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Research Article

CHARACTERIZATION OF ACTINOMYCETES AGAINST PHYTOPATHOGENIC FUNGI OF *GLYCINE MAX* (L.)

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ABSTRACT

Objective: This study was conducted to evaluate the antifungal activity of actinomycetes.

Methods: The cross-streak plate method and agar well methods were used for the screening of actinomycetes for determination of antifungal activity of actinomycetes against phytopathogens of the soybean crop.

Results: A total of 80 strains of actinomycetes were isolated from the soils of different habitats of Chambal region, Madhya Pradesh, evaluated for their ability to inhibit plant pathogens, i.e., *Macrophomina phaseolina*, *Fusarium oxysporum*, *Colletotrichum truncatum,* and *Rhizoctonia solani in vitro*. Entire isolates were screened for their antifungal activity by agar well method against phytopathogenic fungi. After screening, out of these, only one actinomycetae ACITM-1 showed antifungal activity against *M. phaseolina, F. oxysporum, R. solani,* and *C. truncatum.*

Conclusion: This study proves that actinomycetes isolated from soil have good antifungal activity against the fungal pathogens of the soybean crop.

Keywords: Antifungal activity, Saprophytic, Phytopathogens, Soybean, Metabolite.

INTRODUCTION

Most of the actinomycetes described are soil micro-organisms and are active in the decomposition of plant tissue, and thereby in the recycling of carbon and nitrogen [1]. Actinomycetes are diverse group of Gram-positive bacteria that usually grow by filament formation. They belong to the order actinomycetales [2]. They are free-living, saprophytic bacteria, and a major source for the production of antibiotics [3], widely distributed in natural and manmade environments and play an important role in the degradation of organic matter [4,5]. Micro-organisms are virtually unlimited sources of novel compounds with many medicinal and agricultural applications. Actinomycetes, among them, hold a prominent position due to their ability to produce numerous different metabolites such as antibiotics, enzymes, and inhibitors [6]. Actinomycetes are known to be greatest sources of bioactive metabolites [7].

Fungal phytopathogens pose serious problems worldwide in the cultivation of economically important plants. In addition, many also produce mycotoxins, which are harmful to humans and livestock. Biological control has been described as a nonhazardous strategy to reduce crop damage caused by plant pathogens when compared to the exclusive use of the chemical control of plant diseases. Thereby, reducing the use of agrochemicals and also maintains the crop productivity without damage to the ecosystem. Several rhizospheric bacteria and actinomycetes are used as biological control agent. They have provided many important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive compounds. Therefore, actinomycetes hold a prominent position due to their diversity and proven ability to produce new compounds.

METHODS

Collection of soil samples

Soil samples were collected from different sites such as garden, agricultural field, playground, and poultry farms. Soil samples were carefully taken with spatula down to a 10 cm depth into the soil. The samples were stored in sterile plastic bags then labeled and stored at 4°C until use. All the samples were dried at room temperature before isolation.

Isolation of actinomycetes

Actinomycetes were isolated by serial dilution technique on different media such as starch casein agar (SCA), soil extract agar, International *Streptomyces* Project medium, nutrient agar, glycerol asparagine agar, starch agar, yeast extract malt extract agar, actinomycetes isolation agar media, and incubated for 6-7 days at 30°C [8]. The isolated actinomycetes culture were further purified on respective fresh media and stored in biochemical oxygen demand incubator for further use.

Isolation of test phytopathogens

The fungal pathogens *Rhizoctonia solani*, *Fusarium solani*, *Macrophomina phaseolina, Sclerotium rolfsii,* and *Colletotrichum truncatum* were isolated originally from different naturally diseased plant leaves collected from different soybean agricultural fields in Gwalior. The isolated fungi were grown on potato dextrose agar (PDA) plates and incubated at 28°C for 4-6 days. Detected isolates were then transferred into slant of PDA and kept at 4°C for further studies. Pure cultures of the isolated fungi were identified according to the cultural properties, morphological, and microscopical characteristics of each fungus [9-11].

Screening for antifungal activity of actinomycetes

Among 80 isolated actinomycetes, only one actinomycete (ACITM-1) was selected on the basis of screening. All isolates were screened for their *in vitro* antifungal activity against pathogenic fungi. The screening was done on the basis of the primary and secondary method. Primary screening was done by the cross-streak plate method [12]. In this method, first prepared the PDA media and poured into the plate then transferred the fungi at the corner of the plate and put actinomycetes at another corner of the plate and incubated it for 4 days at 37°C [13,14].

Secondary screening was done by the well agar diffusion method. In this method, two different types of media were prepared, i.e., starch casein broth and PDA then selected ACITM-1 were inoculated into two flasks of starch casein broth. Flasks were incubated for 5 days in orbital

shaker then prepared the PDA media and made two wells in each plate using well cutter and each well was loaded with 150 µl of starch casein broth in the wells and put all tested fungi in each plate. Plates were incubated for 6-7 days at 25°C. A dead zone was formed around the well [15].

Cultural, morphological and biochemical characteristics of actinomycetae (ACITM-1)

Cultural characteristics such as color of aerial mycelium, color of substrate mycelium, and pigmentation of the selected actinomycetes isolates were recorded on starch agar medium and SCA medium. Gram-staining was done for morphological characterization [16,17].

Biochemical characterization

After preliminary studies, the isolate which was found to be positive was selected for biochemical studies. Biochemical tests generally used were gelatin hydrolysis, starch hydrolysis, acid production from different sugars, hydrogen sulfide production test, motility test, citrate utilization test, indole test, methyl red test, Voges-Proskauer test, and catalase test [18-20].

Molecular characterization and phylogenic analysis

The selected isolate ACITM-1 was subjected for 16S rRNA molecular sequencing. The genomic DNA was isolated, extracted, and amplified using high fidelity PCR. PCR product was sequenced bi-directionally using the 16S primers. The 16s rRNA gene fragment was amplified using universal primers (forward primer-5'-GCCTAACACATGCTGG-5' and reverse primer-5'-GTATTACCGCGGCTGCTGG-5') analyzed by performing basic local alignment search tool (BLAST) search tool. The cluster omega was used for phylogenetic and molecular evolutionary analysis [21,22].

RESULTS

Isolation and screening of actinomycetes

Out of 80 isolated actinomyecetes only actinomycete, ACITM-1 was selected which produced secondary metabolite against phytopathogenic fungi. The maximum and fast growth of selected actinomycetes were found on SCA media (Table 1).

Cultural, morphological, physiological, and biochemical characteristics of isolates

Morphological and biochemical characterization

Based on morphological and biochemical characteristics as shown in Tables 2-4, isolate ACITM-1 was to be closely related to be genus *Streptomyces.* This Gram-positive organism has of gray aerial mycelia and white substrate mycelium. When observed under the scanning electron microscope (SEM) appeared rod-like structures (chains of cells) and often branched to form a network of filaments (mycelium), smooth surface of the spore (5 µm in length) developed on the terminal of the aerial mycelium (Figs. 1 and 2).

Molecular identification and phylogenic analysis

The 16S rRNA gene sequences from strains closely related to *Streptomyces* sp. were retrieved from the GeneBank database using BLAST. The phylogenetic tree in Fig. 3 shown that *Streptomyces* ACITM-1 formed a close distinct line with clade encompassed by *Streptomyces chilikensis* strain.

Determination of the antifungal activity

The antifungal activities of the actinomycetes isolate against fungal pathogens were showed in Table 4. This study showed that the ACITM-1 isolate has good antifungal activity against *M. phaseolina, F. oxysporum, R. solani, and C. truncatum,* respectively, whereas there was no activity against *S. rolfsii* (Table 5).

DISCUSSION

Very few studies of isolating actinomycetes with antimicrobial potential have been reported from this part of the world, of which actinomycetes

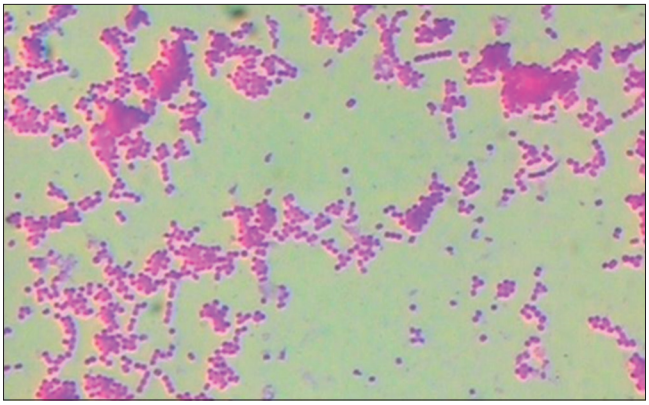

Fig. 1: Microscopic view of actinomycetes under ×100 resolution

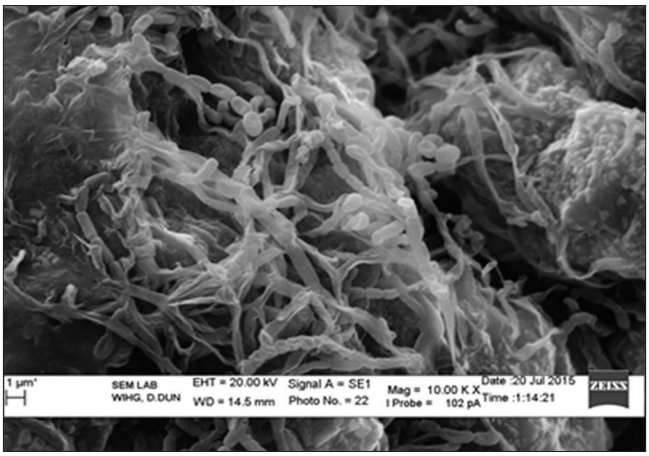

Fig. 2: Scanning electron microscopy of actinomycetes (ACITM-1)

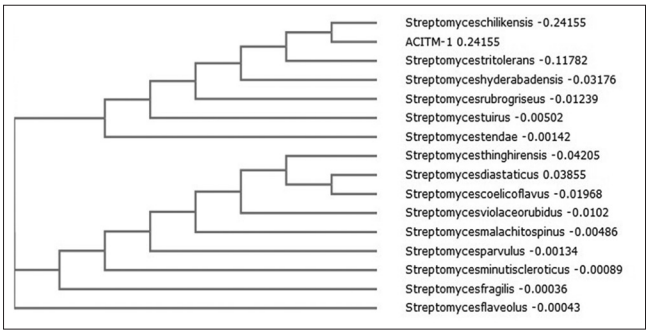

Fig. 3: Phylogenic tree based on 16s rRNA gene sequence showing relationship between ACITM-1 and related members of the genus *Streptomyces*

with antagonistic potential were collected mainly from Gwalior-Chambal region. Total 80 actinomycetes from the soil of different areas of this region were isolated. All actinomycetes screened for antifungal activity against *R. solani*, *F. solani*, *M. phaseolina, C. truncatum,* and *S. rolfsii.* Kumar and Kannabiran (2010) used different media for isolation of actinomycetes, and among the three different media, the SCA was proved to be effective for isolation [23]. In total, 7 different media were used in this study for the isolation of actinomycetes, among them the SCA proved to be very effective in isolation of actinomycetes when compared to other media.

Screened isolate had inhibition zones of 0-22 mm against the fungal pathogens of soybean. The ACITM-1 isolate was shown to be the most inhibitive to *M. phaseolina.* The Gram-staining was performed for the

Table 1: Cultural characteristics of the actinomycetes ACITM-1 on different ISP media

Media used	Growth	Aerial mycelium	Substrate mycelium
Yeast extract malt agar	Good	Grey	White
Starch agar	Good	Grey	White
Starch casein agar	Excellent	Grey	White
Soil extract agar	Good	Grey	White
ISP medium	Good	Grey	White
Actinomycetes isolation agar	Good	Grey	White
Glycerol asparagines agar	Good	Grey	White

Table 2: Morphological characteristics of ACITM-1

Morphological characteristics	ACITM-1
Hyphae	Present
Mycelium	Present
Gram staining	+ve
Spore chain	Spiral
Spore mass colour	White
Substrate mycelium colour	Yellow

Table 3: Biochemical characteristics of ACITM-1

Biochemical characterization	ACITM-1
Citrate utilization test	+ve
Hydrogen sulfide production test	-ve
Catalase production test	+ve
Nitrate reduction test	+ve
Gelatin liquefaction test	-ve
Casein hydrolysis test	+ve
Starch hydrolysis test	-ve
Methyl red	+ve
Voges-proskauer test	-ve
Indole production test	+ve

Table 4: Utilization of different carbon sources by ACITM-1actinomycetes

Carbohydrate test	ACITM-1
Glucose	AG
Xylose	G
Fructose	AG
Arabinose	AG
Sucrose	G
Ribose	G
Galactose	G
Maltose	AG
Rhaminose	G
Raffinose	G

AG: Acid gas, G: Gas

Table 5: Antifungal activity of actinomycetes of ACITM-1

Fungi	Zone of inhibition (mm)
Macrophomina phaseolina	22
Collectotrichum truncatum	16
Fusarium oxysporum	20
Rhizoctonia solani	18
Sclerotium rolfsii	-

morphological view which conformed the spore chain formation of actinomycetes and the spore chains were spiral type and each had more than 15 spores per chain which was observed by Augustine, *et al.* [24] and the SEM confirms the smooth spore chain formation colonies of actinomycetes [25]. The morphological, physiological, and biochemical characteristics of ACITM-1 classified it into *Streptomyces* spp. The 16S rRNA gene sequence classified ACITM-1 as *S. chilikensis.* Many researchers have shown that *Streptomyces* spp. produce valuable bioactive metabolites with broad spectrum activities such as antibacterial, antifungal, antibiotic, antiparasitic, antitumor, antiviral, insecticidal, and herbicidal [26,27]. Future studies are needed to characterize the secondary metabolites of ACITM-1 isolate and its antagonistic mechanisms to fully extend its potential as a biocontrol agent for phytopathogens of glycine max and other plant pathogens.

CONCLUSIONS

The present research was that actinomycetes showed significant antagonistic activities against important phytopathogenic fungi of soybean and reported the first time from soil of chambal region. It is suggested that further studies on actinomycetes present in the chambal region's soil could provide novel species as well as novel bioactive compound. It has been observed that the isolated actinomycete has inhibited the growth of four test phytopathogenic fungi. It is expected that the current attempt of isolation, characterization and the study on soil actinomycetes may be useful for identification of new antibiotics effective against challenging pathogens. Thus, these promising actinomycetes isolate obtained from the present study may be commercially formulated as effective biocontrol agents for the management soil-borne fungal pathogens of soybean. These isolates were further selected for interaction studies between pathogen *in vivo* under field conditions for effects on plant growth performance, disease incidences and induction of systemic resistance in soybean.

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REFERENCES

1. Thongchai T, Chanaphat S, Wanwikar R, Phutdhawong WS. Antifungal activity of 3-methylcarbazoles from *Streptomyces* sp. LJK109; An endophyte in *Alpinia galangal*. J Appl Pharm Sci 2012;2(3):124-8.
2. Okami Y, Hotta K. Search and discovery of new antibiotics. In: Goodfellow M, Williams ST, Mordarski M, editors. In: Actinomycetes in Biotechnology. London: Academic Press; 1988. p. 37-67.
3. Atta HM, Dabour SM, Desoukey SG. Sparsomycin antibiotic production by *Streptomyces* sp. AZ-NIOFD1: Taxonomy, fermentation, purification and biological activities. J Agric Environ Sci 2009;5(3):368-77.
4. Sateesh V, Patil N, Rathod J. Selective isolation and antimicrobial activity of rare actinomycetes from mangrove sediment of Karwar. J Ecobiotechnol 2011;3(10):48-53.4.
5. Silambarasan S, Kumar EP, Murugan T, Saravanan D, Balagurunathan R. Antibacterial and antifungal activities of *Actinobacteria* isolated from Rathnagiri hills. J Appl Pharm Sci 2012;2(10):99-103.
6. Hamedo HA, Makhlouf AH. Identification and characterization of actinomycetes for biological control of bacterial scab of *Streptomyces scabies* isolated from potato. J Biol Agric Healthc 2013;3(13):142-53.
7. Singh N, Rai V. Optimization of cultural parameters for antifungal and antibacterial metabolite from microbial isolate; *Streptomyces rimosus* MTCC 10792 from soil of Chhattisgarh. Int J Pharm Pharm Sci 2012;4(4):94-101.
8. Kalyani AL, Ramya KM, Annapurna J. Isolation and characterization of antibiotic producing actinomycetes from marine soil samples. Int J Curr Pharm Res 2012;4(2):109-12.
9. Pranee P, Rillapat K. Screening of chitinolytic actinomycetes for biological control of *Sclerotium rolfsii* stem rot disease of chilli. Songklanakarin J Sci Technol 2012;34(4):387-93.
10. Loliam B, Morinaga T, Chaiyanan S. Biocontrol of *Phytophthora infestans* fungal Pathogen of seedling damping off disease in economic plant nursery. Hindawi Publ Corp Psyche 2012;12:1-6.
11. Ara I, Bukhari NA, Perveen K, Bakir MA. Antifungal activity of some actinomycetes isolated from Riyadh soil, Saudi Arabia: An evaluation for their ability to control *Alternaria* caused tomato blight in green house pot trial. Afr J Agric Res 2012;7(13):2042-50.
12. Saxena A, Upadhyay R, Kumar D, Kango N. Isolation, antifungal

activities,characterization of soil actinomycetes. J Sci Ind Res 2013;72(08):491-7.
13. Chaudhary HS, Yadav J, Shrivastava AR, Singh S, Singh AK, Gopalan N. Antibacterial activity of actinomycetes isolated from different soil samples of Sheopur (A city of central India). J Adv Pharm Technol Res 2013;4(2):118-23.
14. Jain PK, Jain PC. Isolation, characterization and antifungal activities of *Streptomyces* sampsonii GS 1322. Indian J Exp Biol 2007;45(2):203-6.
15. Bharti A, Kumar V, Omprakash G, Bisht, GS. Antifungal activity of actinomycetes isolated from Garhwal regions. J Sci Eng Tech Manag 2010;2(2):1-9.
16. Nanjwade BK, Chandrashekhara S, Ali MS, Prakash SG, Fakirappa VM. Isolation and morphological characterization of antibiotic producing actinomycetes. Trop J Pharm Res 2010;9(3):231-6.
17. Raja A, Seetha S, Sharma V. Preliminary studies on antifungal activity of actinomycetes isolated from ice point. Int J Adv Res 2013;1(8):17-22.
18. Aneja KR. Biochemical Activities of Microorganisms, Experiments in Microbiology Plant Pathology & Biotechnology. 4th ed. New Delhi: New age International (p) Ltd Publisher; 2003. p. 245-74.
19. Sazci A, Radford A, Erenle K. Detection of cellulolytic fungi by using Congo red as an indicator: A comparative study with the dinitrosalicyclic acid reagent method. J Appl Bacteriol 1986;61(6):559-62.
20. Jadon R, Pandey S, Singh V. Screening of potential actinomycetes isolates against selective pathogenic microorganism. Int J Adv Technol Eng Sci 2014;2(1):631-42.
21. Mangamuri UK, Muvva V, Poda S, Kamma S. Isolation, identification and molecular characterization of rare actinomycetes from mangrove ecosystem of Nizampatnam. Malays J Microbiol 2012;8(2):83-91.
22. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 2007;24(8):1596-9.
23. Kuma RS, Kannabiran K. Antifungal activity of *Streptomyces* VITSVK5 spp against drug resistant *Aspergillus* clinical isolates from pulmonary tuberculosis patients. J Mycol Med 2010;20:101-7.
24. Augustine SK, Bhavsar SP, Basrisalehi M, Kapadnis BP. Isolation characterization and optimization of antifungal activities of an actiomycetes of soil origin. Ind J Exp Biol 2004;42(9):928-32.
25. Kokare CR, Mahadik KR, Kadam SS. Isolation of bioactive marine actinomycetes from sediments isolated from Goa and Maharastra Coast lines (West coast of India). Ind J Mar Sci 2004;33(3):248-56.
26. Baltz RH. Renaissance in antibacterial discovery from actinomycetes. Curr Opin Pharmacol 2008;8(5):557-63.
27. Liu H, Qin S, Wang Y, Li W, Zhang J. Insecticidal action of Quinomyci A from *Streptomyces* sp KN-0647, isolated from a forest soil. World J Microbiol Biotechnol 2008;24:2243-8.