculated with pathogen; inoculation with test isolates and inoculation with both test isolate and fungal pathogen. Percentage disease incidence was recorded while disease intensity was calculated using a 0-6 scale (Mathew and Gupta, 1996).

Field Trial

Two non-streptomyces (ARHS/Mn 3 and ARHS/Mn 7) and two streptomyces strains- ARHS/PO26 and ARHS/PO27 were selected for *in vivo* evaluation of the growth promoting activity on *Vigna radiata*. For seed coating the seeds were soaked in cell suspension overnight. For preparation of cell suspension 7 days old broth culture were centrifuged at 10000rpm for 10 min and the cell pellet was dissolved in 250ml sterile distilled water and tween-20. Growth measurement were observed 15 days after inoculation and dry biomass were measured after three months of inoculation.For growth promotion average root length, shoot length, total height, fresh weight and dry weight were measured against control.

Biochemical analyses

Leaves of *Vigna* plants treated with actinomycetes were used for all biochemical analyses. Leaves were collected for assay 15days after inoculation.

Enzyme assays

Peroxidase (POX; EC1.11.1.7.)

Extraction and assay of peroxidase was done following the method described by Chakraborty *et al* (1993). The plant tissues were macerated to powder in liquid nitrogen and extracted in 5 ml of chilled 0.05(M) sodium phosphate buffer (pH 6.8) containing 2 mM β-mercaptoethanol. One ml of 0.2(M) Na-phosphate buffer (pH 5.4), 1.7 ml dH_2O, 100µl crude enzyme, 100 µl O-dianisidine (5mg/ml methanol) and 0.1 ml of 4mM H_2O_2 were used in the reaction mixture. Activity was assayed spectrophotometrically at 465 nm by monitoring the oxidation of O-dianisidine in presence of H_2O_2.

Chitinase (CHT, EC 3.2.1.14)

Chitinase was extracted and assayed from leaves following the method of Boller and Mauch (1988). 10µl of 1M Na-acetate buffer (pH 4), 0.4 ml enzyme solution and 0.1 ml colloidal chitin were used in the reaction mixture. Incubation was done for 2 hrs at 37°C and centrifuged at 10,000 rpm for 3 min. 0.3 ml supernatant, 30µl of 1M $K\text{-}PO_4$ buffer (pH 7.1) and 20µl Helicase (3%) were mixed and allowed to incubate for 1 h at 37°C. 70µl of 1M Na-borate buffer (pH 9.8) was added to the reaction mixture. The mixture was again incubated in a boiling water bath for 3 min and rapidly cooled in ice water bath. 2 ml DMAB (2% di methyl amino benzaldehyde in 20% HCl) was finally added and incubated for 20 min at 37°C.The amount of GlcNAc released was measured spectrophotometrically at 585 nm and activity was expressed as µg GlcNAc released /min/ g fresh wt. tissue.

Phenylalanine Ammonia Lyase (PAL, EC 4.3.1.5)

Enzyme was extracted and assay was done following the method described by Bhattacharya and Ward (1987). The assay mixture contained 500µl crude enzyme, 300µl of 0.3mM borate buffer (pH 8.0), 300µl of 2% L-phenylalanine and 1.9 ml distilled water. The mixture was allowed to incubate for 1 hr at 40°C and then absorbance value was measured at 290 nm. The enzyme activity was described as the amount of cinnamic acid produced from L-phenyl alanine by the enzyme from 1 g tissue/min.

β- 1,3-glucanase (β-GLU, EC 3.2.1.39)

β-1,3-glucanase was extracted and assayed from leaf samples following the method of Pan *et al.* (1991).The reaction mixture consisted of 62.5µl crude enzyme and 62.5µl 4% laminarin which was incubated at 40°C for 10 min and 375µl DNSA (dinitro salicylic acid) added to the mixture following incubation for 5 min on a boiling water bath. Finally the colored solution was diluted with 4.5 ml water and the amount of glucose liberated was determined spectrophotometrically. Activity was expressed as µg glucose released /min/g tissue.

Results and Discussion

Actinomycetes isolates were characterized by morphologically as well as biochemically. Out of the isolated isolates, two non-streptomyces and two streptomyces actinomycetes isolates showed positive result for biochemical tests.On the basis of *in vitro* plant growth promoting activities and phosphate solubilising activities *in vitro* two non-streptomyces strains (ARHS/Mn 3 and ARHS/Mn 7) and two streptomyces strains- ARHS/PO26 and ARHS/PO27 were selected for further studies (Table 1, Fig. 1). ARHS/PO26 and ARHS/PO27 were identified and morphological identification were confirmed by the National Centre for Fungal Taxonomy, Delhi as *Streptomyces griseus* (NCFT 2578.08; NAIMCC-B-00916) and *Streptomyces griseolus* (NCFT 2579.08).

Isolates were tested for *in vitro* antagonistic effect against *Sclerotium rolfsii*. ARHS/Mn 3 and *Streptomyces griseolus* (ARHS/PO27) were comparatively more effective to control *S. rolfsii*. ARHS/Mn3 and *S. griseolus* (ARHS/PO27) could inhibit 68 % and 59.7% growth of *Sclerotium rolfsii* (Table 2, Fig. 2). Results revealed that among the isolates tested, sclerotial blight disease development was markedly reduced with prior applications of isolates of *S. griseolus* (ARHS/PO 27) and ARHS/Mn 3 in comparison to *S. griseus* (ARHS/PO26) and ARHS/Mn7 (Table 3). Increase in the growth was observed in terms of increase in height of

Table 1: Biochemical tests of two non-streptomyces actinomycetes (NSA) and two streptomyces isolates

Isolates Code	Catalase test	Indole test	Starch hydrolysis	Phosphate solubilising activity
ARHS-Mn3	+	+	+	+
ARHS-Mn7	+	+	+	+
Streptomyces griseolus (ARHS/PO27)	+	+	+	+
Streptomyces griseus (ARHS/PO26)	+	+	+	+

Mn- Rhizosphere soil of *Mangifera indica* (25°32'12"N .88°24'45" E); PO- Rhizosphere soil of *Solanum tuberosum* (26°33.676'N 89°03.149'E).

Table 2: *In vitro* antagonistic activity of Actinomycetes isolates against *Sclerotium rolfsii*

Isolates	% of inhibition of *Sclerotium rolfsii*
ARHS-Mn 3	68.00
ARHS-Mn 7	56.00
Streptomyces griseolus (ARHS/PO27)	59.70
Streptomyces griseus (ARHS/PO26)	57.00

Table 3: Evaluation of isolates on the development of sclerotial blight incidence of *Vigna radiata*

Treatments	Disease Index* *of Vigna radiata*
S. rolfsii	6.35
S. rolfsii + *S. griseus* (A/RHS/Po26)	0.84
S. rolfsii + *S. griseus* (A/RHS/Po27)	0.81
S.rolfsii+ ARHS/MN 3	0.80
S.rolfsii+ ARHS/Mn 7	0.85

*0 = No symptoms; 1= Small roots turn rotten lesion appeared at the collar region; 2= Middle leaves start wilting and 10-20% of root turn brown; 3= Leaves wilted and 20-40% roots become dry with browning of shoot; 4= Extensive rotting at the collar region of roots, 60-70% of roots and leaves wilted, browning of shoot over 60%; 5= 80% roots affected while the root along with the leaves withered and shoot becomes brown more than 80%; 6= Whole plants die. Average of 3 separate inoculated plants (15 days after inoculation)

Evaluation of selected isolates on the growth and development of *Vigna radiata* was conducted in *in vivo* conditions. Marked increase in attributes of parameters in growth of *V. radiate* was noticed when actinomycetes were applied in the rhizosphere of plants. saplings, number of shoots and number of leaves and roots. Growth parameters were recorded from 15 days onwards (Fig. 3 and 4). Better growth enhancement was observed by *S. griseolus* (ARHS/PO27) in comparison to other streptomyces isolate. ARHS/Mn 3 also showed promoted better growth in mung bean in respect to control as well as ARHS/Mn 7 strain.

Activities of POX, PAL, chitinase and glucanase were also observed after application of bacterial strains. POX acitivities were increased more in ARHS/Mn 3 and ARHS/Mn 7 treated plants. In PAL activity, *Streptomyces griseolus* (ARHS/PO27)strain showed better results in comparison to other strains and control. Similarly, chitinase and glucanase activities were increased after application of ARHS/Mn 3 strain (Fig. 5).Induced systemic resistance (ISR) is effective against different types of pathogens but differs from systemic acquired resistance (SAR) in that the inducing PGPR does not cause visible symptoms on the host plant (Van Loon *et al.*, 1998). Pieterse *et al.* (2002) confirmed that to protect themselves from the disease, plants have evolved sophisticated defense mechanisms in which the signal molecules salicylic acid, jasmonic acid and ethylene often play crucial roles. The phenomenon of SAR suggests that there is a signal that originates at the site of elicitor (biotic or abiotic) application and moves throughout the plant. The activation of SAR turns the compatible plant-pathogen interaction into an incompatible (Uknes *et al.*, 1992) one. This resistance was correlated with the accumulation of pathogenesis related (PR) proteins, generally assumed to be markers of defense response (Ward *et al.*, 1991).

It can be concluded from the results of the present study that ARHS/Mn 3 and *S.*

*griseolus (*ARHS/Po27) can be used as good growth promoters as well as biocontrol agents against *S. rolfsii* in *Vigna radiata* in comparison to other selected non-streptomyces and streptomyces actinomycetes strains. Non-streptomyces actinomyces (NSA)

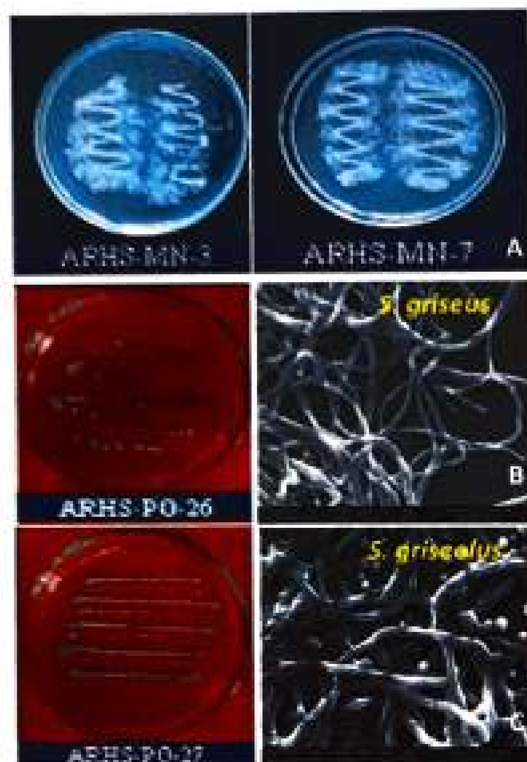

Fig. 1: Actinomycetes strains on starch casein nitrate agar (SCN) medium (A); Scanning electron microscopic view of *Streptomyces griseus* (ARHS/PO26) (B) and *Streptomyces griseolus* (ARHS/PO27) (C).

Fig. 2: Antagonistic activity of streptomyces and non-streptomyces actinomycetes isolates against *Sclerotium rolfsii*

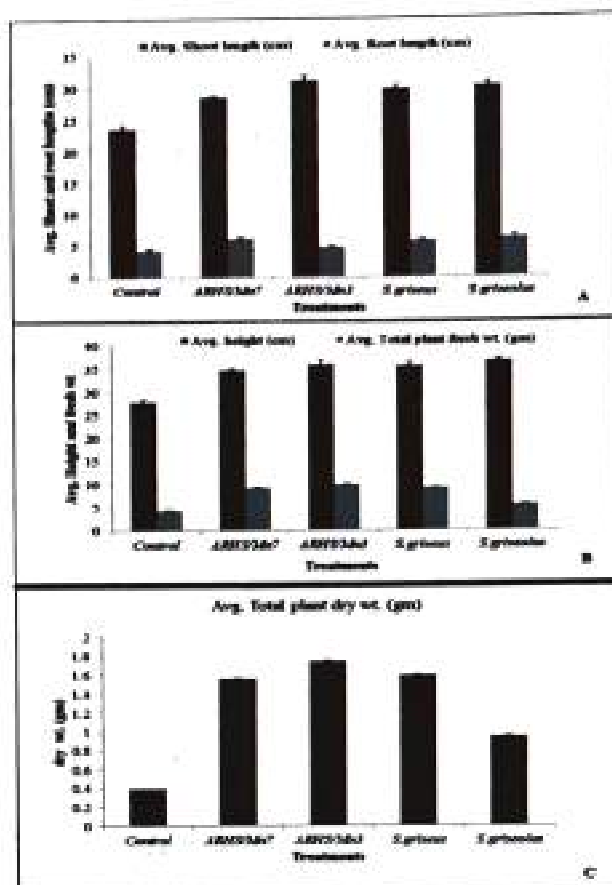

Fig. 3: Growth promotion in *Vigna radiata* after 15 days of treatment with streptomyces and non-streptomyces actinomycetes

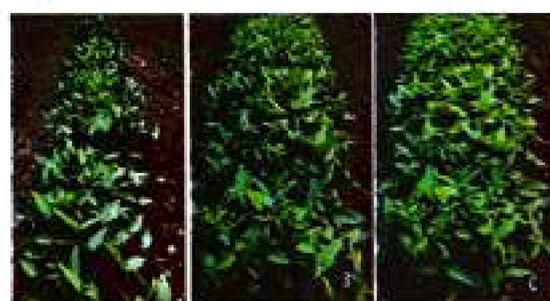

Fig. 4: Growth enhancement of *Vigna radiata* after application of ARHS/Mn 3 strain (B) and *Streptomyces griseolus* (ARHS/PO27) (C) in comparison to control (A).

have great potential as candidates for the biocontrol of soil-borne fungal plant pathogens and also as plant growth promoters. With better understanding and screening of NSA, successful candidates from among NSA for biocontrol and plant growth promotion could be sourced.

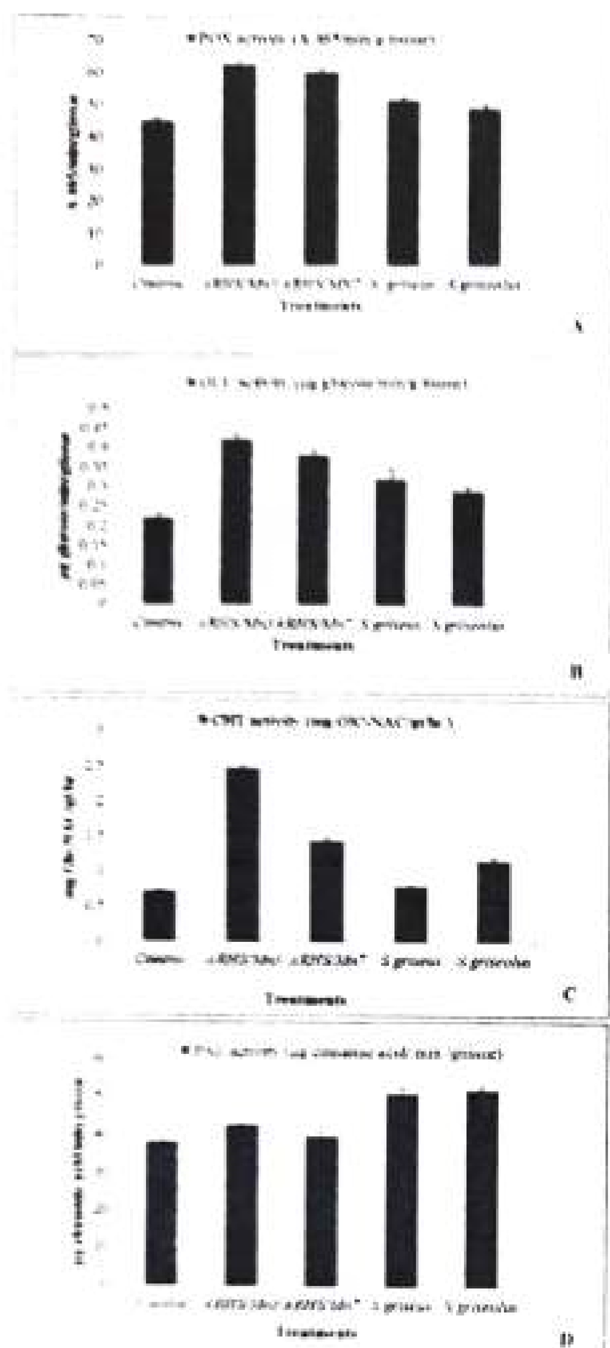

Fig. 5: Changes in defense enzyme activities in *Vigna radiata* after application of streptomyces and non-streptomyces actinomycetes isolates.

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