8

Potential of actinomycetes for the biocontrol of *Phytophthora* foot rot in black pepper (*Piper nigrum* L.)

Suseela Bhai, R., Prameela, T.P., Mahantesh, V. and Anandaraj, M.

INTRODUCTION

Foot rot disease of black pepper caused by *Phytophthora capsici* is a major threat to black pepper. Chemical measures to combat the disease are widely adopted but recurrent applications are required to save the crop. Hence it is always advisable to go for long term strategies such as biocontrol methods which would be ecofriendly and economical. Among the biocontrol agents, actinomycetes have great scope because of their potential in producing bioactive compounds or enzymes which are known for cell wall degradation or antibiosis. Actinomycetes are gram positive bacteria belonging to the order Actinomycetales and are the most important group of secondary metabolite producers. Among the genera, Streptomyces are the major producers of antibiotics and about half of the 10,000 known antibiotics are produced by species of *Streptomyces* (Prashith *et al.*, 2010). Actinomycetes are reported to produce enzymes such as cellulase, xylanase, amylase, lipase, collagenase, protease, chitinase lignase, *etc.* and their biological activities include antibacterial, antifungal, antiprotozoal, antihelmintic, antiviral, insecticidal, antioxidant, cytotoxic and anti-inflammatory (Prashith *et al.*, 2010). The beneficial activities of the actinomycetes also include their biocontrol potential against pathogens of crop plants. Polyene antimycotics, are class of antimicrobial polyene compounds obtained from some species of *Streptomyces* sp. Amphotericin B, nystatin, and natamycin are examples of some of the polyene antimycotics. There are several attempts to exploit the antifungal activity of actinomycetes against fungal pathogens such as *Fusarium*, *Verticillium*, *Botrytis*, *Colletotrichum* and even *Phytophthora* (Long *et al.*, 2009, Prashith *et al.*, 2010, Intra *et al.*, 2011,) *Ref.*). In this study, an attempt was made to exploit the biocontrol potential of actinomycetes towards *P. capsici*, the soil born oomycetes pathogen causing foot rot disease of black pepper.

METHODOLOGY

Isolation of actinomycetes

Soil samples were collected from black pepper and ginger growing tracts of Kerala and Karnataka. The samples were air dried for 12 days. 1 g of air dried soil was suspended in 99 ml sterile distilled water and incubated on a rotary shaker (220 rpm) at 30 °C for 1-2 h and the suspension was incubated in a water bath for 10-15 min at 50 °C. This suspension was subjected to dilution plating in Actinomycete Isolation Agar which contains sodium caseinate - 2.0 g/L, L-asparagine - 0.10 g/L, sodium propionate - 4.0 g/L, di-potassium phosphate - 0.5 g/L, $MgSO_4$ - 0.10 g/L, $FeSO_4$ - 0.001 g/L, agar - 15.0 g/L at pH- 8.1 ± 0.2 and incubated for 10-12 days at 28 °C. Thick, hard and comparatively small discrete colonies characteristics of actinomycetes that appeared on the plate was selected, subcultured and maintained on slants of the same media (Barreto *et al.*, 2008) for further studies.

Evaluation of antagonistic (antinomycete) activity

For evaluation of antagonistic (antioomycete) activity, the actinomycetes isolates were streaked onto PDA plates on both sides at a distance of 3 cm from the periphery and incubated for 5 days. On the 5th day, 5 mm mycelial plugs from 72 h old culture was cut from the periphery of the test organisms, *viz.* three different isolates of *P. capsici* and one isolate each of *P. plmivora* and *P. parasitica* and were placed at the centre of the actinomycete inoculated plate and incubated again at 24 ± 1 °C for 72-96 h. A control plate for each test organism was simultaneously incubated and the radial growth of the test organisms was measured and compared with control and the percentage inhibition was calculated.

Enzymatic screening

Cellulase activity

All the nine isolates were screened for their cellulase producing ability in carboxymethyl cellulose agar medium ($NH_4H_2PO_4$-1 g/L, KCl 0.2 g/L, $MgSO_4.7H_2O$-1 g/L, Yeast extract-1 g/L, carboxymethyl cellulose-26 g/L, Agar 3 g/L). This was done by streaking the actinomycetes cultures in two parallel lines in the media and incubated for 5 days at 28 °C. After incubation the carboxymethyl cellulose agar medium containing actinomycetes was flooded with 0.1 per cent Congo-red solution and left for 15 min, then washed with 1 M NaCl solution and observed for the clear zone around the streak lines.

Amylase activity

All the nine isolates were screened for their amylase producing ability in starch agar medium (Peptone-5g/L, Beef extract-3g/L, Soluble starch-4g/L, Agar-15g/L). As done above, the actinomycetes cultures were streaked in two parallel lines in the media and incubated for 5 days at 28 °C. After incubation the starch agar media was flooded with Gram's iodine solution and observed for the clear zone around the streak lines.

Morphological and cultural characterization

Morphological and cultural characters of the selected actinomycetes were studied by inoculating the isolates into sterile starch casein agar (SCA) media containing soluble starch-10.0 g/L, casein-0.3 g/L, KNO_3-2.0 g/L, $MgSO_4$ $7H_2O$-0.05 g/L, $CaCO_3$-0.02 g/L, $FeSO_4.H_2O$-0.01 g/L, KH_2PO_4-2.0, agar-17.0, pH-7.0 ± 0.2. The medium was sterilized and poured into sterile petri dish. After solidification of the media the culture of the selected strains were streaked on the media surface aseptically and incubated at 27 °C for 7 days. Morphological characteristics such as colony characteristics, type of aerial hyphae, pigmentation etc. was observed.

Microscopic characterization

Gram staining

Seven day old cultures of actinomycetes were used for microscopic characterization. Smear of the cultures were prepared on a clean glass slide and allowed to air dry and then heat fixed. The heat fixed smear was flooded with Gram's crystal violet solution and after one minute it was washed with water and flooded with mordant Grams iodine solution. The smear was decolourised with 95 per cent ethanol, washed with water and counter stained with safranin (0.5 per cent) for 45 seconds. This was washed with water and dried and examined under oil immersion. The isolates were morphologically identified using Bergey's Manual of Determinative Bacteriology (Robert *et al*., 1954).

RESULTS AND DISCUSSION

A total of 30 isolates were obtained in dilution plating which was purified and maintained. The isolates varied in their colony morphology and color of the aerial mycelium and pigmentation in the medium (Fig. 1). All the isolates were tested *in vitro* against *Phytophthora* isolates from black pepper and nine isolates with more than 50 per cent inhibition were short listed (data not shown). These nine short listed isolates were further tested against three *P. capsici* isolates (stem, leaf and root isolates) and one isolate each of *P. palmivora* and *P. parasitica*. The isolates showed varying degrees of inhibition towards different *Phytophthora* isolates. The mean inhibition ranged from 31.99-90.70 per cent (Table 1). Among the isolates Act 1 was found highly inhibitory to all the isolates showing more than 50 per cent inhibition (60-90 per cent) (Table 1) whereas other isolates differed in their degree of activity. For example. Act 6 showed no inhibition to *P. capsici* 1 and its inhibitory effect ranged from 0-48.52 per cent only (Table 2). Microscopic observation of the dual cultured plates showed disintegration of the cytoplasm (Fig. 3) which indicated the antibiotic nature of the organism by the production of toxic metabolites. Similar work has been done by Intra *et al*. (2011). They isolated 304 actinomycetes from rhizospheric soils and evaluated against *Colletotrichum capsici* and *Saccharomyces cerevisiae* to short list 54 isolates active against the organism.

Screening for enzymatic activity revealed that all the isolates except Act 6 were amylase positive whereas none of them were found cellulase positive. Morphological characteristics such as colony characteristics, type of aerial hyphae, pigmentation etc are given in Table 2. Gram staining revealed the gram positive nature of actinomycetes. Microscopic examination of the slide smear showed hook like hyphae characteristic of *Streptomyces* species in most of the isolates (Fig. 2). The isolates were tentatively identified as belonging to the family Streptomycetaceae of Actinomycetales.

Among the enzymes studied, cellulase activity is important since the oomycetes pathogen *P. capsici* contains D-glucan and cellulose instead of chitin as constituents in their cell wall. However none of the isolates in the present study were found cellulase positive. Similarly the production of amylase

Table 1: *In vitro* inhibitory effect (%) of short listed Actinomycetes against species of *Phytophthora*

Actinomycetes	*P. cap* 110-03	*P. cap* 298-165	*P. cap* 398-93	*P. palmivora* 98-01	*P. parasitica* 99-188
Act1	87.50	89.69	69.67	86.30	60.00
Act2	81.00	85.29	63.60	81.11	46.66
Act3	73.40	76.53	43.83	73.07	41.17
Act4	78.72	75.51	77.21	74.35	29.41
Act5	66.67	57.62	39.34	56.18	39.02
Act6	0.00	16.69	48.52	10.67	29.95
Act7	87.50	89.69	44.02	82.02	73.17
Act8	62.50	20.00	39.34	60.67	39.02
Act9 (VC11)	90.70	92.85	31.99	80.89	58.53
CD at 5% = 1.37					

Table 2: Colony characteristics of short listed Actinomycetes

Actinomycetes isolates	Aerial mass colour	Soluble pigments	Reverse side pigments	Melanoid pigments	Cellulase	Amylase
Act1	White changing to grey	+	Grey	+	-	+
Act 2	White to creamy	-	Creamy	-	-	+
Act3	Grey	-	-	-	-	+
Act4	White to grey	-	White	-	-	+
ACt5	Grey	-	Off white	-	-	+
Act6	Grey	-	Yellowish	-	-	-
Act7	White to grey	-	Grey	-	-	+
Act8	Off white grey	-	Off white	-	-	+
Act9	Yellow to white	-	Yellow	-	-	+

enzyme may be helpful for the utilization of naturally occurring starch thus enhancing the saprophytic ability of the organisms (Janice *et al*., 2003). The amylase activity was shown by all the isolates except isolate Act 6, which indicated the competitive saprophytic ability of these isolates and is supported by the reports of Jeffrey (2008) and Prashith *et al*. (2010).

The inhibitory effect of the tested actinomycetes towards *P. capsici* may be due to secondary metabolites produced by the isolates which are toxic to the pathogen. Prashith *et al*. (2010) gave a detailed review of the fascinating diversity and potential biological activities of metabolites produced by various actinomycetes. This includes a macrolide antibiotic Brasilinolide A, produced by *Nocardia brasilliensis* active agaisnt *Aspergillus niger* and another complex polyene antibiotic produced by *Streptomyces* species active against *Botrytis cinera*. Others are Oligomycin A isolated from *Streptomyces libani* that showed high level activity against pathogenic fungi and Bifilamycin B1 and C1 produced by *S. halsteadii* K122. Similarly isochainin from an actinomycete strain designated as Ap1 was found inhibitory to *F. oxysporum* f. sp *albedenis* and *Verticillium dahliae* (Prashith *et al*., 2010). Nanjwade *et al*. (2010) also isolated and characterized antibiotic producing actinomycetes.

The actinomycetes isolates shortlisted in the present study were found to be highly effective in inhibiting different isolates of *P. capsici* from black pepper stem, leaf and root

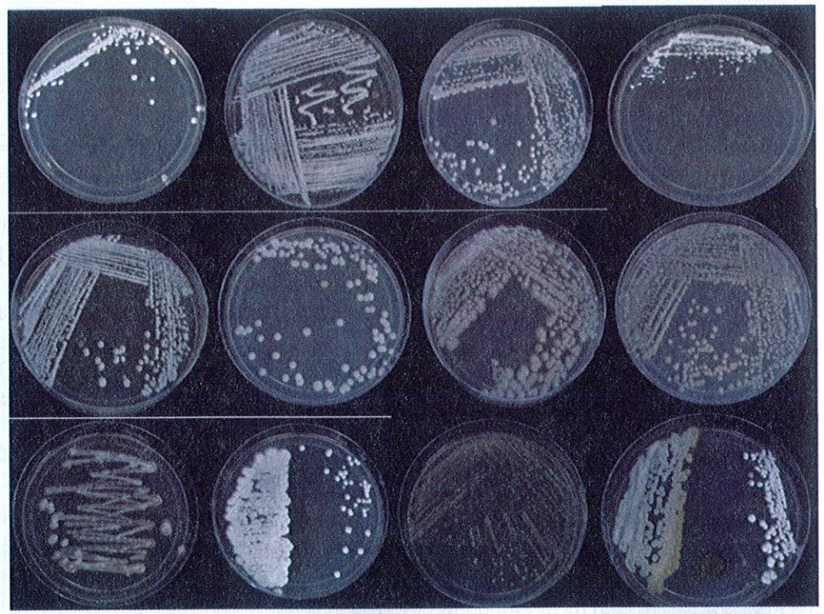

Fig. 1: Morphology of actinomycetes.

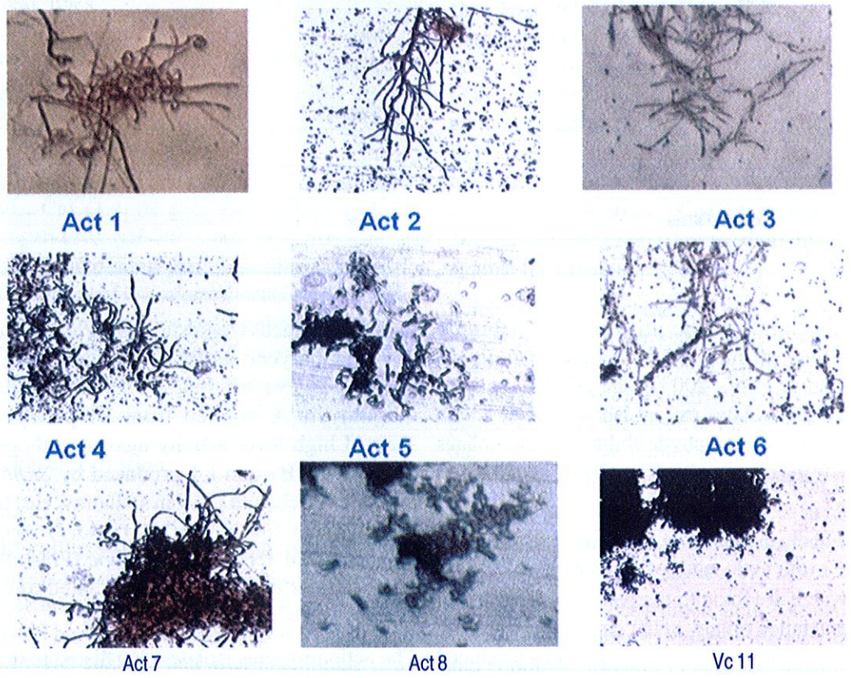

Fig. 2: Microscopic characteristics of short listed isolates.

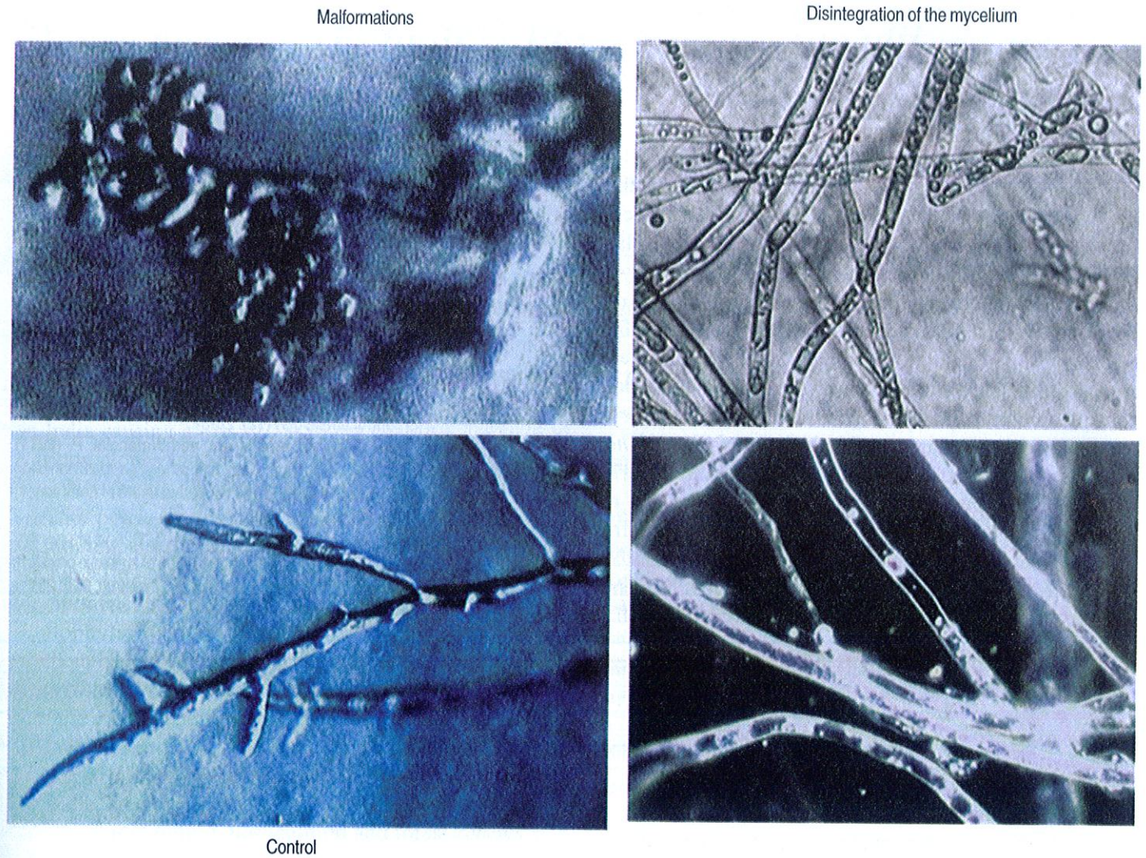

Fig. 3: Mode of inhibition (*Antibiosis*) of pathogen.

and also *P. palmivora* and *P. parasitica*. The *in vitro* inhibition clearly revealed that the antagonistic activity is due to antibiosis. These potential actinomycetes isolates were identified based on the morphological and microscopic characters as belonging to the family III Streptomycetaceae of Actinomycetales. Further study is warranted to effectively utilize these actinomycetes for combating diseases under field conditions and also against other soil borne pathogens of spice crops.

SUMMARY

Actinomycetes are an enormous reservoir for antibiotics and bioactive metabolites, and many are found as excellent biocontrol agents against plant pathogens. Antifungal activity of actinomycetes isolated from different parts of Kerala and Karnataka has been studied. The cultures were isolated using selective media. A total of 30 actinomycetes were isolated and subjected to preliminary screening *in vitro* by dual culture method against *Phytophthora capsici* from black pepper. The degree of antifungal activity varied greatly among the isolates. Nine isolates were shortlisted based on percent inhibition of the pathogen. These were further tested for their antagonism against five isolates of *Phytophthora* which included three isolate of *P. capsici* (soil, root and leaf isoltes) and one isolate each of *P. palmivora* and *P. parasitica*. All the *Phytophthora* isolates were from black pepper. The percentage inhibition of *Phytophthora* sp. by these actinomycetes ranged up to 89.69. Screening for enzymes *viz*. amylase and cellulase showed that all the shortlisted isolates except Act 6 were amylase positive whereas none of them were found cellulase positive. The potential of these isolates can be exploited for biocontrol of *Phytophthora* foot rot in black pepper.

REFERENCES

Barreto, T.R., Silva, C.M.D., Soares, A.C.F. and de Souza, J.T. (2008). Population densities and genetic diversity of actinomycetes associated to the rhizosphere of Theobroma cacao. *Brazilian Journal of Microbiology* **39:** 464-470.

Macagnan, D., Romeiro, R.D.S., de Souza, J.T. and Pomella, A.W.V. (2006). Isolation of actinomycetes and endospore-forming bacteria from the cacao pod surface and their antagonistic activity against the witches' broom and black pod pathogens. *Phytoparasitica*, **34:** 122-132.

Jeffrey, L.S.H. (2008). Isolation, characterization and identiifcation of actinomycetes from agriculture soils at Semongok, Sarawak. *African Journal of Biotechnology*, **7**(20): 3697-3702.

Prashith, K., T.R., Shoba, K.S. and Onkarappa. (2010). Fascinating diversity and potent biological activities of Actinomycetes metabolites. *Journal of Pharmacy Research*, **3**(2): 250-256.

Robert, S.B., Murray G.D. and Smith, N.R. (1957). Bergey's manual of determinative bacteriology, seventh edition, The Williams and Wilkins Company, Baltimore.

Long, Y.I., Chong-gang, X., Ying-fang, Z. and Qiong, W.U. (2009). Preliminary report about screening of antagonistic Actinomycetes against *Phytophthora capsici* and its control effect. *J VI* **(12)**: 28-32.

Bungonsiri, I., Isada, M., Takuya, N., Yasuhiro, I. and Watanalai, P. (2011). Identification of actinomycetes from plant rhizospheric soils with inhibitory activity against *Colletotrichum* spp. the causative agent of anthracnose disease. BMC Research News, **4:** 98.

Nanjwade, B., Chandrashekhara, K. Ali, M.S., Prakash, S.G. and Manvi, F.V. (2010). Isolation and morphological characterization of antibiotic producing actinomycetes. *Tropical Journal Pharmaceutical Research*, **9**(3): 231.