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Research Article

Biological control of damping off (*Pythium aphanidermatum*) using *Streptomyces lavendulae* isolate 21 on tobacco seedbeds

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ABSTRACT: Soil actinomycetes, particularly *Streptomyces* spp., possess antagonistic activity against a wide range of plant pathogens as they are potent producers of antibiotics. In the present study 40 isolates of actinomycetes were isolated from tobacco soils of India and screened against *Pythium aphanidermatum in vitro*. Among them, ten *Streptomyces* isolates showed high antagonistic activity. *Streptomyces lavendulae* isolate 21 showed strong inhibition zone (24mm) of the pathogen. Experiments *in vivo* using spray of mixed spore suspension of *P. aphanidermatum* and *S. lavendulae* isolate 21 on tobacco seedbeds resulted in marked reduction of disease severity, significant improvement in dry weight, leaf area and nutrient content. The treatment *P. aphanidermatum S. lavendulae* recorded 32 per cent increase in number of transplantable seedlings against *P. aphanidermatum alone, while the treatment Streptomyces alone* recorded 1.87 times increase in number of transplantable seedlings against control.

KEY WORDS: Biocontrol, *Pythium aphanidermatum*, *Streptomyces,* tobacco

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INTRODUCTION

Streptomyces are important microorganisms known to produce important biologically active substances such as vitamins, alkaloids, plant growth factors, enzymes and enzyme inhibitors (Omura, 1986 and Shahidi *et al*., 2004). Soil *Streptomycetes* are the major contributors to the biological buffering of soils and have a role in decomposition of organic matter conducive to crop production (Gottlieb, 1973, Keiser, *et al*., 2000). The search for new principles in biocontrol of plant pathogens different from the conventionally used fungicides, is common (Fruh *et al*., 1996, Knight *et al*., 1997). Tobacco (*Nicotiana tabacum* L.) is an important commercial crop earning sizable foreign exchange and internal revenue. It is susceptible to several fungal, bacterial and viral diseases both in nursery and transplanted crop. *Pythium* spp. are essentially soil-borne pathogenic fungi, which cause seed rot and damping-off in many crops, including chilli and tomato apart from tobacco (Shah-Smith and Burns, 1996). Present focus on management of plant diseases has been shifted from chemical pesticides to more eco-friendly biopesticides in order to reduce environmental pollution and to minimize the risk of development of fungicide-resistant strains of plant pathogens. Despite its drawbacks, biological control of plant diseases offers long lasting economical benefits.

The main objective is to develop bacterial antagonists against major plant pathogens of tobacco which can be developed as biofungicides and in the present study promising *Streptomyces* isolates were screened against damping-off disease of tobacco.

MATERIALS AND METHODS

Tobacco seeds @ 0.5g/ m^2 seedbed were sown thickly in pots containing farm soil. After sowing, the pots were kept under shade and watered daily to favour the incidence of damping off. After 15 days, seedlings showing damping-off symptoms were collected and the pathogen, *P. aphanidermatum* (Edson) Fitzp. was isolated by tissue segment method (Rangaswami,1958) on potato dextrose agar medium (PDA). It was purified by single hyphal tip method and maintained in potato dextrose agar slants. (Yella *et al.,*2006*)*. Soil samples were collected from tobacco soils of different agro-climatic zones such as Southern light soils (SLS) of Prakasam district, Northern light soils (NLS) of west Godavari district and Traditional black soils (TBS) of East Godavari district. Several samples

were randomly selected from various localities using an open-end soil borer (20 cm in depth, 2.5 cm in diameter) as described by Lee and Hwang (2002). Samples weighting 10 g each were air-dried for 7–10 days and then passed through a 0.8 mm mesh sieve and were preserved in polyethylene bags at room temperature before use The soil samples were pretreated with calcium carbonate and then dried at 45°C for 1 h in order to reduce the incidence of bacteria and molds. This modified procedure was found to be suitable for the isolation and identification of *Streptomyces* spp. (Pirous *et al*., 1999). Soil dilution plate technique was employed for the isolation and enumeration of *Streptomyces* spp using asparagines-glycerol salts agar (AGS) medium (Pridham and Lyons 1980). Suppression of growth of bacteria and molds was observed in AGS medium supplemented with streptomycin and amphotericin-B (Williams and Davis, 1965)**.** Pure cultures of actinomycetes isolated from soil were identified up to generic level by comparing the morphology of spore bearing hyphae spore chain as described in Bergey's manual (Locci, 1989), following the direction mentioned in the methods and manual of international cooperative project for description and deposition of cultures of *Streptomyces* (ISP) (Shirling and Gottlieb, 1966). Identification procedures of the active isolates were carried out as described by Saadoun and Gharaibeh (2002). The slide culture method was employed for morphological studies (Williams and Cross, 1971) Casein glycerol agar (CGA) medium was prepared from basic ingredients as described by Kuster and Williams (1964) and used to culture streptomycetes.

Screening studies were conducted with 40 pure *Streptomyces* isolates. To evaluate the antifungal activity against the pathogen, bioassays were performed by agar disk method (Shahidi Bonjar, 2003). Antifungal activity around the *Streptomyces* agar disks was evaluated using slightly modified indices of Lee and Hwang (2002) describing as (1) no inhibition = mycelial growth not different from control (2) weak inhibition = partial inhibition of mycelial growth, measured as a diameter of 5-9 mm (3) moderate inhibition = almost complete inhibition of mycelial growth, measured as a diameter of 10-19 mm (4) strong inhibition = complete inhibition, in which most mycelia did not grow, measured as a diameter of >20 mm and (5) Controls included plain agar disks. *Streptomyces* isolate 21 was grown in submerged culture of CG medium on rotary shakers under 130 rpm at 29ºC. To monitor the activity, small aliquots of culture media were taken every 24 h for 108 days and the activity was evaluated by well diffusion-method (Dhingra and Sinclair, 1995; Acar and Goldstein 1996). From ten active *Streptomyces* isolates one showed high antagonistic activity and their colonies were characterized morphologically and physiologically. To measure the MIC values, two-fold serial dilutions of 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78 mg ml^{-1} of the crude extract were prepared in DM (distilled water, methanol) solvent and assayed by well diffusion-method as described by Shahidi Bonjar (2004). The MIC was defined as the lowest concentration able to inhibit any visible fungal growth. Each experiment was replicated twice. To evaluate the relative polarity of the active principle (s) samples were made separately with distilled water, methanol and chloroform adding each @ 2 ml per 20 mg pulverized-crude sample. The samples were vortexed for 20 min, centrifuged at 3000 rpm for 15 min using a bench top low speed centrifuge. Supernatants and pellets were separated, at 50ºC and assayed at concentration of 20 mg $mL^{-1}$by agar diffusion-method (Shahidi Bonjar, 2004). To confirm the chloroform sensitivity, 15-20 mL of chloroform was added to 300 mg pulverized crude and were maintained on rotary shakers under 130 rpm at 29ºC for 24 h, vortexed and divided to three small tubes. After separation and drying of supernatants and pellets equal volumes of chloroform were added to each tube, vortexed and centrifuged and dried under reduced air. The quantitative experiment was conducted twice. Then tubes were placed in a desiccator for 24 h. All samples were tested by well diffusion method. To measure the stability of the active crude in soluble states 0.1 mg m L^{-1} samples were prepared in distilled water and placed in small vials. These samples were kept at room temperature and tested using agar diffusion-method for anti *P. aphanidermatum* activity at 14 days intervals, until the activity persisted. Small aliquots 10 mg mL^{-1} of soluble crude were exposed to temperatures ranging from 30, 40, 50, 60, 70, 80 and 90°C for 10 min and cooled to monitor the effect of temperature on bio activity. Heated oil was used for temperatures higher than 90°C. Bioactivity of treated samples wsa evaluated using well diffusion method. Control included incubation of an untreated sample at 28°C (Nawani and Kapadnis, 2004). Small blocks of inhibition zones (1 mm^3) of *S. lavendulae* against *P. aphanidermatum* were transferred to fresh PDA plates and incubated for 7 days at 26-28°C. During incubation, growth or lack of growth of the fungus was investigated both visually and microscopically. Rejuvenation of growth was indicative of fungistatic and lack of growth represented fungicidal properties of the antagonist. Seeds of tobacco var VT 1158 were grown in ceramic pots containing sterilized sand and farm yard manure. Seeds were planted 3–4 cm below soil surface per pot. In three leaves-stage, the seedlings were separated to receive four treatments *viz.,* (1) pathogen alone, (2) S. *lavendulae* isolate 21, (3) pathogen plus *S. lavendulae* isolate 21 and (4) control (untreated) and replicated ten times. Spore suspension of the pathogen was prepared by adding 2–3 mL sterile distilled water to petri dishes of well grown lawn culture of the

Table 1. Morphological and physiological characterization of *Streptomyces lavendulae* isolate 21

Arial mycelium color	Melanoid pigments	Reverse color	Soluble pigments	Spore chain	Spore surface	L- Arabinose
Yellow white	0	0	0	F	Sm	–
Xylose D–	Inistol I–	Mannitol D–	Fructose D–	Rhamnose	Sucrose	Raffinose
–	–	–	–	–	–	–

F: Flexuous; Sm: Smooth; 0: Negative; –: No utilization

Table 2. Bioassay results of solubility tests of the antifungal principle(s) of *S. lavendulae* isolate 21 against *P. aphanidermatum* in fractions of different solvents indicated by well diffusion method at 20 mg mL^{-1} of dry crude sample

Solvent	Fraction *	Activity	Inhibition zone (mm)
Water	S, P	Traceable	19.50
Methanol	S, P	Traceable	22.24
Chloroform	S, P	Traceable	16.18
S Em ± CD at 5% CV%	–	–	0.05 0.16 0.67

*S: Supernatant, P: Pellet

Table 3. Effect of *Streptomyces* on damping off, growth characteristics, leaf area and nutrient content of tobacco seedlings

Treatment	Disease severity %	Dry wt of the seedlings (g)	Healthy transplantable seedlings	Leaf area cm^2	N%	P%	K%
Control	58.3	2.10	37.0	157	2.76	0.20	2.33
P. aphanidermatum	100	1.33	13.7	115	2.05	0.16	1.45
Streptomyces	38.9	5.43	106.3	469	3.63	0.32	3.35
P. aphanidermatum + *Streptomyces*	60.2	3.10	57.7	308	2.95	0.24	3.18
S Em ±	1.58	0.07	3.37	9.80	0.03	0.01	0.05
CD at 5%	5.4	3.90	11.67	33.9	0.11	0.0	0.19
CV%	4.28	3.90	17.68	6.46	2.09	7.25	3.85

pathogen and kept in small beakers. A conidial suspension of *P. aphanidermatum* 4 x 10^5 conidia mL^{-1} was sprayed in the treatments 1 and 3. *S. lavendulae* isolate 21 @ 20 mg / mL was sprayed in treatments 2 and 4. Controls received water spray only. The severity of damping-off disease was recorded regularly up to 30 days after sowing, on the basis of per cent root area affected by the pathogen utilizing a 0-5 scale (Bharat and Bhardwaj, 2001) where different grades denote the per cent root rot area infected as follows: 0 – no symptoms, 1 - 1-10%, 2 – 10-20%, 3 – 21-50%, 4 – 51-75% and 5 – 76-100%.

The tobacco seedlings were pulled and washed to remove the soil and incubated for 48 h at 60°C to measure the dry weights. The experiment was repeated twice and means recorded.

RESULTS AND DISCUSSION

Ten isolates of actinomycetes were found to be active in dual culture methods. Two of them showed suppressive reaction to *P. aphanidermatum*, S. *lavendulae* isolate 21 exhibited complete inhibition of >20mm zone followed by *Streptomyces* isolate16 which showed the inhibitory effect of 17mm. Bioassay also showed similar results with S. *lavendulae* isolate 21 showing inhibition zone of 23mm followed by *Streptomyces* isolate16 which showed the inhibitory zone of 19mm against *P. aphanidermatum* measured in agar disk-method. In both methods, S. *lavendulae* isolate 21 showed best antifungal effects on the pathogen. Subhashini and Padmaja (2009) also reported similar results.

Activity reached maximum after 5 days in rotary cultures. In shaker cultures, this interval was used to harvest cultures to prepare crude extract for use in further investigations. Isolate 21 was identified as *Streptomyces lavendulae* based on morphological and biochemical characterization (Table 1). Gram staining test revealed that *Streptomyces lavendulae* isolate 21 is Gram stain positive.

In well diffusion-method, MIC of the crude sample was determined as 3.12 mg m L^{-1}against *P. aphanidermatum*. Solubility results apparently show, multiple active principles, involved since the activity was traceable both in polar and non polar solvents of distilled water, methanol and chloroform. Stability of the active crude sample in distilled water at room temperature (27°C ±1) was about five months, through agar diffusion-method against *P. aphanidermatum*.

Bioactivity of active isolate diminished to zero at 150ºC. Transfer of blocks from inhibition zones to fresh PDA plates revealed subsequent growth of the pathogen which was indicative of fungistatic activity of *S. lavendulae* 21.

The results of biological activity of *S. lavendulae* isolate 21 against *P. aphanidermatum* revealed 60.2% disease severity in treatment of plants with pathogen plus *Streptomyces* against 100% in disease inoculated treatment. Maximum dry weight of 5.43g was recorded in the treatment *Streptomyces* alone followed by the treatment *S. lavendulae* in combination with *P. aphanidermatum* which showed 3.1g dry weight and least dry weight was recorded with *P. aphanidermatum* inoculated treatment. Similar result was obtained in case of number of transplantable seedlings also as reported by earlier workers in case of tomato (Ratnam and Traquair 2002). Significant improvement in the NPK content of the tobacco seedlings was observed due to the application of *Streptomyces* alone followed by the treatment *Streptomyces* in combination with *P. aphanidermatum.* Lowest content of the nutrients was recorded in the pathogen alone applied treatment. Similar findings were reported by AbdEL- Ghany *et al*., (2010).

In sustainable agriculture natural biofungicides are safe and pro environment. Since most of the synthetic fungicides harm the ecosystem to some extent, their usage should be restricted and safer strategies of biological control techniques may be promoted. Present research findings suggest the potential of S. *lavendulae* isolate 21 against *P. aphanidermatum* for development as biofungicide.

REFERENCES

Ghany, A., Bouthaina, F., Arafa, R., Rahmany, E. L., Tomander, A., and Shazly, M. 2010. Effect of soil microorganisms on soil properties and wheat production under North Sinai conditions. *Journal of Applied Sciences Research*, **4**: 559–579.

Acar, J. F. and Goldstein, F. W. 1996. Disk susceptibility test. In: *Antibiotics in Laboratory Medicine*. 4th Edn. William and Wilkins Co, Baltimore, 51 pp.

Bharat, N. K. and Bhardwaj, L. N. 2001. Interactions between VA mycorrhizal fungi and *Dematophora necatrix* and their effect on health of apple seedlings. *Indian Journal of Plant Pathology*, **19**: 47–51.

Dhingra, A. D. and Sinclair, J. B. 1995, pp. 287–296. In: *Basic plant pathology methods*. CRC Press, USA.

Fruh, T., Chemla, P. Ehrler, J. and Farooq, S. 1996. Natural products as pesticides: Two examples of stereoselective synthesis. *Pesticide Science*, **46**: 37–47.

Gottlieb, D. 1973. General consideration and implications of the Actinomycetes, pp. 1–5. In: Sykes, G. and Skinner, F. A. (Eds.). *Actinomycetales: Characteristics and Practical Importance*, Academic Press, London, UK.

Keiser, T., Bibb, M. J., Buttner, M. J., Chater, K. F. and Hopwood, D. A. 2000. General introduction to actinomycete biology, pp.1–31. In: *Practical Streptomyces Genetics*. John Innes Foundation, Crowes, Norwich, England.

Knight, S. C., Anthony, V. M., Brady, A. M., Greenland, A. J., Heaney, S. P., Murray, D. C., Powell, K. A., Schulz, M. A., Sinks, C. A., Worthington, P. A. and Youle, D. 1997. Rationale and perspectives on the development of fungicides. *Annual Review of Phytopathology*, **35**: 349–372.

Kuster, E. and Williams, S. T. 1964. Selection of media for isolation of *Streptomyces*. *Nature*, **202**: 928–929.

Lee, J. Y. and Hwang, B. K. 2002. Diversity of antifungal actinomycetes in various vegetative soils of Korea. *Canadian Journal of Microbiology*, **48**: 407–417.

Locci, R. 1989. *Streptomycetes* and related genera, pp. 2451–2469. In: *Bergey's manual of systematic Bacteriology*, Vol. **4** (Williams ST edition), Williams and Willkins, London, UK.

Nawani, N. N. and Kapadnis, B. P. 2004. Production dynamics and characterization of the chitinolytic system of the *Streptomyces* sp. NK1057, a well equipped chitin degrader. *World Journal of Microbiology and Biotechnology*, **20**: 47–494.

Omura, S., 1986. Philosophy of new drug discovery. *Microbiologica Review*, **50**: 259–279.

Pirous, T., Karbasian, M. A. and Goodfellow, M. 1999. Isolation of some aerobic *Actinomycetes* species from the soil of Zahedan country, South East of Iran. *Iran Journal of Medical Sciences*, **24**: 65–67.

Pridham T. G. and Lyons, A. J. 1980. Methodologies for *Actinomycetes* with special reference to *Streptomyces* and *Streptoverticella*, pp. 169-170. In: Dietz, A. and Thayer, D. W. (Eds.). *Actinomycete taxonomy.* SIM Special publication nember 6.

Rangaswamy, G. 1958. *Diseases of crop plants in India*. Prentice hall of India Pvt. Ltd., New Delhi, 504 pp.

Ratnam, S. and Traquair, J. A. 2000. Formulation of a *Streptomyces* biocontrol agent for the suppression of *Rhizoctonia* damping – off in tomato transplants. *Biological Control*, **23**: 245–253.

Saadoun, I. and Gharaibeh, R. 2002. The *Streptomycetes* flora of Jordan and its potential as a source of antibiotics active against antibiotic-resistant Gram-negative bacteria. *World Journal of Microbiology and Biotechnology*, **18**: 465–470. Shahidi Bonjar, G. H. 2003. *In vitro monitoring of antibacterial properties of soil Streptomyces*. Research project report. Department of Plant Protection, College of Agriculture, Bahonar University of Kerman, Iran. 45 p.

Shahidi Bonjar, G. H., Fooladi, M. H., Mahdavi, M. J. and Shahghasi, A. 2004. Broadspectrum, a novel antibacterial from *Streptomyces* spp. *Biotechnology*, **3**: 126–130.

Shahidi Bonjar, G. H. 2004. New approaches in screening for antibacterials in plants. *Asian Journal of Plant Sciences*, **3**: 55–60.

Shah Smith, D. A. and Burns, R. G. 1996. Biological control of damping off of sugar beet by *Pseudomonas putida* applied to seed pellets. *Plant Pathology* **45**: 572–582.

Shirling, E. B. and Gottlieb, D. 1966. Methods for characterization of *Streptomyces* species. *International Journal of Systemic Bacteriology*, **16**: 313–340.

Subhashini, D. V. and Padmaja, K. 2009. Isolation of *Streptomyces* from tobacco soils that show antimicrobial activity. *Journal of Biological Control*, **23**: 417–419.

Williams, S. T. and Cross, T. 1971. Isolation, purification, cultivation and preservation of *Actinomycetes*. *Methods in Microbiology,* **4**: 295–334.

Williams, S. T. and Davis, F. L. 1965. Use of antibiotics for selective isolation and enumeration of *Actinomycetes* in soil. *Journal of General Microbiology*, **38**: 251–262

Yella, R. R., George, G. and Phoolan, A. K. 2006. A simple technique for producing oospores in *Pythium myriotylum*, causing soft rot of ginger. *Indian Phytopathology,* **59**: 368–369.