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Antifungal activity of some medicinal plants on Soybean seed-borne *Macrophomina phaseolina*

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ABSTRACT

Soybean *Glycine max* (L.) Merr. seeds, pods and seedlings are susceptible to fungal attack due to its rich nutrient content. The most commonly isolated soybean (JS-335) seed-borne fungi were *Fusarium* spp., *Macrophomina phaseolina.*, *Pythium* spp., *Aspergillus* spp., *Phoma* spp., and *Phomopsis* spp. *Macrophomina phaseolina* is an important plant pathogenic fungus that causes charcoal rot of soybean and infects more than 500 hosts. In humid climates, the fungus causes a post emergence damping-off of soybean seedlings leading to 50% of crop losses. The objective of this work was to study the efficacy of the botanicals on soybean seed-borne *Macrophomina phaseolina.* Among the 10 botanicals screened, *Datura metel* (L.) methanol leaf extract showed the most promising activity against *Macrophomina phaseolina*.

INTRODUCTION

Soybean (*Glycine max* (L.) Merill) is a versatile food plant used in its various forms. It is capable of supplying most nutrients (Potter *et al*., 1998). Worldwide production of soybean was projected to be 250.39 million mt in 2009-10 (USDA 2009). USA stands first in the production followed by Brazil, Argentina, China and India. In India, soybean estimated yield is 9.81 mt from 9.21 m ha. In developing countries like India, most of the crop yield is reduced due to pathogenic fungi (Agrios 2005). Soybean can substitute for meat and to some extent for milk (Endres *et al*., 2003). Fungi are significant destroyers of foodstuffs and grains during storage rendering them unfit for human consumption by retarding their nutritive value and often by producing mycotoxins (Paster and Bullerman 1988). Soybean is an important host of the *M. phaseolina* which causes charcoal rot, seedling blight, dry root rot, ashy stem blight and dry weather wilt disease (Mengistu *et al.*, 2011). The estimated average annual loss due to charcoal rot in soybean was estimated to be 30-50% (Wrather *et al*., 2003). The grains stored for consumption purposes cannot be treated with fungicides, pesticides and bactericides due to resistance developed by pathogens (Khater 2012). The fungicides, pesticides and bactericides are non-biodegradable in nature and are extremely toxic in nature (Yadav 2010). The main effects are genotoxicity (Bolognesi 2003), hepatotoxicity (Cecchi *et al*., 2012), reproductive disorders (Richard *et al*., 2005) and immunosuppression (Singh *et al*., 2011) Wild and Gong, 2010. It is therefore necessary to search for novel antifungal agents for control measures that are cost-effective, non-toxic, eco-friendly and which eliminate or reduce the incidence of these important pathogens, so as to prevent bio-deterioration of soybean seeds. Throughout the history of mankind, plant kingdom had a rich repository of medicines. According to the WHO, more than 65-80% of the world population relies on traditional herbal medicine for their primary healthcare (Akerele, 1993). In modern times, plants reflect the validity of many traditional uses regarding the value of natural products in health care**.** Plants cannot escape or hide when attacked by herbivores and pathogens. Therefore, in order to protect themselves against herbivores and pathogens, they have evolved to produce secondary metabolites (Wallace, 2004).

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These secondary metabolites have a broad-spectrum antifungal, antibacterial, antiviral, anti-tumor and cytotoxic potential (Mackeen *et al*., 2000; Jing *et al*., 2012). Plants are the sources of natural pesticide that make excellent leads for new pesticide development (Tripathi and Shukla, 2010; Kuri *et al*., 2011). The present investigation focuses on isolation and identification of soybean seed-borne *M. phaseolina* and *In vitro* studies were carried out to test the antifungal efficacy of ten plant extracts on preventing the growth of *M. phaseolina*. The finding of the present investigation is an important step towards soybean protection strategies for antifungal activity against seed-borne *M. phaseolina*.

MATERIAL AND METHODS

Plant materials

Ten widely available plants (Table 1) were selected based on an ethnobotanical survey. Leaf samples were collected early morning during the flowering season in March/April 2010 and 2011 from Dhanvantrivana, Bangalore University and Gandhi Krishi Vigyan Kendra (GKVK) University of Agricultural Sciences, Bangalore). An authenticated voucher specimen of the plant was deposited in the herbarium of Molecular diagnostic laboratory, Department of Microbiology and Biotechnology, Bangalore University, Bengaluru, Karnataka, India (Table 2). The freshly collected leaves were washed with tap water, and then rinsed in de-ionized water, oven dried at 35 - 40^{o} C for 48 hours and pulverized using a sterile electric blender to obtain a powdered form and it was sieved and stored in sterile polyethylene sample bags prior to use (Sateesh, 1998).

Table. 2: Herbarium deposition number.

Plant name	Deposition number
Azadirachta indica (A. Juss.)	MBMDL 167
Boerhavia chinensis (L.)	MBMDL 55
Delonix regia (Raf.)	MBMDL 56
Datura metel (L.)	MBMDL 50
Euphorbia pulcherrima (Wild.)	MBMDL 58
Jacaranda mimosifolia (D. Don.)	MBMDL 59
Hibiscus rosa-sinensis (L.)	MBMDL 44
Ficus religiosa (L.)	MBMDL 45
Ficus benghalensis (L.)	MBMDL 46
Acalypha indica (L.)	MBMDL 47

Preparation of aqueous extract

The aqueous extract of each plant material was prepared by soaking 10 g of powdered samples in 200 ml of sterile distilled water in 500 ml Erlenmeyer flask at room temperature for 24 hours. The supernatant was filtered through double layered muslin cloth and centrifuged at 4000 g for 30 min. It was filtered through Whatman No. 1 filter paper. The extract was preserved aseptically in a brown bottle at 5^{o} C prior to use.

Preparation of Solvent extracts

The organic solvent extraction was carried out by sequential extraction of the plant material (1:10 w/v) with different solvents (hexane, chloroform and methanol) according to their increasing polarity by using Soxhlet apparatus for 48 hours at a temperature not exceeding the boiling point of the respective solvent. The yields of positive plants are represented in the (Table 3). The extraction yield was calculated as: Yield (%) = (WE/WP) × 100 where WE and WP are the weights of the dried extract and the dried powder before extraction respectively (Ponnusamy *et al*., 2010).

Table. 3: The extract yield of the plants in different solvent.

Name of the Plant	Extraction solvent	Yeild (%)
Datura metel (L.)	Water	0.6
	Methanol	3.3
	Chloroform	1.1
	Hexane	0.4
Azadirachta indica (A. Juss.)	Water	0.7
	Methanol	2.9
	Chloroform	1.2
	Hexane	0.3

Test organism

Potato Dextrose Agar (PDA) medium was used for isolation of seed-borne *M. phaseolina.* Soybean seed samples (JS 335) were surface disinfected with Sodium hypochlorite (1%) solution for about 2 min at room temperature and placed in Petri plates (10 seeds/plate). The plates were subjected to SBM ISTA (1996). On the seventh day incubation, seeds were observed under stereobinocular microscope. *M. phaseolina* was identified based on their growth, mycelial structure and spore morphology using standard manuals (Bressano *et al*., 2010). *M. phaseolina* is a soil-borne/seed-borne fungus that belongs to phylum Ascomycota and class Dothideomycetes. It is highly variable by production of both pycnidia and sclerotia in culture media and host tissues. The pycinidial state of the fungus was initially named *Macrophoma phaseolina*, later renamed as *Macrophoma phaseoli* and then as *Macrophomina phaseoli* by Ashby in 1927. Finally, the name *Macrophomina phaseolina* was settled by Goidanich (Girish *et al*., 2012). The microsclerotial state was first described as *Rhizoctonia bataticola* (Taub.) The pycnidiospores are ellipsoid to ovoidvarying form 100 µm to 250 µm. During the sclerotial formation, 50-200 individual hyphal cells aggregate to give multicellular bodies named microsclerotia.

Determination of antifungal activity

The agar disc diffusion method was employed to determine the antifungal activity. Potato dextrose broth was inoculated with *M. phaseolina* which serves as culture inoculum. A swab of the fungal suspension was spread on to the petri plates containing PDA media (Rankovic *et al*., 2010). Each solvent extract was dissolved in DMSO to final concentration of 10 mg/ml. Sterile filter paper discs (6mm in diameter) impregnated with 10µl of different concentration of plant extracts were placed equidistantly around the margin of the plate. Standard reference compound, Deltamethrin was used as positive control and the fungal plates were incubated at 28^{o} C for seven days. The extracts showing higher inhibitory activity compared to the standard compound was selected for further phytochemical studies. The diameter of zone of inhibition with the corresponding positive plants was represented in (Table 4). Antimicrobial activity was indicated by the presence of clear inhibition zone around the discs.

Table. 1: Antifungal activity of the selected plant leaf extracts on soybean seed-borne *M. phaseolina.*

Name of the Plant	Family	Solvent Used	Result Positive	Negative
Azadirachta indica (A. Juss.)	Meliaceae	W, M, C & H	M, C	W, H
Boerhavia chinensis (L.)	Nyctaginaceae	W, M, C & H	-	W,M,C,H
Delonix regia (Raf.)	Cesalpiniaceae	W, M, C & H	-	W,M,C,H
Datura metel (L.)	Solanaceae	W, M, C & H	M, C	W, H
Euphorbia pulcherrima (Wild.)	Euphorbiceae	W, M, C & H	-	W,M,C,H
Jacaranda mimosifolia (D. Don.)	Bignoniaceae	W, M, C & H	-	W,M,C,H
Hibiscus rosa-sinensis (L.)	Malvaceae	W, M, C & H	-	W,M,C,H
Ficus religiosa (L.)	Moraceae	W, M, C & H	-	W,M,C,H
Ficus benghalensis (L.)	Moraceae	W, M, C & H	-	W,M,C,H
Acalypha indica (L.)	Euphorbiaceae	W, M, C & H	-	W,M,C,H

W: Water, M: Methanol, C: Chloroform, H: Hexane

Table. 4: Diameter of inhibition zone in mm at different concentration µg/disc.

Name of the plant	Name of the different solvent extract	10	20	30	40	50	60	70	80	90	100
Datura metel	Methanol extract	-	13.03 ±.06e	13.4 ±.26e	14.03 ±.15d	14.33 ±.23d	14.96 ±.06c	15.16 ±.15bc	15.73 ±.11ab	16.03 ±.06a	16.23 ±.15a
	Chloroform extract	-	-	-	-	10.16 ±.11kl	10.76 ±.06jk	11.03 ±.15ij	11.36 ±.25ghij	11.83 ±.06fg	12.2 ±.1f
Azadirachta indica	Methanol extract	-	-	-	-	-	-	11.1 ±.06hij	11.33 ±.06ghij	11.4 ±.1ghi	11.766 ±.06fgh
	Chloroform extract	-	-	-	-	-	-	-	9.06 ±.06m	9.76 ±.06l	9.96 ±.06l

Each value is mean of three replicates; ± indicates standard error from mean value. (-) nil. Different letters within a column indicate significant differences according to Scheffe's test ($P < 0.05$).

Statistical analysis

The Antifungal assay was carried out in triplicate and the values were expressed as mean ± Standard error (SE). Statistical analysis was done using three-way ANOVA followed by Scheffe post hoc test using SPSS software (version 20). Difference were considered significant when $P<0.05$.

RESULT AND DISCUSSION

Among the ten plants tested, the leaf extracts of *D. metel* and *A. indica* exhibited good antifungal activity against *M. phaseolina*. The extract of *D. metel* showed significantly higher inhibitiory activity than the extract of *A. indica.* Both of the extracts exhibited antifungal activity in a dose dependent manner. Methanol extracts exhibited higher degree of antifungal activity as compared to chloroform extracts.

Methanol extract of D. metel showed minimum inhibitory concentration (MIC) of 20 µg/disc (13.03±0.06 mm) where as that of A. indica showed MIC of 70 µg/disc (11.1±0.06 mm). The results of the antifungal screening of the crude extracts of plants were shown in table 4.).

The remaining plant leaf extracts did not show significant inhibition on *M. phaseolina*. This result indicates that the leaf extracts of *Datura metel* and *Azadirachta indica* leaf have fungicidal properties which inhibit the growth of soybean seed-borne *M. phaseolina*.

A similar finding have had been reported recently by Javaid and Saddique (2011) and Dubey *et al*., (2009) who found mycelial growth inhibition of *M. phaseolina isolated from* soil-borne and diseased roots of soybeantreated with *D. metel* and *A. indica*. So the extracts of *D. metel* may be utilized as phytofungicide to control the *M. phaseolina* on soybean seed.

CONCLUSION

Datura metel is a weed that grows naturally and can be seen commonly in the backyards of houses and on pavements across India. The methanolic leaf extract of *D. metel* have a greater effect than that of chloroform extract. It indicates that the active plant compounds are readily extracted in methanol when compared to other solvents. The factors responsible for this high susceptibility of the fungi to the *D. metel* leaf extract are not exactly known, but may be attributed to the presence of secondary plant metabolites which is soluble in methanol. Therefore further detailed study on phytochemistry of *Datura metel* is needed for isolation and purification of secondary metabolites which may further yield significant antifungal agents against *M. phaseolina*.

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