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Symbiotic bacteria, *Xenorhabdus* spp. of entomopathogenic nematodes: Source of antifungal compounds against four plant pathogens

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Nematodes that kill the insects are called as Entomopathogenic nematodes (EPNs). These nematodes are in association with symbiotic bacteria (*Xenorhabdus* spp. and *Photorhabdus* spp.) which are known to secrete different bioactive compounds and exhibit broad spectrum biological activities *viz.*, insecticidal, antifungal, antibacterial properties. Entomopathogenic nematodes, *Steinernema* and *Heterorhabditis* were isolated from soil using *Galleria mellonella* baited traps at fourteen locations representing different cropping agro-ecosystems in Gandhi Krishi Vignana Kendra campus, University of Agricultural Sciences, Bangalore, Karnataka, India. Bacteria isolated from nematodes were identified as *Xenorhabdus* spp. and designated as School of Ecology and Conservation one to fourty four. Two symbiotic bacterial cultures, School of Ecology and Conservation-6 and School of Ecology and Conservation-10 were used for *in vitro* evaluation against four plant pathogenic fungi. These two cultures have shown a good insecticidal activity when tested on second instar larvae of diamond black moth *Plutella xylostella* L. under *in vitro* condition, compared to other isolated bacterial cultures. Hence, these cultures were selected to know its fungistatic activity against four plant pathogenic fungi i.e., *Fusarium oxysporum* (Vanilla), *Alternaria solani* (Tomato), *Sclerotium rolfsii* (Brinjal) and *Aspergillus niger* (Groundnut). SEC 6 culture was found to be best for inhibiting the growth of *Sclerotium rolfsii* (82.41) followed by *Aspergillus niger* (51.73), *Fusarium oxysporum* (48.29) and *Alternaria solani* (45.10).

Key words : *Alternaria solani*, *Aspergillus niger*, *Fusarium oxysporum*, *Heterorhabditis* spp, *Sclerotium rolfsii*, and *Steinernema* spp.

INTRODUCTION

A number of biological agents have been developed or are in the process of being developed for controlling some important plant diseases. Secondary metabolites from microbial fermentation offer a good source of bioactive compounds for controlling plant diseases. Soil is the natural habitat for EPNs where they are associated with various insects. They can be extracted from soil by baiting with susceptible insects, a simple and efficient soil sampling baiting technique with *Galleria mellonella*. (Bedding and Akhurst, 1975). The bacteria, *Xenorhabdus* and *Photorhabdus* spp of family Enterobacteriaceae are symbionts of Entomopathogenic nematodes, *Steinernema* (Rhabditida: Steinernematidae) and *Heterorhabditis* (Rhabditida: Heterorhabditidae) known to produce novel secondary metabolites, which have shown broad spectrum of biological properties ranging from antimicrobial to insecticidal property (Chen *et al.,* 1994). In recent days it has become difficult to control many plant pathogenic fungi by conventional/ synthetic fungicides. Because of development of resistance due to heavy selection pressure by synthetic fungicides application by the farmers in disease management. Therefore, in this connection a study was conducted for *in vitro* evaluation of supernatant of symbiotic bacteria against some plant pathogenic bacteria.

MATERIALS AND METHODS

Isolation of plant pathogenic fungi :

The diseased crop plants *viz*., vanilla infected by *Fusarium oxysporum*, tomato by *Alternaria solani*, groundnut by *Aspergillus niger* and brinjal by *Sclerotium rolfsii* were brought to laboratory from the field. The leaf, stem or root tissues of infected plants were cut into small bits of 5-6 mm size and surface sterilized with 0.1 per cent sodium hypochlorite solution prepared using distilled water for one minute and washed repeatedly

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thrice in sterile water to remove traces of sodium hypochlorite and were placed on sterile potato dextrose agar (PDA) plates under aseptic conditions. The plates were incubated in the BOD incubator at temperature of 28±1^{0}C for growth of fungi on the media. The radiated hyphal tips from plant tissues on growth media were transferred to sterile PDA slants. However, pure culture of *Sclerotium rolfsii* was obtained from infected roots of brinjal following above mentioned technique under aseptic condition. All fungi isolated from infected plants were maintained on sterilized PDA medium with antibiotic streptocycline added, pathogenicity test was performed using Koch's postulates.

Isolation of entomopathogenic nematodes (EPN) from soil :

The EPNs were trapped from soil by using Greater Wax Moth, *Galleria mellonella* larvae, a susceptible host following baiting method (Bedding and Akhurst, 1975). The baited traps were prepared using small plastic vials of 50 ml capacity with wire mesh on either sides with final instar larvae (8th instar) of *G. mellonella* inside. The *G. mellonella* baited traps were installed in different locations covering diverse habitats in Gandhi Krishi Vignana Kendra campus, University of Agricultural Sciences, Bangalore, Karnataka, India. The traps were sampled on fourth and seventh day after installation and samples were brought to laboratory for isolation of nematodes. The nematodes from infected *G. larvae* were collected in the distilled water (Fig. 2) following White's trap method (White, 1925).

Fig. 1 : Field installation of *Galleria mellonella* baited trap EPNs from soil

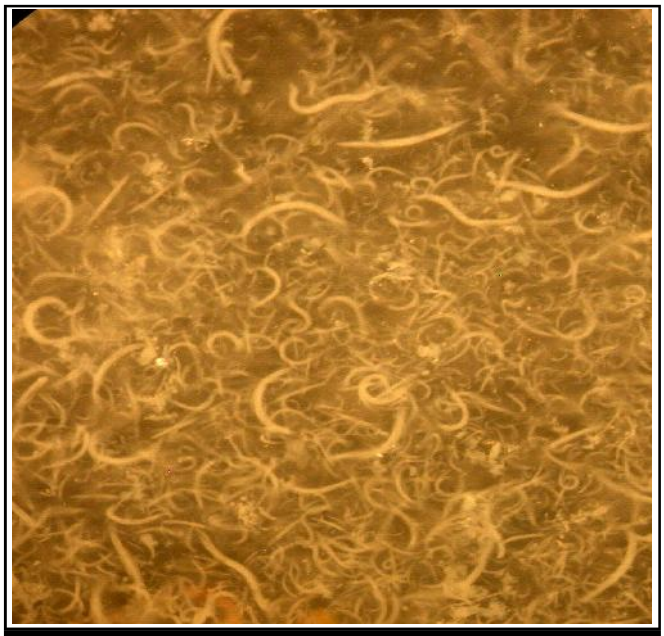

Fig. 2 : Entomopathogenic nematodes released from infected lrvae into the distilled water

Isolation of symbiotic bacteria from entomopathogenic nematodes :

The nematodes collected from field infected larvae of *G.* mellon*ella* were used for isolation of symbiotic bacteria. The infective juveniles were surfaced sterilized with 0.1 % hyamine solution and rinsed 2-3 times with distilled water in order to remove traces of hyamine. Three to five final instar larvae of greater wax moth were released into Petri plate with moist filter paper disc with infective juvenile's suspension and it was incubated at 28±1^{0}C for 2 days. It was monitored constantly for larval mortality and dead larvae were surface sterilized with 70-80% ethyl alcohol and rinsed 3-4 times with sterilised water. Later, the larvae were passed over the flame using sterile forceps for few seconds. They were dissected and a drop of haemolymph from insect cadaver was streaked on sterile plated NBTA medium *i.e.*, (37 g Nutrient agar,

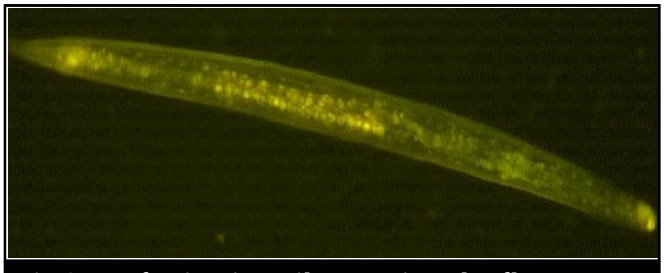

Fig. 3 : Infective juvenile carrying the fluorescence symbiotic bacteria

supplemented with 25 mg Bromothymol blue, 4 ml of 1% 2, 3, 5 Triphenyl–tetrazolium chloride in 1000 ml distilled water). Petri plates were sealed with parafilm and incubated at 28^0 C in the dark for 24 hrs. The primary form of bacteria absorbs bromothymol blue dye and appears blue (Fig. 4). Single colony of the bacterium stained blue were then selected and streaked on to new plates of nutrient agar and sub-cultured continuously until colonies of uniform size and morphology were obtained.

Fig. 4 : Culture of *Xenorhabdus* sp on media

Based on the morphology, colony characters, Gram staining reaction and biochemical analysis, the bacteria isolated from the infective juvenile of entomopathogenic nematodes were identified as *Xenorhabdus* spp. and designated as SEC-1 to SEC-44.

In vitro screening of locally isolated bacterial supernatant against isolated plant pathogenic fungi:

The symbiotic bacterial cultures were grown in nutrient broth for 72 h at 28^0C and at 120 rpm, in the incubator shaker. Later 72 h old grown bacterial cultures in the nutrient broth were centrifuged at 10000 rpm at 4^0C for 15 minutes to obtain supernatant. Fifteen ml bacterial supernatant was incorporated into the 100 ml sterile melted Potato dextrose agar (PDA) medium and poured into sterile Petri plates. Sterile PDA medium plates without bacterial supernatant were maintained as control. Five mm mycelial discs of isolated pure plant pathogenic fungal cultures were placed at the centre of Petri plates on PDA medium and incubated at 28 $\pm 1^0$C for growth of inoculated fungi. Three replications were maintained for each treatment and observation on growth of fungi was recorded 10 days after inoculation. The data on growth of fungi was converted to percentage in each treatment and per cent growth inhibition over standard check was worked out for each treatment (Fig. 5).

Fig. 5 : *In vitro* evaluation of symbiotic bacterial supernatant against four isolated plant pathogenic fungi

RESULTS AND DISCUSSION

The supernatants of two symbiotic bacterial isolates, *Xenorhabdus* spp. and *Steinernema* spp. coded as SEC 6 and SEC 10 were tested for antifungal activity against four plant pathogenic fungi *in vitro*. The symbiotic bacterial isolates, SEC 06 and SEC 10 were found to exhibit potent antifungal activity in the preliminary screening of 44 native symbiotic bacterial isolates (SEC 01 to SEC 44) against plant pathogenic fungi. Therefore, an effort was extended to quantify the antifungal activity of these two potent symbiotic isolates (SEC 06 and SEC 10) against four plant pathogenic fungi *viz., Sclerotium rolfsii, Alternaria solani, Aspergillus niger* and *Fusarium oxysporum*.

Antifungal activity across test plant pathogenic fungi:

Collective mean (±sd) per cent inhibitions of plant pathogenic fungi by supernatants of symbiotic bacterial isolates were found to be significantly different between them ($F_{3,8}$=482.9; p<0.001). Significantly strong per cent growth inhibition was observed in the colonies of fungi *viz., Sclerotium rolfsii* and *Alternaria solani* with collective means of 62.87±1.34 and 44.98±2.46 per cent

Table 1: *In vitro* evaluation of symbiotic bacterial supernatants against four isolated plant pathogenic fungi

Organisms	Bacterial Culture-6 supernatant	Bacterial culture-10 supernatant	Pooled mean
Fusarium oxysporum	48.29±1.52 (47.80±0.05)	13.78±1.96 (29.85±0.21)	31.03±1.76 (38.82±10.36)b
Alternaria solani	45.10±1.81 (53.29±1.49)	44.87±3.12 (53.18±2.02)	44.98±2.46 (53.23±1.45)a
Aspergillus niger	51.73±0.89 (47.49±0.46)	0.15±0.06 (13.63±0.16)	25.94±0.47 (30.56±19.54)c
Sclerotium rolfsii	82.41±1.79 (65.27±1.27)	43.34±0.90 (41.31±0.36)	62.87±1.34 (53.29±13.85)a
Pooled mean	56.88±25.53 (53.46±7.72)a	25.53±1.51 (34.49±15.62)b	
Bacterial isolates		$F_{1,8}$=1375.6; p<0.001	
Test plant pathogenic fungi		$F_{3,8}$=482.9; p<0.001	
Bacterial isolates x Test plant pathogenic fungi		$F_{3,8}$=192.2; p<0.001	

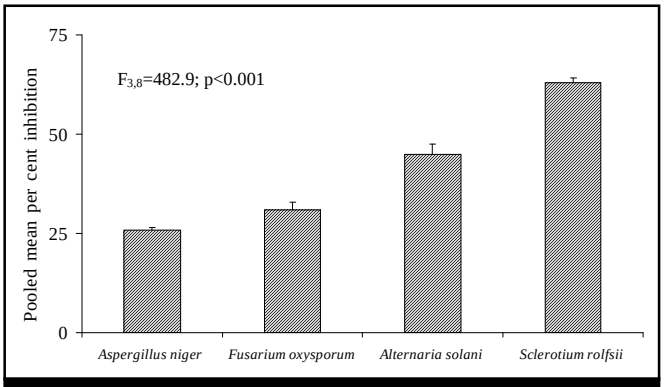

Fig. 6 : Collective mean per cent growth inhibition by supernatants of two symbolic bacterial isolates across plant pathogenic fungi

inhibition, respectively. However, per cent growth inhibition was not significantly different between them. This suggest that two plant pathogenic fungi were found to be susceptible to supernatants of symbiotic bacterial isolates. Similarly, per cent growth inhibition was significantly lower in the colonies of test plant pathogenic fungi, *Aspergillus niger* and *Fusarium oxysporum* showed less susceptibility to supernatants of bacterial isolates with collective mean inhibition of 25.94±0.47 and 31.03±1.76 per cent, respectively and were significantly different each other (Table 1, Fig. 6).

The mean (±sd) per cent growth inhibition of interaction between supernatants of symbiotic bacterial isolates and test pathogenic fungi was also found to be significantly different ($F_{3,8}$=192.2; p<0.001), suggested the susceptibility of *S. rolfsii* to supernatants of both the symbiotic bacterial isolates. This was followed by *Aspergillus niger, Fusarium oxysprum* and *Alternaria solani*.

The symbiotic bacteria, *Xenorhabdus* spp. of entomopathogenic nematodes are known to secrete insecticidal toxins, which are lethal to insect pests (Ffrench-Constant and Bowen, 1999). Apart from insecticidal toxins, a symbiotic bacterium secretes secondary metabolites exhibiting antimicrobial actions (Chen *et al*., 1994). The cumulative per cent mean inhibition of fungal growth was also significantly different among four test plant pathogenic fungi, which indicate that the response of fungi is not uniform to the bioactive compounds of symbiotic bacterial isolates. *Sclerotium rolfsii* was most susceptible to supernatants of bacterial isolates than *Alternaria solani*, which is less susceptible. This clearly explains the existence of diverse bioactive compounds in the supernatants with broad spectrum activity against wide range of pathogens.

Conclusion :

EPNs, symbiotic bacteria and their secondary metabolites are increasingly being viewed as an exhilarating field of research in ecology, biodiversity, evolution, biochemistry and molecular genetics. The symbiotic bacterial isolates produce novel antimicrobial, antifungal and exoenzymes in addition to novel insecticidal toxins, but many of these symbiotic bacterial species and strains of EPNs are still unexplored.

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