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BIOEFFICACY OF RHIZOSPHERE ANTAGONISTIC STREPTOMYCES SP FOR THE MANAGEMENT OF BROWN LEAF SPOT OF RICE

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Abstract

Bioefficacy of rhizosphere antagonistic Streptomyces sp for the management of Brown leaf spot of rice isolate the native Streptomyces sp from rhizosphere of rice plants from different locations. The in vitro efficacy of native rhizosphere Streptomyces sp against brown spot pathogen of rice was done by dual plate technique and their compatibility with other biocontrol agents and to assess the efficacy of Streptomyces sp in enhancing the plant growth checked by roll towel method. The effect of antagonistic Streptomyces against brown spot pathogen of rice under field condition

Keywords: Rice- Streptomyces - Helminthosporium oryzae Introduction

In Tamil Nadu, rice production is limited very much due to various diseases caused by fungi, bacteria and virus. In Cauvery delta, Brown spot of rice is very common in rice crop both in kuruvai and samba/thaladi seasons. The disease cause significant yield reduction in rice crop. Among the number of disquieting diseases, brown spot of rice caused by Helminthosporium oryzae is most disastrous and reported in all rice expanse of the world (Singh and Singh, 2000). Although this disease has been abandoned and ignored as a major rice disease, but history reveals that the famous Bengal famine in 1942 occurred due to this ruinous problem which caused significant losses to humanity (Padmanabhan, 1973). Brown spot fungus attacks the plant at any growing stage, causing seedling and adult plant infection. It appears on the foliage as scattered brown spot that coalesces and result in the whiting and yellowing of the leaves. Moreover, it inhibits germination, causing rotting of seeds, roots and coleoptiles. Poor germination and poor seedling vigor further cause considerable economic loss (Mia and Nahar, 2001; Naeem *et al.*, 2001; Malavolta *et al.*, 2002)

The use of chemicals for the disease management has been found to be with limited success. The chemicals used at the ear head stage lead to environmental pollution, residual toxicity and development of resistance by the pathogen besides necessitating repeated application involving more expenditure on plant protection.

Several studies have reported the potential of actinomycetes to control diseases caused by diverse phytopathogens such as *Alternaria solani* and *Helminthosporium* oryzae

(Chattopadhyay and Nandi, 1982), Phytophthora, fragariae var rubi (Valois et al., 1996), Macrophomina phaseolina (Hussain et al., 1990), Sclerotium rolfsii (Pattanapipitpaisal and Kamlandharn, 2012), Sclerotinia sclerotiorum (Baniasadi et al., 2009) Alternaria brassicicola (Tahvonen and Avikainen, 1987). Actinomycetes constitute a morphologically diverse group, distinguished from other Gram-positive bacteria by their filamentous growth and GC-rich DNA (Lacey, 1997). Actinomycetes are metabolically and morphologically more complex than sessile bacteria (Doumbou et al., 2001). Metabolically, they are prolific producers of an array of secondary metabolites, including antimicrobial agents, plant growth hormones and siderophores.

With this background, the present study is proposed to isolate, screen effective Streptomyces for the management of brown spot of rice. Objectives 1. To collect and isolate the native Streptomyces sp from rhizosphere of rice plants from different locations2.To study in vitro efficacy of native rhizosphere Streptomyces sp against brown spot pathogen of rice and to check their compatibility with other biocontrol agents and to assess the efficacy of Streptomyces sp in enhancing the plant growth checked by roll towel method 3.To study the effect of antagonistic Streptomyces against brown spot pathogen of rice under field condition

Materials and methods

Isolation of pathogen

Soil collected from the Tamil Nadu rice Research institute field was filled in 15 cm diameter earthen pots. Rice seeds were sown thickly in the pots. After sowing, the pots were kept under shade and watered daily to induce damping off incidence. After 14 days, seedlings showing damping off symptom were collected and the pathogen was isolated by tissue segment method using potato dextrose agar medium (Rangasamy, 1972). The isolates were purified in plain agar medium by single hyphal tip method (Riker and Riker 1936). Two isolates obtained from affected seedlings grown on nursery soil and among the two isolates based on virulence assay, the virulent isolate was used for further studies.

Identification of Pathogen

The mycelial disc from the purified pathogen was dissolved in water and mycelium containing water droplets was taken on a microscopic slide and was exposed under 100 X magnification. The mycelia and spore characters were recorded and based on the spore morphology the pathogen was identified up to species level.

Isolation of Actinomycete *Streptomyces* from the soil

The soil samples were collected from rhizosphere regions of rice from different places. The samples were sieved and 2 g soil samples were taken for isolation. The soil samples were suspended in 25 ml basal salt solution (5.0 g/l KH₂PO₄ and 5.0 g/l NaCl) and shaken in rotary shaker (150 rpm) at 28 °C for 30 min. The soil suspensions were diluted and heated at 50 °C for 6 min. Subsequently 0.1 ml of diluted soil suspensions were spread into Starch-Casein agar (Benjaphorn et al., 2008) plates which were supplemented with 50 μ g/ ml of filter sterilized cycloheximide to inhibit fungal growth and incubated at 28 °C for 12-14 days. Colonies on the agar plates were picked on the basis of their morphological character. A total of fifteen isolates were isolated from the samples collected from different geographical locations.

In vitro testing of actinomycete Streptomyces

The antagonistic effect of actinomycete *Streptomyces* against *H.oryzae* was assessed by dual culture method on PDA medium (Dennis and Webster, 1971). In sterilized petriplates medium was allowed to settle for 1 hour. Then the actinomycete *Streptomyces* were streaked at the periphery of the petriplate

and incubated for 48 hours for good growth. After 48 hours 9 mm disc of *H.oryzae* was placed just opposite to the bacterial streak. Three replications were maintained. The growth was measured at every six hours interval. From these the most effective actinomycete *Streptomyces* antagonist was selected for further studies

Biochemical characterization

Biochemical characteristics of isolated actinomycetes was determined by the method described by Shirling and Göttlieb (1966). All tests were performed at room temperature.

Starch hydrolysis

Actinomycetes isolate was streaked on solidified starch agar medium and incubated for 5 days. The Petriplate was flooded with iodine solution for 30 seconds after incubation and drained. The amylase positive activity was indicated by the formulation of yellow zone around the colonies.

Gelatin hydrolysis

Actinomycetes isolate was tested for the production of gelatinase, which is proteolytic exo enzyme and capable of hydrolyzing gelatin. Solidified gelatine agar plates were streaked with the actinomycetes isolates and incubated for 5 days at 30°C. Hydrolysis was confirmed by flooding the plates with mercuric chloride solution

Casein hydrolysis

The isolate were streaked on skim milk agar) plates and incubated at room temperature for 5 days. Hydrolysis of casein was confirmed by flooding the plates with mercuric chloride solution and the plates were observed for the presence of clear zone surrounding the colonies and considered for positive reaction.

Hydrogen sulfide production test

Sulfide indole motility (SIM) agar deep tubes were stab inoculated with actinomycetes isolates and incubated at 35°C for 4-5 days. Black colouration along the line of stab inoculation indicates H_2S production. Hydrogen sulphide production was carried out according to (Cowan, 1974).

Indole production test

The actinomycetes isolates were inoculated into glucose tryptone broth and incubated for 5 days. About 0.3 ml of Kovac's reagent was added and mixed well. After incubation, the reddening of the alcohol layer within a few minutes indicates indole production by the culture.

Methyl red Voges proskauer test

MR-VP tests were performed to differentiate bacteria that produce acid from those that produce acetoin, a neutral product. The actinomycetes isolates were inoculated into MR-VP broth and incubated at 35°C for 4-5 days. The positive Methyl red test was indicated by change in colour of broth from yellow to red by the addition of methyl red indicator after incubation. Positive test of Voges Proskauer was indicated by the development of red colour in MRVP broth by addition of Baritts' reagent

Urease test

Urease test of actinomycetes isolates was performed on urea agar containing the pH indicator phenol red. The actinomycetes isolates were inoculated and incubated for 5 days. The development of red colour in the broth indicates the positive reaction for the test.

Efficacy of actinomycete *Streptomyces* against *H.oryzae* under glass house conditions.

Preparation of bio-formulation

Actinomycetes cultures were grown in molasses broth for 20 days. Then the 400 ml broth was mixed with 1 kg of talc powder and 5 g of Carboxy methyl cellulose. Allowed to dry in shade for 2 days to get 20% moisture level. Fifteen gram of each bio formulations were used for pot culture studies.

In vivo studies

An experiment was conducted in glass house to test the efficacy of effective actinomycetes *Streptomyces* in reducing the damping off disease incidence in rice variety ADT 46. Two delivery methods were adopted 1. Seed treatment and 2. Soil application.

Seed treatment with effective isolates of actinomycetes *Streptomyces*

Seeds of rice variety Co1 were treated with actinomycete *Streptomyces* isolate @ 10g/Kg of seed with small amount of sterile distilled water. Treated seeds were sown in pathogen inoculated soil at the rate of 25 seeds per pot. Each treatment was replicated three time and the pots were uniformly irrigated daily.

Soil application of effective isolates of actinomycete *Streptomyces*

Actinomycete *Streptomyces* was added to the soil @ 10 g/pot, five days prior to the addition of pathogen inoculum and the pathogen inoculum was added one day prior to seed sowing. Fungicide was soil drenched one day prior to sowing. Rice seeds ADT 46 was used for sowing at the rate of 25 seeds per pot. Pots were uniformly irrigated daily. Three replications were maintained in each treatment.

Compatibility among bacterial strains

Streptomyces strains were tested for their compatibility among each other by following the method described by Fukui *et al.*, (1994). The compatibility was determined for *Streptomyces* and other biocontrol strains by using Nutrient Agar (NA) medium. The bacterial strain *Streptomyces sp* was streaked horizontally on NA medium and the test strain was streaked vertically from the streak and incubated at room temperature. Compatibility was tested by overgrowth or by inhibition of *Streptomyces* and *test* strains by incubating at room temperature and by making observations over a period of 72 h.

Results and discussion

Biological control of plant pathogens using

antagonistic plant growth promoting microorganisms has been considered a more natural an-d environmentally acceptable alternative to the existing chemical control methods (Weller et al., 2002; Bhattacharyya and Jha, 2012). It has been suggested that antagonistic microorganisms isolated from the root or rhizosphere of a specific crop may be better adapted to that crop and may offer better control of diseases than microorganisms originally isolated from other plant species (Cook, 1993). Several studies have reported the potential of actinomycetes to control plant diseases (Chattopadhyay and Nandi 1982; Tahvonen and Avikainen 1987; Hussain et al., 1990; Hodges et al., 1993; Valois et al., 1996; Gyenis et al., 2003; Baniasadi et al., 2009; Pattanapipitpaisal and Kamlandharn, 2012; Adhilakshmi et al., 2013). In the present study actinomycetes were isolated from the rhizosphere soil collected from different parts of Tamil Nadu, India. These isolates were tested for their efficacy in suppressing mycelial growth of H.oryzae and Streptomyces sp in vitro.

Survey for collection of Streptomyces sp

Actinomycetes was isolated from rhizosphere soils of rice collected from different parts of Tamil Nadu, India. Soil samples was suspended in sterile water (10%) and agitated for 30 min at 420 rpm. The supernatant was serially diluted and plated on the Ken Knight's medium (Allen, 1953). The strains was identified based on Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984). The culture was named as Str1-7Survey for collection of Brown spot of rice The diseased rice leaf showing the typical symptom of Brown spot was collected from different districts of Tamil Nadu. The infected portion of leaf showing the brown spot symptoms was cut into small bits, surface sterilized in 0.1 per cent mercuric chloride solution for 30 sec., washed in repeated changes of sterile distilled water and placed on to sterilized PDA medium poured in sterilized Petri dishes. The plates was incubated at room temperature (28 \pm 2° C) for five days and observed for the fungal growth the fungus was purified by single spore isolation technique and the purified isolates were maintained on PDA slants for further studies. The isolates from different districts was assigned number and maintained for further studies.(Adhi)Isolation and characterization of actinomycete **Streptomyces** Actinomycetes are important soil microorganisms and are best known for their ability to produce antibiotics. Evidence indicates that actinomycetes are quantitatively and qualitatively important in the rhizosphere, where they may influence plant growth and protect plant roots against invasion by root pathogenic fungi (Crawford et al., 1993). In the present study a total of fifteen isolates of actinomycete Streptomyces have been isolated and characterized based on the morphology and also molecular means. In the study all the isolates were maintained on the Starch Casein medium. The possibility of exploiting Streptomyces for the management of the soil borne diseases have been discussed a long ago by many authors (Elizabeth et al., 1999; Handelsman and Stabb, 1996). (pg) Observation of brown spot spores under light microscope

The morphological characters viz., growth, colour, septation of the mycelium, conidia, size (length and width) and shape of the conidia was observed. The measurement of 100 spores was observed under the microscope in respect of each isolate by using light microscope the spore of the brown spot was slightly curved, widest at the middle and tapering toward the hemispherical apex, where their width approximates half the median width. Mature conidia are brownish with a moderately thin peripheral wall.



Brown spot of Rice

Observation of Streptomyces sp under light microscope

The morphological characters of *Streptomyces* sp conidia was observed. The measurement of 100 spores was observed under the microscope in respect of each isolate by using light microscope the spore of the Streptomyces was Smooth Conidia and



Fig 2: Conidia Of <i>Streptomyces</i> Sp
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SNo	Areas	Isolates	Percent disease incidence		
1. Aduthumi		ADTt	28.84 (32.46)c		
2.	2. Thanjavore TNJ		76.53 (61.00) *		
3.	3. Trichy THY		22.89 (28.52) *		
٩.	Nagapattinam	NGM	64.41 (53.37)*		
5.	Madurai	MDU	30.12		

 Table 1. survey for the incidence of Brown

 spot incidence in cauvery delta zone]

Field surveys were conducted in 5 districts of Tamil Nadu. A total of 20 leaf sample were collected from the above districts. The Per cent Disease Index was worked out for each sample. The results revealed that the PDI ranged from

S.No	Rhizosphere isolates	Mycelial growth Inhibition (mm)	Inhibition over control
1.	Str1	20.00	56.52
2.	Str2	18 30	60.21
3.	Str3	14.00	69.56
4.	Str4	20.16	56.17
5.	Str 5	10.00	76.26
6.	Stró	27.00	4130
7.	Str7	32.00	30.43
8.	Control	46.00	

28.84-.76.53

Table 2: In vitro efficacy of bio control Agents against Helminthosporium oryzae

Seven *Streptomyces* isolates were screened against *Helminthosporium oryzae* to test their efficacy *in vitro*. Among the isolates, Str 5 showed a maximum per cent inhibition of 78.26 followed by Str3 isolate with 69.56 per cent. The contol plate recorded the mycelial growth of 46 mm growth.

The studies on the persistence of efficacy of actinomycetes *Streptomyces* against the radial growth of *H. oryzae* revealed a bipolarized activity. The reason for the change in inhibitory activity of *Streptomyces* sp isolate could be decrease in antibiotics production at the later hours or the degradation of secondary metabolites into a non toxic form in the later hours of incubation. From this study it is implied that the isolate showing the inhibitory effect in the early hours of incubation is not effective in the later hours of promising agent for management of disease.

Pridham et al., (1956) made an extensive study using antagonistic Streptomyces culture filtrates to control foliage diseases. Prapagdee et al. (2008) reported on the inhibition of growth of C. gloeosporioides as a response to the culture filtrates of Streptomyces hygroscopius accompanied by marked cellular changes including hyphal swelling, distortion and bulbous roundedness on hyphal structure. Getha and Vikineshwary (2002) also agreed with the earlier woks observed bulging of hyphal tips of Colletotrichum mycelium treated with cell free culture filtrate of S. hygroscopius. Chattopadhyay (1982) also confirmed that the when culture filtrate of S. longisporus was highly inhibitory to *H. oryzae*

The present study revealed that the actinomycete *Streptomyces* isolate KVT was

highly inhibitory to the pathogen *P. aphanidermatum* due to the production of an antifungal substance, which was liberated in the culture media.

Biochemical test for Streptomyces sp

Identified isolate *Streptomyces* sp. produced amylase and protease enzyme that were confirmed by starch, casein hydrolysis test,

S.No	Types of test	Characteristics of actinomycete isolates					Observation	
		Strl	Str2	Str3	Str4	Str5		
1	Methyl red test	ł	+	4	÷.	+	The positive Methyl red test was indicated by change in colour of both from yellow to red by the addition of methyl red indicator after incubation.	
2.	Voges proskaner test	ě	80	200	- 1 2		The positive Methyl red test was indicated by change in colour of broth fiom yellow to red by the addition of methyl red indicato rafter incubation.	
3.	Indole production	2		9245	5	20	The reddening of the alcohol layer within a few minutes indicates indole production by the culture.	
4.	Starch hydrolysis	t	+	+	+	ť	For the hydrolysis of starch the production of clear zz around the growth actinomycetes.	
5.	Casein hydrolysis	8	+	1923	84	2	The plates were observed for to ne of case in hydrolysis a rout the growth of actinomycetes.	
б.	Gelatin hydrolysis	5	t	852	+	2	For the presence of hydrolyz zone around the growth actinomycetes.	
1.	Cittate utilization	3	Ц.	1 T	and the	No.	Plates were observed for th zone of casein hydrolysis around the growth of actinom ycetes.	
8	H2 S Production	+	+	÷	÷	÷	Black colouration along the line of stab inoculation indicates HS production.	
9.	Urease production	+	+	+	÷	÷	The development of red colour in the media indicates the positive reaction for the test	

Indole, Vogues Proskauer and Methyl red test of Streptomyces sp.

Table 1. Biochemical characterization of *Streptomyces* sp

Characterization of actinomycetes

The above five strains were identified based on Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984). All the five isolates were found to be Gram positive. The growth of all the five isolates were maximum in molasses medium and less growth on glycerol medium. The maximum growth was observed when the isolates were incubated at 37 °C. The pH 7 was found to be suitable for the growth of all isolates. All the five isolates showed good growth up to 7% NaCl. The isolates showed good growth on glucose and maltose and moderate growth on galactose and fructose and less growth on mannitol and sucrose. Biochemical tests indicated that all the isolates showed positive results in Starch hydrolysis, Citrate utilization, H₂S Production, Nitrate reduction, Urease production and Methyl red test and negative results in Indole production and Voges proskauer test. The isolates namely PDK and ANR showed positive results in Casein hydrolysis, Gelatin hydrolysis and the strains CBE, MDU and SA showed negative results in Casein hydrolysis and Gelatin hydrolysis.

Compatibility of Actinomycete with other microorganism

Bioagent	Ac tinomyc ete Isola tes				
	Strl	Str2	Str3	Str4	Str5
Pseudomonas fluorescens	÷	÷.	t)	÷.	÷
Bacillus subtilis	÷	+	+	+	ť
Bacillus megalerium var phosphaticum	ŧ	ł	+	ł	ŧ
<i>Rhizob ium</i> strain TNAU 14	ł	t	t	ť	ť
Azosp irillum brasiliense strain 204	Ŧ	+	ŧ	+	Ŧ



1. Pseudomonas fluorescens 2. Bacillus subtilis 3. Bacillus megaterium va r phosphaticum 4. Rhizobium

Treatments	Seed germination* (%)	Root length* (cm)	Shoot length* (cm)	Seedling vigpur	
Str1	32	720	9.00	518.40	
Str2	24	6.78	8.64	370.08	
Str3	19	4.00	5.90	188.10	
S tr4	14	394	5.00	125.16	
Control	10	3.12	4.00	71.20	

The Streptomyces sp isolates were found to be compatible among them and compatible with 1. Pseudomonas fluorescens 2. Bacillus subtilis 3. Bacillus megaterium var phosphaticum 4. Rhizobium strain TNAU 14 5.Azospirillum brasiliense strain 204 as evidenced by the mutual absence of growth inhibition

Table 2. Compatibility of Streptomyces sp



with other microorganism

TNAU 14 5. Azospirillum brasiliense strain 204

Table 3. Effect of *Streptomyces sp* on seed germination and seedling vigour of rice by Roll Towel Method.

Vigour index = Seed germination (%) x Seedling Length (Shoot + Root Length (cm))

Assess the efficacy of *Streptomyces* sp in enhancing the plant growth checked by roll towel method

The effect of *Streptomyces* and Mancozeb fungicide on Brown spot seed infection, seed germination and seedling vigour in the variety ADT 46 was assessed by standard blotter method and roll towel method and the results are presented in table 3. All the biocontrol agent and fungicide treatments have significantly reduced *H.oryzae* seed infection in rice when compared to untreated control. The

Streptomyces sp treatment was found significantly superior than biocontrol agents in reducing the *H.oryzae* seed infection. Seed germination, root and shoot length and seedling vigour was significantly higher in all the treatments when compared to control.

Among the different bioagents tested for their efficacy in the management of seed-borne infections of *H.oryzae* seed treatment with *Streptomyces* 1, showed least seed infection of 46.72 per cent with maximum per cent germination of 95.3 per cent followed by *Streptomyces* 2 with per cent seed infection, per cent germination and vigour index of 518.40

The effect of antagonistic *Streptomyces* sp against brown spot pathogen of rice under Pot culture conditions.

Tr.No	25 25	Post inoculation spray		
	Trea tment	Mean PDI	Percent decrease over control	
T,	Seed Treatment (ST)	3.75	87.14	
Τ,	S oil Application (SA)	4.12	85.88	
T,	T 1+ T2	6.95	76.18	
T4	Foliar spray (<i>Streptomyces</i> sp.)	5.24	82.04	
T,	Foliar spray of cow dung shury	7.68	73.68	
Té	Mancozeb	12.92	55.72	
	Control	29.18		

In order to verify the efficacy of *Streptomyces* sp against brown spot pathogen a study was conducted at glass house condition along with Mancozeb as the contact fungicide. The bio control agent *Streptomyces* sp were applied as soil application, seed treatment and foliar spray and with chemical treatment. The study was conducted post inoculation spray after the inoculation of the pathogen. The data from the table 4 revealed that *Streptomyces* sp , were found to be more effective than fungicide. *Streptomyces sp* under post inoculation spray

recorded 87.14 percent decrease over control in seed treatment followed by soil application respectively this was followed by *Streptomyces s*p which were applied as combination

Tr.No	Treatment	Dose	Mean PDI	Yield
T,	Seed Treatment (ST)	10 g / kg	46.65	4116
T ₂	Soil Application (SA)	2.5 kg / ha	40.36	4743.3
T,	T1+T2	As above	48.14	5153.3
T4	Foliar spray (Streptomyces sp.)	0.2 %	41.47	5020.0
T,		20 per cent	47.70	5033.3
T ₆	Mancozeb	2 g/lit	45.59	4743.00
T,	Control	E.	39.62	2746.6
		CD(0.05)= 12	96	
		Se(d)= 5.93	5	

Table 4: Antagonistic *Streptomyces* sp against brown spot pathogen of rice under Pot culture conditions.

Efficacy of *Streptomyces sp* on the incidence of brown spot diseases of rice under field condition

A field trial was conducted during *Thaladi* 2015-16 for the management of brown disease in rice with rhizosphere inhabiting *Streptomyces* sp. Seed treatment @ 10 g/kg + soil application @ 2.5 kg/ha provided the maximum control of brown spot disease (PDI 48.14) and was on par with foliar spray of 20% cow dung slurry (PDI of 47.70%) when compared to control (PDI 39.62). However, maximum yield of 5153 kg/ha was recorded by Seed treatment @ 10 g/kg + soil application @ 2.5 kg/ha compared to the control yielded only 2746.6

The results are in agreement with findings of Reddi and Rao (1971) who reported the antagonistic activity of *Streptomyces* against *H.oryzae* in rice. The earlier workers reported the exploitation of *Streptomyces* in controlling the disease incidence in crop plants (Yuan and Crawford, 1995; Bélanger, 2002; Sabaratnam, 2002). A strain of *Streptomyces* sp. isolated

from the rhizosphere of field-grown tomato has been reported to suppress damping-off of tomato which was caused by *P. aphanidermatum* (Sabaratnam and Traquair, 2002). Tahvonen, (1982) reported that the isolates of Streptomyces spp. were assessed in Finland for 20 years for their ability to control fungal diseases. Streptomyces have been implicated in antagonism of a variety of plant pathogens. Trejo-Estrada et al., (2008) isolated Streptomyces violaceusniger from turf grass rhizosphere and it inhibited seven soil borne pathogens viz., Fusarium oxysporum, Pythium ultimum, Pythium aphanidermatum, Gaeumannomyces graminis, Colletotrichum graminicola, Rhizoctonia solani, Microdochium nivale and Sclerotinia homeocarpa. The findings suggest that the possibility of exploitation of Streptomyces spp in control of soil borne diseases including P.aphanidermatum.

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