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Crude toxin extract from culture filtrate of *Phomopsis azadirachtae* infecting neem and its phytotoxicity

K Girish [1,2,*], **BS Shankara** [1], **KA Raveesha** [1,3]

[1] *Department of Studies in Microbiology, University of Mysore, Mysore, India*
[2] *Department of Microbiology (P.G.), Maharani's Science College for Women, Mysore, India*
[3] *Department of Studies in Botany, University of Mysore, Mysore, India*

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Abstract
Die-back disease of neem caused by *Phomopsis azadirachtae* is presently a major devastating disease of neem resulting in almost 100% loss of fruit production and drastic reduction in evergreen canopy. The crude phytotoxin of *P. azadirachtae* was extracted from culture filtrate using methanol and chloroform. TLC revealed the presence of four compounds in the crude phytotoxin on exposure to UV. The bioassay of the crude phytotoxin against neem seed resulted in complete inhibition of seed germination at both 500 and 1000 ppm of extract. A progressive decrease in the neem callus growth on exposure to increasing toxin concentration along with necrosis of tissues at higher concentrations of toxin was observed. These results suggest the presence of toxic compounds in the culture filtrate of *P. azadirachtae.*

Keywords: Callus, Crude phytotoxin extract, Die-back, Neem, *Phomopsis azadirachtae*, Seed germination, TLC.

INTRODUCTION

Die-back of neem (*Azadirachta indica* A. Juss.) is caused by *Phomopsis azadirachtae* Sateesh and Shankara (1997). The diseased trees show characteristic twig blight symptoms and also inflorescence blight and fruit rot. The pathogen is seed borne (Girish *et al.*, 2007). This disease results in almost 100% loss of fruit production and drastic reduction in evergreen canopy, and thus affects the availability of seeds (Shankara *et al*., 1998). Neem seeds have many medicinal and biopesticidal ingredients and are the major commercial product of neem. The die-back of neem is spreading at an alarming rate reducing the life expectancy and seed production.

Phytotoxins are secondary metabolites produced by plant pathogenic microorganisms (fungi and bacteria) and are low molecular weight substances. They are toxic to plants and play an important role in host-pathogen interactions and in disease expression (Svabova and Lebeda, 2005; Amusa, 2006). During the last decade there was a remarkable development in the studies on the role of fungal toxins in plant pathogenesis. Many fungal metabolites are known to be phytotoxic (Yoder, 1980; Desjardins and Hohn, 1997).

Pathogens are not affected by the same phytotoxins that they produce (Amusa, 2006), but these compounds can cause electrolyte leakage from the host cells and other modes of toxicity to host plants (Mackay *et al*., 1994), thus helping in disease manifestation. Toxic fungal metabolites also induce adverse effects on plants such as suppression of seed germination, malformation and retardation of seedling growth (Lynch and Clark, 1984).

Toxigenic pathogen species are present in all main taxonomic groups of fungi (Svabova and Lebeda, 2005). Production of a wide variety of phytotoxins by many phytopathogenic fungi was reported (Sugawara *et al*., 1998; Geraldo *et al*., 2006). This includes many phytotoxin producing *Phomopsis* spp. (Lanigan *et al*., 1979; Kunwar *et al*., 1987; Avantaggiato *et al*., 1999; Shankar *et al*., 1999). Culture filtrates of many phytopathogenic fungi are known to contain phytotoxic metabolites (Lanigan *et al*., 1979; Haegi *et al*., 1994) and such toxins in culture filtrates of phytopathogenic fungi can be isolated (Bashan *et al*., 1995; Avantaggiato *et al*., 1999). *In vitro* studies on the effect of phytotoxin against host tissues can be carried out using tissue culture (Dahleen and McCormick, 2001;

Corresponding author:
K. Girish, Ph.D.
Department of Microbiology (P.G.),
Maharani's Science College for Women,
JLB Road, Mysore - 570 005, India
Email: girishk77@yahoo.com

Mohanraj *et al*., 2003). Screening of toxicity on seed germination is also applied as a bioassay for phytotoxin and to select resistant varieties (Kunwar *et al*., 1987; Zeng *et al*., 2001; Amusa, 2006).

Toxicity of culture filtrate of *P. azadirachtae* was reported by Sateesh and Shankara (1997) and Fathima (2004). The present investigations were undertaken to isolate the toxic metabolite from the culture filtrate of *P. azadirachtae* and to study its toxicity on neem seed germination and neem callus growth.

MATERIALS AND METHODS

Isolation and culture of the organism

The infected twigs collected from die-back affected neem trees were cut into 2-3 cm pieces including the middle transition region of healthy and infected portions using sterile blades. Healthy twigs served as control. Both healthy and infected twig pieces were washed separately with running tap water for an hour. Then they were cut into 1-1.5 cm segments with the transition zone at the middle portion. The bark was removed and the segments were washed thoroughly with running tap water and surface-sterilized using sodium hypochlorite solution (with 5% available chlorine), then they were rinsed five times with sterile distilled water. The surface-sterilized twig segments were placed in Petri dishes containing potato dextrose agar (PDA, Himedia, Mumbai, India) amended with 100 ppm of chloramphenicol (20 ml per plate), aseptically at the rate of four segments per plate. The inoculated plates were incubated for 10 days at 26±2ºC with 12 h photoperiod and observed for the growth of the pathogen from the twig segments. The incubation was continued for 15 days to allow sporulation. The spores were identified microscopically and the presence of *P. azadirachtae* was confirmed as per Sateesh *et al*. (1997).

Sub-culturing was done using hyphal tips. Mycelial plugs were removed from the margin and transferred on to fresh PDA plates amended with chloramphenicol at 100 ppm. The inoculated plates were incubated for seven days at 26±2ºC with 12 h photoperiod. 100 ml of potato dextrose broth (PDB, Himedia, Mumbai, India) in 250ml Erlenmeyer flasks was inoculated with a mycelial agar disc drawn from advancing margin of seven-day-old cultures. In total, 2.5 l of medium were inoculated. All the flasks were incubated at 26±2ºC with 12 h photoperiod for 25 days. After incubation, the culture medium was filtered through three layers of cheese-cloth and Whatman no.1 filter paper. Culture filtrate thus collected was filter sterilized using 0.45 µm membrane filter discs (Sartorius, Goettingen, Germany).

Extraction of toxin

The culture filtrate was concentrated to 10% of its original volume by using a flash evaporator at 50ºC (Stierle *et al*., 1992). An equal volume of methanol was added to the concentrated solution, and then extracted by chloroform (double the amount of methanol). The chloroform layer was separated in a separation funnel. The extraction with chloroform was repeated twice. The combined chloroform layers were evaporated at room temperature. 914 mg of dark brownish semi-solid crude extract were obtained. The crude extract was dissolved in 9.14 ml of methanol to have a 10% toxin solution. 8.0 ml of this solution were diluted to 160 ml by adding the solution drop-wise to double distilled water with continuous stirring to obtain a stock toxin solution of 5000 ppm having 5% of final methanol concentration (Mackay *et al*., 1994). 1000 ml of PDB was extracted similarly and the extract obtained (483 mg) was dissolved in 4.83 ml of methanol to have a 10% solution. 4.0 ml of this solution was diluted to 80 ml with double distilled water to obtain a stock control solution of 5000 ppm having 5% of final methanol concentration, as mentioned above. These stock solutions were used for further bioassays. The remaining 1.0 ml of crude toxin extract and 0.83 ml of control solution were used for Thin Layer Chromatography (TLC).

Thin Layer Chromatography (TLC)

TLC was employed using microscopic slides and 20 X 10 cm glass plates with gel silica (Qualigens, Mumbai, India), without fluorescence indicators. 10 µl of crude toxin extract was applied in duplicates on slides and developed using different combinations of chloroform: methanol solvent system (5.0:5.0; 5.5:4.5; 6.0:4.0; 6.5: 3.5; 7.0:3.0; 7.5:2.5; 8.0;2.0; 8.5:1.5; 9.0:1.0), then each slide was analyzed under UV light at 365 nm. 10 µl of control solution was also subjected to TLC as above. The R_f was calculated using the formula:

$$R_f = \frac{\text{Distance (cm) moved by the solute from the origin}}{\text{Distance (cm) moved by the solvent from the origin}}$$

Toxicity of crude extract of *P. azadirachtae* on neem seed germination

20 ml and 10 ml of 5%-methanolic stock toxin solution (5000 ppm) were diluted to 100 ml with double distilled water in separate beakers to obtain 1000 ppm and 500 ppm solutions of toxic metabolites. The toxin solution was filter-sterilized using 0.45µm filter discs (Sartorius, Goettingen, Germany). Freshly harvested healthy neem seeds were washed thoroughly, hard endocarp was dissected out and surface-sterilized using sodium hypochlorite solution (with 5% available chlorine) for 15 min, then the seeds were rinsed well in

sterile distilled water for five times. About 100 surface-sterilized neem seeds were treated with 1000 ppm toxin solution for 24 h by placing them in 25 ml of toxin solution in 100 ml beaker. Similarly, the surface-sterilized neem seeds were treated with 500 ppm toxin solution and 1000 ppm control solution separately. Seeds treated only with the control solution served as control. After treatment the seeds were germinated by the blotter paper and paper towel methods (ISTA, 1993), incubating for 15 days at room temperature with natural alternate day and night photoperiod. Each treatment had four replications. Root length, shoot length and percentage germination were recorded and the vigour index was calculated using the formula given by Abdul-Baki and Anderson (1973).

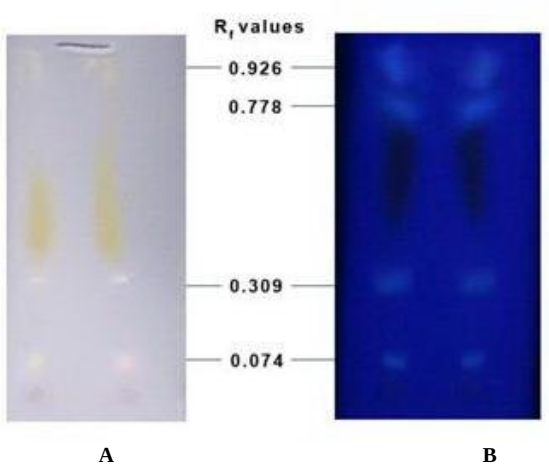

Figure 1: Thin layer chromatography (TLC) of crude toxin extract of *Phomopsis azadirachtae*. A: TLC plates run in 7.5 : 2.5 combination of chloroform: methanol solvent system. B: TLC plates run in 7.5 : 2.5 combination of chloroform: methanol solvent system and inspected under UV light at 365 nm.

Toxicity of crude extract of *P. azadirachtae* on neem callus growth

Neem callus cultures were established as per Sateesh and Shankara (1997). Freshly harvested seeds were washed in running tap water for 30 min after removing the external hard seed coat. They were surface sterilized with 0.1% aqueous mercuric chloride solution for 15 min and rinsed well in sterile distilled water for five times. The seeds were allowed to germinate on basal Murashige and Skoog (MS) medium (1962) for 15 days, then the cotyledonary explants were excised aseptically and transferred to 250 ml tissue culture bottles having 30 ml of MS medium incorporated with one ppm each of 6-benzylaminopurine (BAP) and Kinetin. These bottles were incubated at 26±2^{o}C with 12h photoperiod for 30 days. The calli obtained were sub cultured and maintained on MS medium supplemented with the same concentrations of hormones and with the same incubation conditions, for every 30 days of incubation. These calli were used for testing the phytotoxicity of crude extract of *P. azadirachtae*.

Stock toxin solution was filter-sterilized using 0.45µm filter discs (Sartorius, Goettingen, Germany). MS medium having one ppm each of BAP and kinetin was amended with different concentrations of crude toxin extract, *viz.*, 10, 100, 250, 500 and 1000 ppm separately. Final toxin concentrations were achieved by adding the appropriate volume of 5%-methanolic stock toxin solution to one and half-strength sterilized MS medium (Mackay *et al.*, 1994). About 30 ml of toxin-amended MS medium was transferred to 250ml tissue culture bottles. Calli from actively growing stage *ca.* 100±10 mg were transferred aseptically to each bottle. Inoculated bottles having MS medium amended with control stock solution (1000 ppm) served as control. The inoculated bottles were incubated as mentioned above. Calli were weighed after 30 days of incubation. Each treatment had eight replications. The relative growth was calculated (Gowda and Shankara, 1988) using the formula below:

$$\text{Relative Growth} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}}$$

RESULTS

Thin Layer Chromatography

The crude toxin solution slides developed with the solvent system of chloroform: methanol (7.5:2.5) produced the best separation of toxin solution wherein three spots / bands of light yellow colour were observed on the slides and plates (Fig. 1A). On exposure to UV light at 365 nm the same bands fluoresced, in addition to one more band (Fig. 1B). The R_f of the bands are mentioned in Fig. 1. The control solution slides developed with the same solvent system produced bands unrelated to the toxin-solution bands.

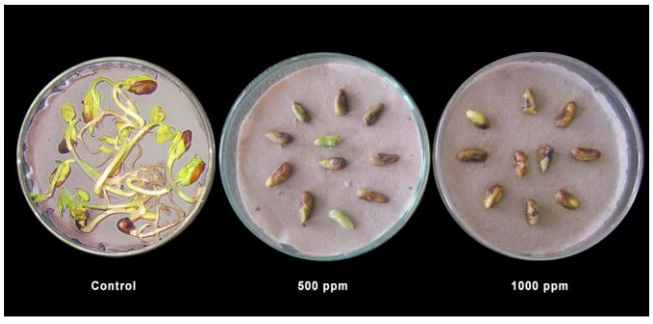

Figure 2: Toxicity of crude toxin extract of *Phomopsis azadirachtae* on neem seed germination

Toxicity of crude extract of *P. azadirachtae* on neem seed germination

The germination of neem seeds exposed to both 500 and 1000 ppm concentrations of toxin solution was completely inhibited wherein the seeds exposed only to control solution exhibited normal germination (Fig. 2).

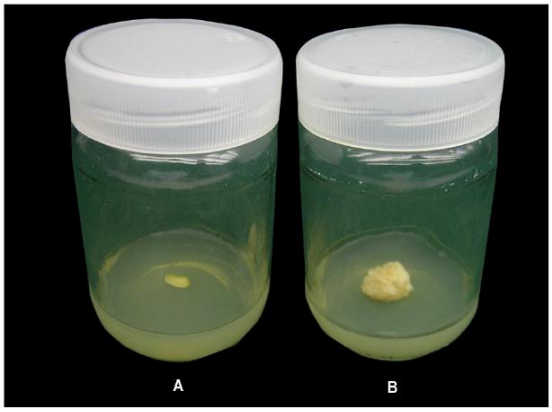

Figure 3: Development of neem callus from cotyledonary explant of neem on MS medium. A: Cotyledonary explant of neem. B: Neem callus after 30 days of incubation.

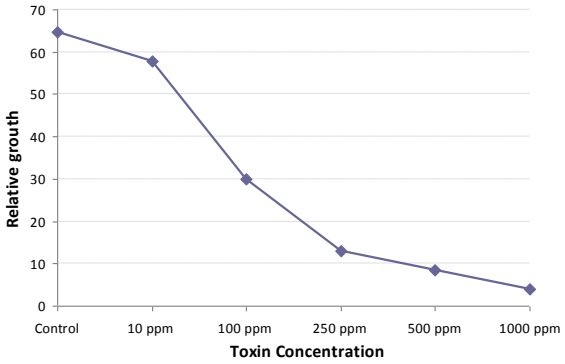

Figure 4: Growth rate of neem callus on MS medium amended with different concentrations of crude toxin extract of *Phomopsis azadirachtae* after 30 days of incubation.

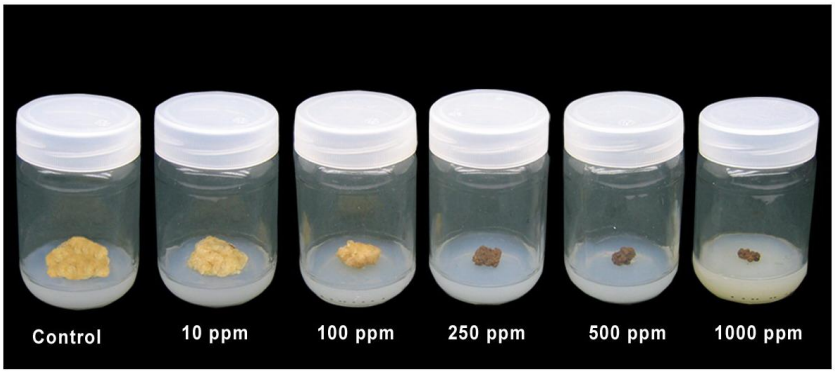

Figure 5: Toxicity of crude toxin extract of *Phomopsis azadirachtae* on neem callus.

Toxicity of crude extract of *P. azadirachtae* on neem callus growth

Cotyledonary explants on one ppm of both BAP- and kinetin-amended MS medium exhibited good callusing (Fig. 3). Neem calli displayed decreased growth with increased concentration of toxin solution showing a negative proportional relationship (Fig. 4). At 10 ppm, calli exhibited growth almost similar to the control. At 100 ppm there was decreased growth of calli and at 250 ppm and above in addition to decreases in growth, calli also showed browning and necrosis (Fig. 5). Thus, with increase in concentrations of crude toxin extract of *P. azadirachtae*, pronounced phytotoxic effects on neem callus were observed.

DISCUSSION

Phytotoxins play an important role in the host plant and pathogen interaction. Many deuteromycetous fungi were reported to release toxic secondary metabolites into the media (Agrios, 2004). The culture filtrates of many *Phomopsis* spp. are known to contain toxic metabolites that were purified and characterized (Mazars *et al*., 1991; Avantaggiato *et al*., 1999; Shankar *et al*., 1999). In the present investigations, *P. azadirachtae* was found to release toxic metabolite into the medium that was isolated from culture filtrate.

Methanol and chloroform solvents used for the extraction of toxic metabolite from culture filtrate proved to be beneficial, in agreement with the earlier reports (Lanigan *et al*., 1979; Filtenborg *et al*., 1983; Shankar *et al*., 1999). The usage of Thin Layer Chromatography (TLC) and UV visualization is in agreement with earlier reports of using the both to detect, identify and partially purify many mycotoxins (Scott *et al*., 1970; Filtenborg *et al*., 1983; Geraldo *et al*., 2006).

The toxin completely inhibited seed germination and significantly reduced the quality of seed. This is in accordance with other similar observations (Kunwar *et al*., 1987; Zeng *et al*., 2001). The phytoxicity of *P. azadirachtae* crude extract was also evaluated for its effect on neem callus. Tissue culture technique provides a controlled environment, where the effect of toxin or any chemical can be evaluated on callus tissues without any interfering external biotic and abiotic factors (Gowda and Shankara, 1988). Callus tissues are more sensitive than intact plants. Thus the tissue culture technique provides a good experimental tool for precise

evaluation of the phytotoxicity of fungal toxic metabolites *in vitro* (Sateesh and Shankara, 1997). The good yield of callus tissues of neem with kinetin and BAP at one ppm is par with that reported by Sateesh and Shankara (1997). Exposure of neem callus to different concentrations of *P. azadirachtae* toxic metabolite revealed its phytotoxicity against neem tissues. A progressive decrease in the callus growth observed with the increasing concentration of toxin is in conformity with the previous reports of many other plant-callus and pathogen-toxin interactions (Mackay *et al*., 1994; Dahleen and McCormick, 2001; Mohanraj *et al*., 2003).

The callus tissue undergoes necrosis and brownish discoloration because of the accumulation of phenolic compounds and their products (Mohanraj *et al*., 2003). Similar browning of callus tissues was observed in the present studies. Other changes in callus that were reported on exposure to phytotoxins include changes in permeability, protein pattern, electrolyte leakage, inhibition of shoot difference and loss of chlorophyll (Mackay *et al*., 1994; Mohanraj *et al*., 2003). The reduction in the callus tissue quality that was observed in the present study with the increase in toxin concentration may be attributed to some of the phytotoxic effects mentioned above.

Tissue culture techniques have produced germplasm with enhanced disease resistance (Daub, 1986). Growing the plant callus in the presence of a fungal culture filtrate or purified fungal toxin is widely used for the selection of disease-resistant lines (Amusa, 2006). Similarly the phytotoxin of *P. azadirachtae* could be used for the selection of die-back resistant lines of neem employing the tissue culture technique.

Results of the present studies revealed the ability of *P. azadirachtae* to produce phytotoxic compound in the culture filtrate and its toxicity on neem tissues. Thus, the involvement of this toxin in the development of die-back symptoms is a possibility. Proper understanding of toxin chemistry and its role in pathogenesis requires further investigations and the current investigations provide a proper base for this.

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