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ISOLATION, IDENTIFICATION AND ANTAGONISTIC POTENTIAL OF *STREPTOMYCES* ANSCA22 FOR BIOCONTROL OF *RHIZOCTONIA BATATICOLA* INFECTION OF SOYBEAN

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Abstract

Streptomyces ANSCa22 isolated from rhizospheric soil of *Capsicum annuum* demonstrated antifungal activity against the pathogen *Rhizoctonia bataticola* by dual culture technique. *Streptomyces* ANSCa22 treatment on *Rhizoctonia bataticola* inoculated Soybean seeds could control *Rhizoctonia bataticola* infection of Soybean in pot study which was evident from the significant increase in the values of average shoot height, pod number, seed weight and plant dry weight after the treatment. *Streptomyces* ANSCa22 treatment of normal soybean seeds without any pathogen inoculation also resulted in positive effect on three out of four tested plant growth parameters as compared to control. Microscopic examination of *Streptomyces* ANSCa22 revealed typical features of *Streptomyces*. Partial sequencing of rRNA gene identified ANSCa22 as *Streptomyces rochei.* This strain was compatible with *Bradyrhizobium japonicum*, exhibited tolerance to calcium carbonate and calcium sulphate upto 5% concentration and indicted varying degree of growth at 7 to 10 pH range. *Streptomyces rochei* ANSCa22 was positive for amylase enzyme.

Key words : Antagonistic *Streptomyces*; *Rhizoctonia bataticola*; Biocontrol.

Introduction

Management of *R. bataticola* infection of Soybean is of major concern in Akola district owing to the prevalence of this fungal phytopathogen. Root rot, Stem rot, Charcoal rot and damping- off are some of the diseases caused by this pathogen resulting in yield losses (Yang and Navi, 2005, Mengistu *et al.*, 2011). In major soybean growing states of India, incidence of *Rhizoctonia* root rot has been reported in the range of 3.29% to 40.33% and in Maharashtra it was in the range of 17.49% to 37.44% during 2012 to 2013. (Belkar. and Gade, 2016).

Chemical fungicides have been commonly employed to control these diseases (Divya and Sudini 2013). However chemical control has always been associated with adverse effects of chemical fungicides on environment and agro- ecosystem (Yang *et al.*, 2011). During past few decades the efforts have been focused on exploring eco-friendly and safe biocontrol agents for management of plant diseases (Ratul and Singh, 2018). Importance of *Streptomyces sp*. for biocontrol of phytopathogens and plant growth promotion has been well established by many researchers. (Gopalakrishnan *et al.*, 2011 b; Viswanathan *et al.*, 2015; Ahmed 2017).

Streptomyces Strains Di944 (Siva, 1999) and *Streptomyces lydicus* 108 (Yuan and Crawford, 1995) have been patented in Landon and United state respectively. Focused studies at International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) of Andhra Pradesh, India (Gopalkrishana *et al.*, 2012) have resulted in promising outputs with respect to agricultural applications of *Streptomyces* spp.

The success of the applications of any microbial agro-input is directly related with the acquaintance of the strain with the physico-chemical and the climatic conditions prevailing in the agro-ecosystem of the region. The present investigations deal with isolation, identification and evaluation of antagonistic and plant growth promoting potential of *Streptomyces* ANSCa22, an indigenous strain of Akola District, Maharashtra, India.

Materials and Methods

Isolation of Streptomyces

Rhizospheric soil sample was collected from *Capsicum annuum* from a field of Kanheri sarap village of Akola district. Streptomycetes were isolated by serial dilution method (Usha and Selvam 2011) on Starch casein agar (SCA). Compact, hard, powdery, slow growing colonies were selected. Pure cultures were maintained in glycerol stock.

Isolation of *R. bataticola*

Soybean plant samples were collected from the fields of North, South, East and West side villages of Akola district. Diseased plants looking different from the normal healthy plants with respect to general appearance, height and colour of leaves were collected in different carry-bags. Surface sterilised small pieces of roots or stems were placed on PDA and incubated for 7 days at 27^0C. Isolated fungal plant pathogens were identified on the basis of colony characteristics and microscopic examination described by **(**Veena *et al.,* 2014).

Antifungal activity by dual culture technique

R. bataticola was inoculated on SCA by spread plate technique. *Streptomyces* isolates were spot inoculated on the same plate. After 7 days incubation colonies showing zone of inhibition against *R. bataticola* were selected.

Antifungal activity of *Streptomyces* ANSCa22 against *R. bataticola* was studied further by inoculating the two cultures on the same SCA plate opposite to each other (Gopalkrishana *et al.,* 2012).

Microscopic examination of selected isolate

Cover slip method was used (Williams *et al.,* 1983) in which half of sterilized cover slip was inserted in the starch casein agar plate. Culture of isolate ANSCa22 was inoculated (straight line) near cover slip on agar. After incubation period cover slip was removed and observed under microscope. The culture was identified on the basis of arrangement of spores, according to Madigan *et al.,* (1987).

Test for Pathogenicity of *R. bataticola*

Inoculum of *R. bataticola* was mixed into sterilized soil at 4% by weight of soil (Lukade, 1992) and was filled in sterilized pots. Surface sterilized Soybean seeds were used for sowing. Soil without inoculum of pathogen was used as control. Readings were noted on 15th day after sowing regarding germination % and number of survived and infected plants. Percent disease incidence was calculated (Srinivas, 2016). *R. bataticola* isolate (R1) demonstrated 100% disease incidence.

Pot study

Fresh biomass (20%) of *Streptomyces* ANSCa22 was mixed with talcum powder. Fresh inoculum of *R. bataticola* (R1) was prepared in potato dextrose broth. Surface sterilized seeds were socked in broth of *R. bataticola* (R1) for 30 minutes and then the seeds were subjected to different treatments.

These treatments included R1 inoculation followed by *Streptomyces* ANSCa22 treatment (100mg/g seeds) (T_1) and Carbendazim treatment (0.2mg/g of seeds) (T_3). Other pots of the sets were R1 inoculated seeds without any treatment (T_4), only *Streptomyces* ANSCa22 treated (100mg/g) seeds without R1 inoculation (T_2) and control (C1) *i.e*. seeds without any treatment. (Sabaratnam, 1999). The effects on seed germination and plant growth parameters were monitored for three months using sterilized pots and soil.

Statistical analysis

Statistical analysis was carried out by using Graph pad prism software version 5. ANOVA and T test were performed (Otto-Hanson *et al.,*2012).

Stress tolerance for salt concentration and pH

Streptomyces ANSCa22 was grown on Starch casein agar media with different concentrations of NaCl (2.5%, 5%, 7.5% and 10%), $CaCO_3$ (2.5% and 5%), $CaSO_4$ (2.5% and 5%) and CaCl2 (2.5% and 5%), and on different pH (7, 8, 9, 10) (Carla *et al.,* 2008, Sunita *et al.,* 2015).

Cellulose degradation

Streptomyces ANSCa22 isolate was spot inoculated on Czepak mineral salt medium and incubated for 2-3 days at 28^0C. After incubation period plate was flooded with 1% Congo red solution and excess dye was drained after 5 minutes. Solution of 1M NaCl was then added repeatedly until colour of Congo red solution disappeared. Clear zone around the colony indicated degradation of cellulose (Carder 1986; Sharma *et al.,* 2001).

Chitin degradation

Streptomyces ANSCa22 isolate was spot inoculated on colloidal chitin agar plate (swollen chitin 2g/lit, ammonium sulphate 0.05g/lit., agar 1.8g/lit. and pH 7) and incubated up to 5 days. Chitinase activity was identified by clear zone around the colony (Palaniyandi *et al.,* 2013).

Hydrolysis of casein

Streptomyces ANSCa22 isolate was spot inoculated on casein agar plate (10g/lit. casein, 10g/lit., glucose, 1.5g/lit., K_2HPO_4 and 15g/lit. agar) (Palaniyandi *et al.,* 2013)

and incubated at 28°C for 5 days, Casein hydrolysis was identified by clear zone around the colony.

Hydrolysis of gelatin

Streptomyces ANSCa22 isolate was spot inoculated on nutrient agar containing 15% gelatin and incubated for 3 to 4 days. After incubation plate was flooded with 1% $HgCl_2$. Clear zone around the colony indicated the hydrolysis of gelatin (Stefka *et al.,* 2004).

Hydrolysis of starch

Streptomyces ANSCa22 isolate was spot inoculated on starch agar plate and incubated at 28°C for 3-5 days. After incubation period plate was flooded with 1% iodine. Clear zone around the colony indicated the amylase activity (Shaw *et al.,* 1984; Santos *et al.,* 2003).

Compatibility with *Bradyrhizobuim japonicum*

Bradyrhizobuim japonicum was inoculated on SCA by spread plate technique. *Streptomyces* ANSCa22 was spot inoculated on the same plate. After 9 days incubation growth of both microorganisms without zone of inhibition against *Bradyrhizobuim japonicum* indicated compatibility of *Streptomyces* ANSCa22 with *Bradyrhizobuim japonicum.*

Identification of Selected *Streptomyces*

Molecular phylogeny of *Streptomyces* ANSCa22 was determined by amplifying and sequencing genomic 16s rRNA region using 8F and 1541R primers. Sequencing facility was availed from Yaazh Xenomics Chennai. The results obtained in this study were used for identification of isolates.

Results and Discussion

Total 12 cultures were isolated from rhizospheric soil of Capsicum *(Capsicum annuum) viz.,* ANSCa22 to ANSCa33. All isolates exhibited typical characteristics of *Streptomyces* colony (compact, hard, powdery, slow growing colony). *R. bataticola* was isolated from infected plant sample. Brown to black colour mycelial growth of *R. bataticola* (R1) was observed on agar. In microscopic examination, dark sclerotia with round to irregular shape and right angle branch to parent hyphae were observed (Fig. 1).

Colony of *Streptomyces* ANSCa22 (Fig. 2) had white colour arial mass and yellow colour reverse side pigment. No melonoid pigment production was seen. Spiral and hook like) spore arrangement was observed in microscopic examination of *Streptomyces* ANSCa22 (Fig. 3). Screening of twelve *Streptomyces* isolates for antifungal activity against R1 by dual cultures technique revealed the presence of antagonistic activity in ANSCa22 (Fig. 4). None of the 11 other isolates were antagonistic for the pathogen R1.

Streptomyces ANSCa22 was studied for the antagonistic potential against the pathogen R1 and also for plant growth promoting potential for soybean in pots. Averages of shoot height, number of pods, seed weight and plant dry weight of the pathogen R1 inoculated seeds were studied after different treatments**.** *Streptomyces* ANSCa22 treatment (T_1) could effectively control *R. bataticola* infection as evident from improvement in all the growth parameters after the treatment (Table 1). Efficacy of this biological treatment was appreciably better than the chemical treatment with carbendazim (T_3)

Table 1: Effect of different treatments on growth parameters of soybean.

Treatments	Average of shoot height (cm)	Average of pod no.	Average of seed weight (gm)	Average of plant dry weight
Control (C1)	36.74	8.800	0.7316	12.70
R.bataticola+ *S.* ANSCa22 (T_1)	36.20	13.00	0.970	16.24
Streptomyces ANSCa22 (T_2)	40.20	14.40	0.981	17.00
R. bataticola+ Carbendazim (T_3)	15.54	4.400	0.5208	6.640
R. bataticola (T_4)	1.480	0.200	0.0320	1.460

Table 2: P values of T test and ANNOVA for results of pot study.

S.No.	Parameters checked	T test (P value)		ANOVA (P value) (Between 5 groups)
		T1vs T4	T1vs C1	
1	Plant height	0.0097	0.7518	0.0012
2	No. of Pods	0.0097	0.0119	0.0003
3	Seeds weight	0.0097	0.0079	0.0002
4	Plant dry weight	0.0097	0.0079	0.0003

Note: T_1- *R. bataticola* and *Streptomyces* ANSCa22 treated seeds, T_4 - *Only R. bataticola* treated seeds and C1- Control.

Table 3: Effect of different salt concentrations on the growth of *Streptomyces* ANSCa22 isolate.

S.No.	Isolates	CaCo3		CaSO4		CaCl2		NaCl	
		2.5%	5 %	2.5%	5 %	2.5%	5 %	2.5%	5 %
1	ANSCA22	++++	++	+++	++	+	-	+	+

Poor growth +, moderate growth ++, good growth +++, luxuriant growth ++++, No growth -

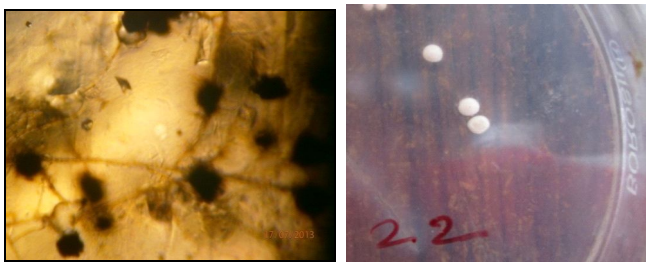
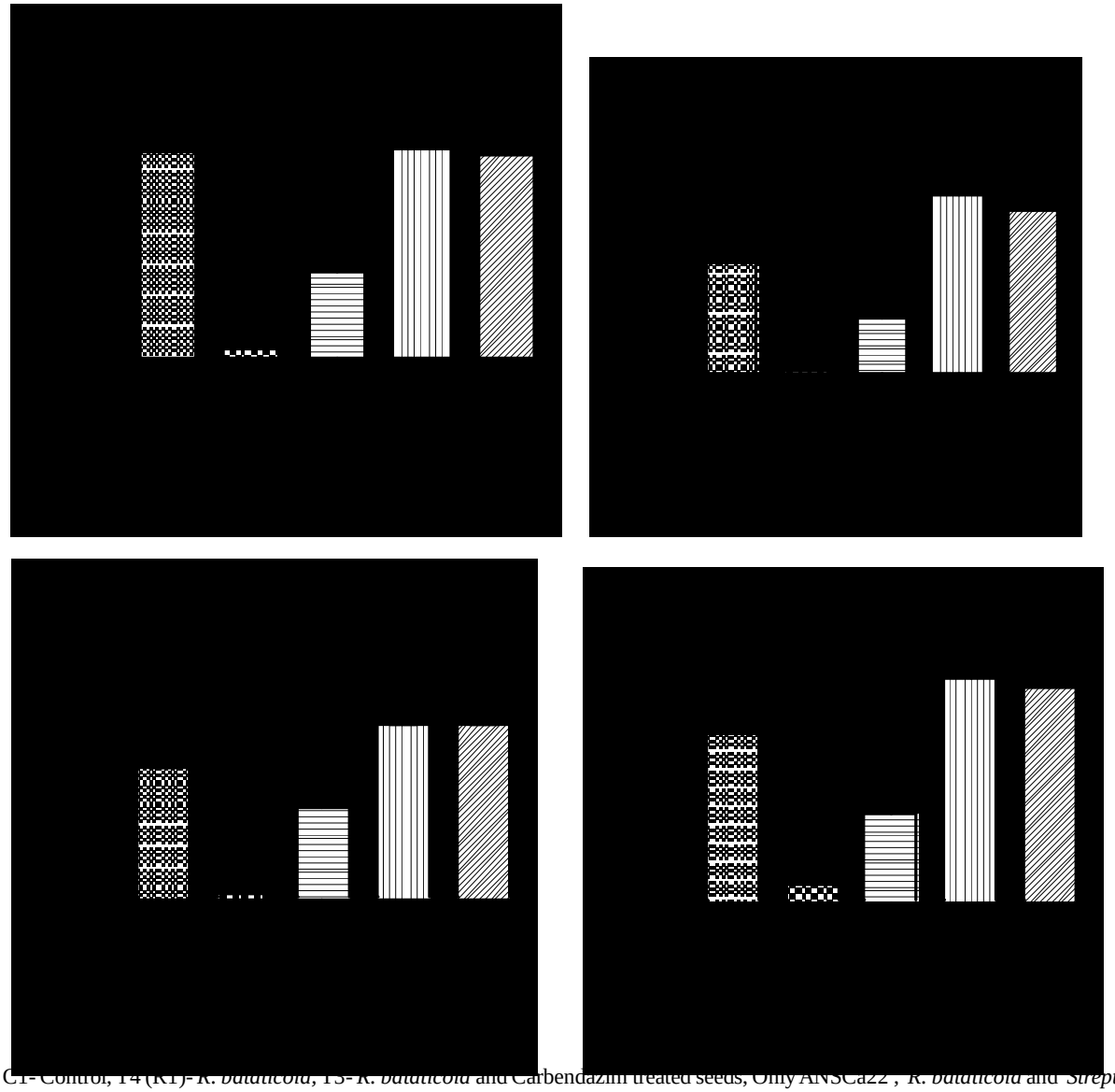


Fig. 1: Microscopic examination of
Fig. 2: *Streptomyces rochei* ANSCa22 *R. bataticola.*

Fig. 3: Microscopic examination of
Fig. 4: Dual plate technique. *Streptomyces rochei* ANSCa22



Note: C1- Control, T4 (R1)- *R. bataticola*, T3- *R. bataticola* and Carbendazim treated seeds, Only ANSCa22 , *R. bataticola* and *Streptomyces* ANSCa22 treated seeds.

Fig. 5: Effect of different treatments on growth parameter of Soybean.

(Fig. 5). Average Shoot height, pod number, seed weight and plant dry weight increased after *Streptomyces* ANSCa22 treatment by 36.1cm, 12, 0.947g and 15.74g respectively and after chemical treatment the increase in the four above parameters was 13.66cm, 4.2, 0.488g and 5.18g respectively.

Streptomyces ANSCa22 treatment (T_2) of normal soybean seeds (without pathogen inoculation) also resulted in remarkable improvement in three out of four tested growth parameters as compared to control (C1)

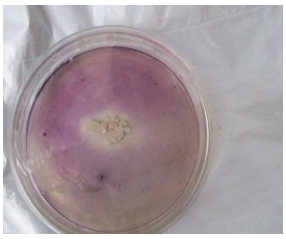


Fig. 6: Compatibility with *B. japonicum*
Fig. 7: Starch hydrolysis.

(Table 1, Fig. 5). This clearly indicated the presence of plant growth promoting potential for soybean in *Streptomyces* ANSCa22.

Significance of the observations was analyzed by T test and ANOVA. P values obtained were in the range of 0.0079 to 0.0119 in T test and 0.0002 to 0.0012 in ANOVA. These readings supported the significance of plant growth promoting potential and antagonistic bioactivities of *Streptomyces* ANSCa22 culture (Fig. 5, Table 2).

The presence of two favourable attributes of antagonism and plant growth promotion in *Streptomyces* ANSCa22 is very beneficial for the field application of the culture. Moreover, *Streptomyces* ANSCa22 appeared to be compatible with *Bradyrhizobium japonicum*. (Fig.6) Many earlier studies have reported the presence of both the antagonistic and plant growth promoting activities together in one culture of *Streptomyces* species (Sabrtnam, 1999, Gopalakrishnan *et al*., 2011b).

Streptomyces ANSCa22 could grow at varying degree at 2.5% and 5% concentrations of NaCl, $CaCO_3$ and $CaSO_4$ table 3 but not at 5% $CaCl_2$ concentration. *Streptomyces* ANSCa22 was also found to grow on pH 7 to 10 (luxuriant growth to poor growth). Thus its application potential appears to be suitable for neutral to alkaline and non saline to slightly saline soils.

Streptomyces ANSCa22 was observed to hydrolyze starch (Fig. 7) but negative results were obtained about chitin, cellulose, gelatine and casein degradation.

Streptomyces ANSCa22 isolate was identified by 16s rRNA gene sequencing. Polymerase chain reaction was carried out using 8F and 1541R primer. The 16s rRNA sequence was blast using NCBI blast similarity search tool. It was identified as *Streptomyces rochei*.

The nucleotide sequence of this organism is

>CONTIG STREPTO

GAGTTGCAGACCCCAATCCGAACTGAGACCGG
CTTTTTGAGATTCGCTCCACCTCGCGGTATCGCA
GCTCATTGTACCGGCCATTGTAGCACGTGTGCA
GCCCAAGACATAAGGGGCATGATGACTTGAC
GTCGTCCCCACCTTCCTCCGAGTTGACCCCGGC
GGTCTCCCGTGAGTCCCCAGCACCACAAGGGCC
TGCTGGCAACACGGGACAAGGGTTGCGCTCGTTGCGG
GACTTAACCCAACATCTCACGACACGAGCTGA
CGACAGCCATGCACCACCTGTACACCGACCACAAGG
GGGACCCTGTCTCCAGGGTTTTCCGGTG
TATGTCAAGCCTTGGTAAGGTTCTT
CGCGTTGCGGTCGAATTAAG
CCACATGCTCCGCCGCTTGTGC GGGCCCCCC
GTCAATTCCTTTGAGTTTTAGCCTTGCGGCCGTACTCCC
CAGGCGGGGCACTTAATGCGTTAGCTG
CGGCACGGACAACGTGGAATG
TTGCCCACACCTAGTGCCCACCGTTTACGGCG
TGGACTACCAGGGTATCTAATCCTGTTC
GCTCCCCACGCTTTCGCTCCTCAGCGTCAGTA
TCGGCCCAGAGATCCGCCTTCGCCACC
GGTGTTCCTCCTGATATCTGCGCATTTCACCGCTA
CACCAGGAATTCCGAT CTCCCCTACCGA ACTCT
AGCCTGCCCGTATCGACTGCAGACCCGGGGTTAA
GCCCCGGGCTTTCACAACCGACGTGACAAGCCGC
CTACGAGCTCTTTACGCCCAATAATTCCG
GACAACGCTTGCGCCCTACGTATTACCGCGGCT
GCTGGCACGTAGTTAGCCGGCGCTTCTTC
TGCAGGTACCGTCACTTTCGCTTCTTCCCTGCTGAAA
GAGGTTTACAACCCGAAGGCCGTCATC
CCTCACGCGGCGTCGCTGCATCAGGCTTTCGCCCA
TTGTGCAATATTCCCCACTGCTGCCTCCCGTAG
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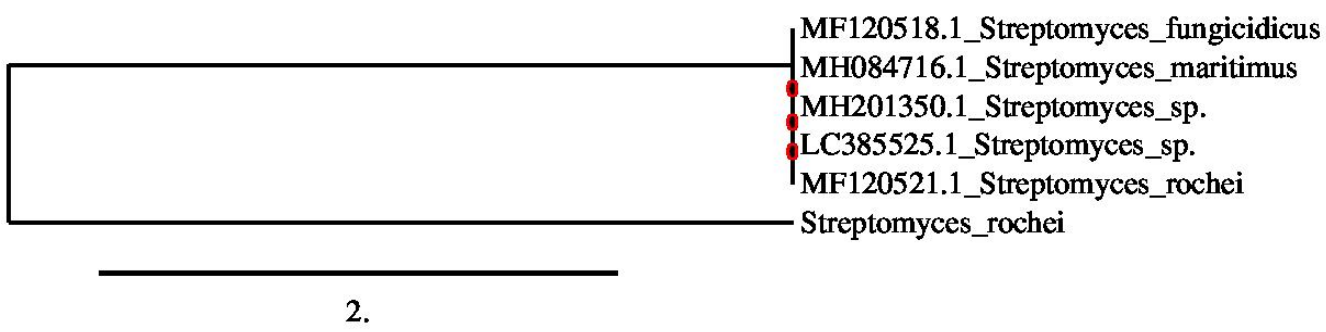

GGGCTCATCCTGCACCGCCGGAGCTTTCGAACC TCGCAGATGCCTGCGAGGATCAGTATCCGGTATTA GACCCCGTTTCCAGGGCTTGTCCCAGAGTGCAGGG CAGATTGCCCACGTGTTACTCACCCGTTCGCCACT AA

Sequence of *Streptomyces rochei* ANSCa22 deposited under the GenBank accession number is SUB4723523 Strep MK116539.

Conclusion

S. rochei ANSCa22 appeared to possess antagonistic as well as plant growth promoting potentials with respect to *R.. bataticola* infection and plant growth of soybean.. Its biocontrol treatment was more effective as compared to Carbendazim treatment for all the growth parameters in pot trails. Hence this culture needs to be explored with intensive studies to evaluate its application potential in fields.

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