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By
Preeti, N. Kumari and V. Sharma

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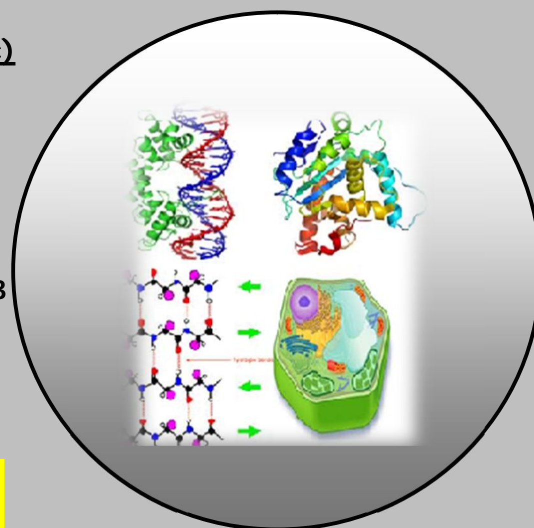

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Detached Leaf Assay for Resistance to *Macrophomina phaseolina* and Isolation of Toxin from Infected Leaves and its Analysis by TLC

Preeti, N. Kumari and V. Sharma
Department of Bioscience and Biotechnology, Banasthali University, Banasthali-304 022 (Rajasthan), India

ABSTRACT
Macrophomina phaseolina, the causal agent of charcoal root rot of chickpea (Cicer arientium) and various leaves of different host of Macrophomina phaseolina was toxigenic when cultured on various media. The crude extract or culture filtrate induced chlorosis and necrosis on leaves, inhibition of germination of seeds and wilting of seedling. A phytotoxin was isolated both from cell free culture filtrate and infected tissue, purified and toxicity identified as phaseolinone, by using thin layer chromatography. The results show patho-physiological role of phaseolinone in disease.
Keywords: M. phaseolina, Cicer arientium, Leaf assay and Phaseolinone.

INTRODUCTION
Several phytopathogenic species of *Macrophomina phaseolina* have been reported to produce phytotoxin metabolite (Bajaj, 1988). A large of fungal phytopathogens produce disease related phytotoxins but the way these toxins interact with the cell machinery has been established only for a few of them [(Ballio, 1991); (Gopalkrishnan *et al.* 2011)]. *M. phaseolina* produces a number of phytotoxins namely asperlin, isoasperlin, phomalactone, phaseolinic acid, phomenon and phaseolinone [(Dhar *et al.*, 1982); (Mahato *et al.*, 1987); (Bhattacharya *et al.*, 1992); (Bhattacharya *et al.*, 1987)]. These toxins cause leaf necrosis on several plants and primarily due to membrane lysis. Mathur (1968) first observed that *M. phaseolina* produces phytotoxic substances in culture that showed disease symptoms similar to that of a pathogen.

Later the toxin was named as phaseolinone (Kombrink and Schmelzer, 2001) which appears to be the most important toxin that induces disease symptoms in plants. It is a non-specific exotoxin that is highly stable and non-biodegradable even at high temperatures (Ergon, 2006). It affects seed germination, seedling growth and cause necrosis in tissue cultures during callusing and regeneration. This toxin is responsible for wilting of seedlings and inhibition of seed germination (Agrios, 2005). Charcoal rot is an important disease and specially manifests during hot, dry weather or when unfavorable environmental conditions stress the plant (Arora *et al.*, 2012). Hot and dry weather promotes infection and development of charcoal rot. Charcoal rot produces symptoms similar to other fungal stalk rots. The characteristic sign of charcoal rot is the production of black microsclerotia in the vascular tissue and inside the ring of stalk**.** The stalk will appear gray to black in color. The causal agent *Macrophomina phaseolina* is a saprophytic parasite and highly variable pathogen (Shanner *et al.*, 1999). Microsclerotia in the soil consititute the greatest source of *Macrophomina phaseolina* inoculums to cause new infections [(Smith *et al.*, 1989); (Kendig *et al.*, 2000); (Baird *et al.*, 2003)]. Host plant resistance offers a potential practical solution. Rapid screening methods to evaluate host resistance and pathogen variability are needed to hasten progress in developing resistant cultivars. An alternative to whole plant evaluations for charcoal root rot resistance is a detached leaf assay (Twizeyimana *et al.*, 2007). Detached-leaf assay is a rapid, economical way to do pathogenicity tests and the procedure has been used to study many host pathogens which have a number of advantages, including accurate quantification of disease development and pathogen reproduction.

MATERIAL AND METHODS

Organism

Macrophomina phaseolina was obtained from IMTECH Chandigarh (MTCC 2165).

Phtotoxicity assay and production of culture filtrate *Macrophomina phaseolina* isolate MTCC 2165 was initially cultured on potato dextrose agar. Several 1cm^2 pieces of agar medium with associated mycelium were used to inoculate 250ml flasks containing 150ml aliquots of Potato Dextrose Broth (PDB) prepared from components using fresh potatoes. Five flasks were inoculated for each culture and shaken at 125 rpm at 24°C for 2 weeks. Culture material was filtered through cheesecloth and pooled and a100ml aliquot was passed through membrane filter (250µm) to obtain cell free filtrate, which was bioassayed for phytotoxicity (Mohammad *et al.*, 2006).

Isolation of toxin from infected leaves and its analysis by TLC

In the present study detached leaf bioassay was performed by using the modified method adopted by (Cowly *et al.*, 2008). 1gm of leaf material was randomly selected from different hosts of *Macrophomina phaseolina* like *Brassica juncea, Brassica oleracea, Vigna mungo, Helianthus annus* and *Cicer arietinum.* Leaf were rinsed with 5-6 changes of sterilized distilled water and sterilized with 0.1N sodium hypochlorite (NaOCl) solution.

Leaves were spread on sterile paper towels in the laminar hood and adaxial side of leaves were aseptically inoculated with 25µl of culture filtrate using auto pipette. Each leaf was placed on a TAKC medium (technical agar 10gm, kinetin 10mg and chloramphenicol discs (3 discs per petriplate). The addition of the hormone kinetin delayed leaf senescence thereby allowing decay symptoms to be attributed to the pathogen (Twizeyimana *et al.*, 2007). The Petri dishes were arranged on shelves in optimum conditions in a culture room maintained at 20°C with alternate 12 hour light and dark conditions. The isolation of toxin from leaf samples was done according to Janardhanan and Hussain (1983). Infected leaves of *Vigna mungo*, *Helianthus annus* and *Brassica oleracea* showing early and advanced stages of infection were collected from inoculated plants and leaf material was homogenized with 100ml of 80% acetone. The homogenate was filtered through Whatman no. 1 filter paper and concentrated at low temperature. The aqueous extract thus obtained was acidified with dilute HCl to pH 2 and extracted thrice with 10 ml of 5% $NaHCO_3$. The aqueous layer was separated, and extracted with ethyl acetate. The ethyl acetate extract was dried and solvent was evaporated at low temperature in vaccuo. The dried residue re-dissolved in methanol was chromatographed by TLC on silica gel using chloroform: methanol (90:10) solvent, parallel extracts obtained from leaves of uninoculated plants and processed in similar manner were used as control. The spots were developed by exposing the TLC plates to iodine vapours (Suvarnalatha *et al.*, 2010).

RESULTS

Culture filtrate applied on leaves of *Brassica juncea*, *Brassica oleracea*, *Vigna mungo*, *Helianthus annus* and *Cicer arietinum* produced spots after 72 hrs of treatment, which enlarged and turned necrotic after 144 hrs. [(Table 1) (Fig 1-5)].

TLC specification

The residue obtained was chromatographed by TLC on silica gel using gel chloroform: methanols (90:10) solvent system along with samples of leaves and seeds from unincoulated plants and processed in similar manner were used as control.

Fig. 1(a)

Fig. 1(b)

Fig. 1 Effect of phytotoxin of crude culture filtrate on *Cicer arientum* leaves applying 25µl at 144hrs of incubation (a) control (b) 144hrs of incubation show necrotic spots.

Phaseolinone in infected plants detected by TLC analysis of toxin isolated from infected leaves indicated the presence of ethanolic $FeCl_3$. The spots were developed by exposing the TLC plates to iodine vapours (Suvarnalatha *et al.*, 2010). TLC plates showed a single spot having the major phytotoxic principle. Other spots were either nontoxic or weakly toxic. The preparation of phaseolinone showed a single spot in thin layer chromatography (TLC) plate (Sett *et al.*, 2000). Phaseolinone was isolated and purified from culture filtrate of *M. phaseolina* as reported earlier (Siddiqui *et al.*, 1979; Dhar *et al.*, 1982).

Fig. 2 (a) **Fig. 2 (b)**

Fig. 2 Effect of phytotoxin of crude culture filtrate on *Helianthus annus* leaves applying 25µl at 144hrs of incubation (a) control (b) 144hrs of incubation show necrotic spots.

Fig. 3 (a) **Fig. 3 (b)**

Fig. 3 Effect of phytotoxin of crude culture filtrate on *Brassica juncea* leaves applying 25µl at144hrs of incubation (a) control (b) 144hrs of incubation show chlorosis and necrotic spots.

HPLC

The infected leaves (1 gm) were homogenized in 50% aqueous methanol and centrifuged at 2000 g for 10 minutes. The supernatant was evaporated at reduced pressure to dryness, redissolved in 1 ml of methanol and filtered. The residue was discarded, and 100µl of filtrate was evaporated to dryness in a test tube, treated with 0.5ml of a chloroform-petroleum ether mixture (1:1), vortexed, and centrifuged at 1500 g for 10 minutes. The clear supernatant was decanted to another tube, evaporated, dried and dissolved in 500µl of assay buffer.

Table 1. Infection parameters of selected leaves evaluated for resistance to *Macrophomina phaseolina* (Tassi Goid) 144hrs after inoculation of detached leaves in Petri dishes.

Name of plants	Evaluation method	Lesion appearance (days)	Necrosis %	Chlorosis	Weight loss
Cicer arientium	Control infected	- 5	- 60-65%	---------- No chlorosis appearance	0.972gm 0.345gm
Vigna Mungo	Control infected	- 2	_ 40%	---------- Chlorosis appearance	0.981gm 0.01gm
Brassica juncea	Control infected	- 1	- 45%	----------- Chlorosis appearance	0.951gm 0.015gm
Brassica olreacea	Control infected	- 6	- 85%	----------- No chlorosis appearance	0.931gm 0.5gm
Helinathus annus	Control infected	- 7	- 70%	------------ No chlorosis appearance	0.987gm 0.45gm

Extracted toxin samples were further analyzed by High Performance Liquid Chromatography (HPLC) (Shimazdu LC-10 A) using reverse phase C-8 column. Conditions of HPLC were as follows: mobile phase: methanol- water (30:70, vol/vol) flow rate: 0.3ml/min, detector wavelength: 250 nm. The toxin samples were filtered using Millipore filter (0.22μm). An infected sample was dissolved in mobile phase and was injected with the instrument's injector. Beside pure phaseolinone, autoclaved crude culture filtrate of *M. phaseolina* was also used as a source of phaseolinone to judge whether presence of other substances interferes or accelerates infection process. (Fig 6-7).

Fig. 4 (a)

Fig. 4 (b)

Fig. 4 Effect of phytotoxin of crude culture filtrate on *Brassica oleracea* leaves applying 25μl at 144hrs of incubation (a) control (b) 144hrs of incubation show necrotic spots.

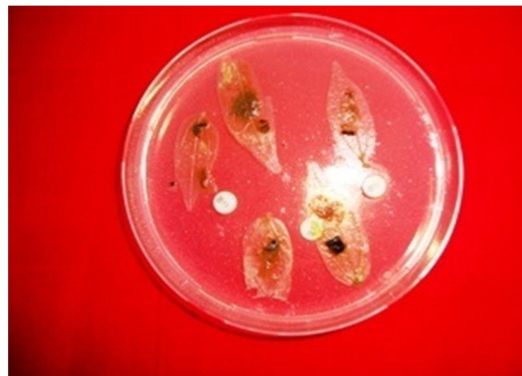

Fig. 5 (a) **Fig. 5 (b)**

Fig. 5 Effect of phytotoxin of crude culture filtrate on *Vigna mungo* leaves applying 25µl at 144hrs of incubation (a) control (b) 144hrs of incubation show chlorosis and necrotic spots.

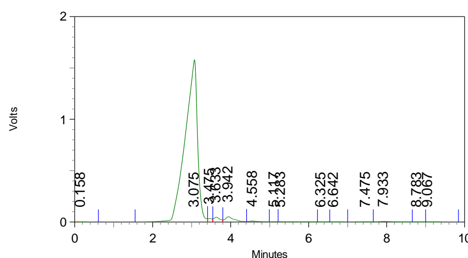

Fig. 6 HPLC chromatogram of crude culture filtrate of *M.phaseolina* used as a source of phaseolinone.

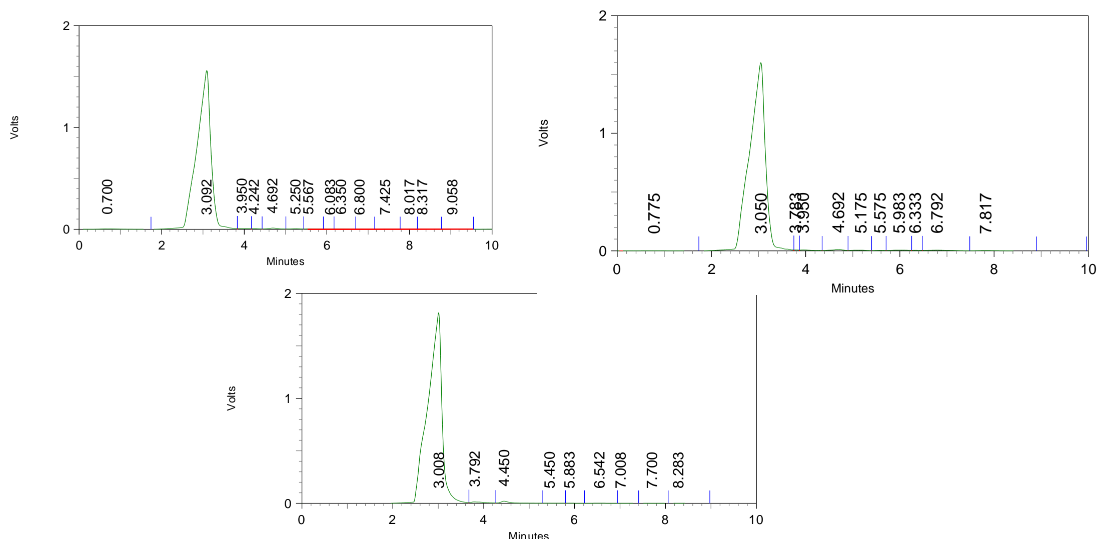

Fig.7 HPLC chromatogram of infected samples of *Brassica oleracea*, *Helianthus annus* and *Cicer arietinum.*

DISCUSSIONS

Detached-leaf assay is a rapid; economical way to do pathogenicity tests and the procedure has been used to study many host-pathogen interaction systems in dicots (Dufresne, 2000).Use of fresh potatoes was found to be necessary to get production of pigment and phytotoxin. The detached leaf assay is a rapid and reliable method to evaluate germplasm, breeding lines and mapping population for *Macrophomina phaseolina* resistance. The method can be also used to monitor symptom development and infection parameters over time. In the detached leaf assay lesions were evident from 6 days after inoculation (Cowley *et al.*, 2008).

In *Brassica olreacea* 85% necrosis, (Twizeyimana *et al.*, 2007), chlorosis and drastic loss of weight seen in *Brassica juncea* and *Vigna mungo*. This also previously reported [(Arjen *et al.*, 2007); (Have *et al.*, 2007)] in tomato for evaluating partial resistance to B. cinerea. The advantage of the detached leaf assay would be an ability to initially screen large populations of genotypes using smaller resources for inoculum, less space for cold room and easier handling, sufficing the problem or need for large greenhouse spaces to maintain populations (Goth, 1997). Selected candidate resistant plants may then be further subjected to screening with replicates on whole plant basis to measure plant recovery as well.

Experimental results indicate that *Macrophomina phaseolina* culture filtrate induced lesions and spotting on leaves, as well as growth inhibition of germinating seeds. The observations of support these conclusions, demonstrating that the pathogen produces several metabolites into culture filtrate, one of which was phaseolinic acid, phaseolinone, an eremophilane sesquiterpenoid specifically an epoxidized analogue of phomenone identification of the compound was done by TLC. The observations of Fulton *et al.* (1965) support these conclusions, demonstrating that the pathogen produces several metabolites into culture filtrate, one of which was phaseolinone identification of the compound was done by TLC. The importance of phaseolinone, the major phytotoxin substance produced by *Macrophomina phaseolina* in disease initiation has been evaluated using nontoxigenic, avirulent mutant which affects plants.

Initiation of infection by *Macrophomina phaseolina* in the presence of phaseolinone clearly indicated that phaseolinone made *Cicer arientium* and host of *Macrophomina phaseolina* susceptible to infection. Based on the present study finally we conclude that isolation of phaseolinone from infected leaves indicate that the toxin was produced by the pathogen during pathogenesis. Thus phaseolinone can be considered as a vivotoxin in the case of charcoal root rot of *Cicer arientium.* The results also support the findings of Mikami *et al.* (1971) on leaf blight of *Datura innoxia*. The difference obtained in TLC pattern of control and infected varieties of various hosts laid foundation for isolation of toxin.

Validation by HPLC

The phaseolinone contents of infected leaves extracts were also estimated by reverse-phase HPLC. The retention time obtained by this method was in good agreement. Quantitation was done by measuring the peak area, which was proportional to the amount of toxin applied (1 to 100ng).

Phytotoxic metabolites have been isolated from culture filtrates of many plant-pathogenic fungi (Durbin, 1981). However, production of such toxins during culture does not necessarily establish their production under conditions of natural infections. Since many of these toxins are produced in very small amounts, demonstration of their production in infected plants has always been a challenging task. Because of its high sensitivity, HPLC is a suitable method of detection and estimation of such toxins (Butler and Bisby, 1960; Morgan, 1989). The correlation between the amount of phaseolinone produced in infected leaves by *M. phaseolina* and the degree of necrosis indicates that the action of the fungus is probably mediated through this toxin.

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Corresponding authors: Professor V. Sharma, Department of Bioscience and Biotechnology, Banasthali University, Banasthali-304 022 (Rajasthan), India

E-mail: vinaysharma30@yahoo.co.uk Fax No: 01438-228365, 228587

Dr. Preeti, Department of Bioscience and Biotechnology, Banasthali University, Banasthali-304 022 (Rajasthan), India

E-mail: preetibiotech87@gmail.com

Dr. Nilima Kumari, Department of Bioscience and Biotechnology, Banasthali University, Banasthali-304022, Rajasthan India

Email: nilima_km@yahoo.com