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Antimycotic Potentiality of the Plant Extract *Bacopa monnieri* (L.) Penn.

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Abstract: Ethanolic extract from the leaves and young shoot tips of *Bacopa monnieri* (Scrophulariopsis) was investigated for their antifungal activities. The growth inhibition of the fungi were determined using disc diffusion assay against sixteen fungal strains, out of which the extract showed significant inhibition of *Rhizoctonia solani*, *Curvularia lunata*, *Alternaria brassicicola* and *Acremonium kiliense*. Solvent extract of the plant material showed inhibitory activity against these microorganisms at 100-200 μg mL^{-1} concentration level. Thin layer chromatographic analysis of the plant extract showed the fractions of alkaloids and natural lipids which may be the important factor in causing the inhibitory effect. The seed treatment by this plant extract showed more effectiveness in reducing disease in rice plants against the damage caused by *Rhizoctonia solani*, as compared to foliar spray treatment.

Key words: Antifungal, biocides, active principles, field applications

INTRODUCTION

Now a days, throughout the world search is going on the environmentally safe, non toxic and economically viable plant based products for remedy of various plant diseases. The majority of the synthetic antimicrobial products used in agricultural purpose are toxic to different biological system as well as environment and they induce the development of resistant strains and affecting plant health. Soil borne plant pathogens are sometimes difficult to control with chemical fungicides or bactericides and the application of biocides may be one of the best alternatives in controlling pathogens. Hence, throughout the world, scientists are concentrating their views for screening of plant sources for their antimicrobial activity as higher plants represent a potential source of novel antibiotic or antimycotic prototype and simultaneously as they are environmentally safe, biodegradable and renewable (Cunat *et al*., 1990; Kurucheve *et al*., 1997; Singh and Maheshwari, 2001; Sengupta *et al*., 2002, 2004; Kishore and Singh, 2005; Koduru *et al*., 2006; Sharma *et al*., 2006). The plants like *Acalypha wilkensiana*, *Azadirachta indica*, *Datura metel*, *Eucalyptus camadulensis*, *E. citridora*, *Allium sativum*, *A. cepa*, *Lecus aspera*, *Ranunculus scleratus*, *Holarrhena antidysentericca*, *Emblica officinales*, *Ocimum sanctum*, *Calotropis pocera* have already been screened out for their antimicrobial properties (Alade and Irobi, 1993; Yossry *et al*., 1999; Ganesan *et al*., 2004; Sharma *et al*., 2005). Recently, Saha *et al*. (2005) showed the control measure of foliar tea disease by the leaf extract of *Polyalthia longifolia*, where as Tripathi (2005) studied the efficacy of fungicides and plant products against stem gall disease of coriander caused by *Protomyces macrsoporus* Unger. On the other hand Aliero *et al*. (2006) studied the biofungicidal activity of the various parts of *Solanum pseudocapsicum* and Bohra *et al*. (2006) established the efficacy of native isolates of fungal and bacterial agents and neem based formulations for management of damping off in brinjal and chilli.

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In the present investigation, leaves and young shoot tips extract of *Bacopa monnieri* have been screened for its antifungal properties against sixteen fungal strains, out of which emphasis was given on one of the most devastating pathogen *Rhizoctonia solani* that causes various important diseases like sheath blight and sheath rot in *Oryza sativa*, which is one of the economically important crops of Asian countries like India and China. So, the damage of rice plants due to this pathogen gives the countries a great economic loss. Hence, keeping in view the adverse effect of the fungicides on the agro-ecosystem, emphasis was given to find out the environmentally safe biocides from *B. monnieri* to control the disease caused by *Rhizoctonia solani* in rice plants.

MATERIALS AND METHODS

Plant Material

The leaves and young shoot tip parts of *Bacopa monnieri* were collected from a natural population around 24 Parganas, West Bengal, India and were authenticated in the Department of Botany, Presidency College, Kolkata, India. The *in vitro* study was carried out in the Mycology and Plant Pathology Research Laboratory, whereas the field experiment was carried out in the Experimental Garden of the above mentioned Department during the period 2004-2006.

Extraction and Screening of Antifungal Activities

Collected plant material was properly cleaned (washed 2-3 times in tap water and surface sterilized with 95% alcohol) and for solvent (ethanol) extraction (20 g 100 mL^{-1}), it was dried (oven dried at 40°C for 4-5 days), dusted and stored in airtight cleaned glass tubes for 7-8 days and then kept in desiccator. The extraction was done in soxlet apparatus and then it was concentrated (one fifth of its volume) in rota evaporator. Finally the extract was filtered by passing it through sterilized bacteriofilter before testing its antifungal properties. The pH value of the extract was determined by dipping the high sensitivity pH paper in the extract. The antifungal activities of the plant extract was tested against *Aspergillus niger* Van Tieghem, *Acremonium kiliense* Gruitz, *Alternaria alternata* (Fr.) Keissler, *Alternaria brassicicola* (Schw.) Wiltshire, *Curvularia lunata* (Wakker) Boedijn, *Colletotrichum capsici* (Synd.) Butler and Bisby, *Cladosporium herbarum* (Pers.) Link, *Fusarium udum* Butler, *Macrophomina phaseolina* Maubl., *Myrothecium roridum* Tode ex Fries, *Penicillium expansum* Link ex Fries, *Penicillum digitatum* Sacc., *Phytophthora parasitica* Dastur, *Rhizopus stolonifer* (Ehrenb. ex Fr.) Lind, *Rhizoctonia solani* Kuehn and *Scopulariopsis* sp. The antifungal activities of the plant extract were assayed by filter paper disc diffusion method (Prescott and Harley, 1996); Brand-Whatman No. 1 and the diameter of paper discs-6 mm). Control plates received only in respective solvent. A multiple copies (3 times) of the prototypes were prepared for each experimental set. The diameter of inhibition zones around the discs was measured after 48-120 h at 28°C (Sengupta *et al*., 2002, 2004).

Screening of Minimum Inhibitory Concentration Values

For testing the Minimum Inhibitory Concentration (MIC) value of the extract, it was dried to powder by complete evaporation of the solvent (ethanol) and from that made a known concentration of the extract (mg mL^{-1}). The stock solution of the extract was then further diluted in a serial dilution to get various strengths (10 to 800 μg mL^{-1}). This experiment was carried out by disc diffusion assay method. After incubation at optimum temperature, the plates were screened for just production of inhibition zones at minimum concentration level. The minimum concentrations for such antimicrobial effect were determined to record their respective MIC values.

Thin Layer Chromatographic Separation of Plant Extract

The extract of *Bacopa monnieri* have various active compounds which have their antimicrobial activities and hence to separate the different fractions of the active principles present in the plant extract, thin layer chromatography (Stahl *et al*., 1969) has been used. The chemical constituents like phenols and phenolic acids, lipids, alkaloids, glycosides, coumarins and terpenoids are selected in this investigation. For conducting the experiment, the quantity of plant extract in capillary tubes (10-15 μL) was applied as spot on TLC plate coated with silica gel G (TLC aluminium sheets, 20×20 cm, silica gel 60 F_{254}, Merck). Different solvent systems and different spraying chemicals were used to separate the probable active principles (Harborne, 1976; Dhingra and Sinclair, 1995; Sadasivam and Manickam, 1996; Wagner and Bladt, 1996). The R_f values of each fraction of the samples were also calculated. For testing the antimicrobial activities of the different fractions of the active constituents present in the plant extract appeared as spots on the TLC plates, they were scrap out separately from the plate and finally their properties have been tested against the test organisms by disc diffusion assay technique, which showed maximum inhibitory capacity against the tested organisms (Sengupta *et al*., 2004).

***In vivo* Antifungal Activities of Plant Extract**

To test the efficacy of the plant extract, a field trial was carried out consecutively for 2 years during the period December-May, 2005 and 2006 with Satabdi variety (IET 4786) of *Oryza sativa*, collected from Chinsura Rice Research Institute, Hoogly, West Bengal, India. For seed treatment, the earthen pots were sterilized with 1% formalin solution and filled with autoclaved soil. Before sowing the sterile seeds in the earthen pots, they were treated with the aqueous plant extract at 200 μg mL^{-1} concentrations for 6 h first (as by repeated experiment, this concentration showed maximum effectiveness) and later after dried it, treated with mycelial suspension of the *R. solani* for another 6 h at 10^6 mL^{-1} concentration (as this concentration was used in disc diffusion assay technique) (Set 4). Seeds without any treatment served as control (Set 1) and seeds treated with only plant extract (to test the phytotoxicity of the plant extract) and only with mycelial suspension of the fungal pathogen respectively (each for 6 h) were Set 2 and Set 3. In each set 75 seeds were placed and the sizes of the pots were 2.9½×4.2½. The experiment was conducted with three replication with randomised block design (Singh and Maheshwari, 2001; Laha and Venkataraman, 2001; Tripathi, 2005). Later all the sets were kept under normal environmental condition for 21-28 days. In foliar spray treatment, all the sterile seeds in four sets (75 seeds in each set) were sowing in the sterile pots filled with sterile autoclaved soil and kept in sterilized condition. After 14 days, Set 1, 2 and 3 were sprayed with sterile water, aqueous plant extract (200 μg mL^{-1} concentration) and mycelial suspension of the *R. solani* (10^6 mL^{-1} concentration), respectively. Finally the last one (Set 4) treated with the aqueous plant extract first and later with mycelial suspension of the fungal pathogen twice at 7 days intervals (Laha and Venkataraman, 2001; Sharma and Tripathi, 2001). The experiment was conducted with three replications. Later all the sets were kept under normal environmental condition for 14-21 days. After the incubation periods of both the cases disease index (%) was calculated by counting the number of infected or diseased plants.

RESULTS AND DISCUSSION

The results of disc diffusion assay technique showed that the extract of *Bacopa monnieri* have maximum antifungal activities against *Curvularia lunata* and *Rhizoctonia solani* (each 18 mm diameter of inhibition zones) which is slightly higher than the activities against *Alternaria brassicicola* and *Acremonium kiliense* (16 and 15 mm, respectively), whereas the minimum activities were observed against *Colletotrichum capsici* and *Scopulariopsis* sp. (7 mm each). The antifungal

Table 1: Minimum Inhibitory Concentration (MIC) value of *Bacopa monnieri* (L.) Penn. against some fungal pathogens

Concentration of MIC values (μg mL^{-1})	*Acremonium kiliense*	*Alternaria brassicicola*	*Curvularia lunata*	*Rhizoctonia solani*
10	-	-	-	-
50	-	-	-	-
100	7	-	8	8
200	8	8	9	14
400	10	10	10	15
800	11	12	12	15

- = No inhibition zones

Table 2: Antimicrobial properties of different fractions of extract of *Bacopa monnieri* (L.) Penn

Probable active principles (as revealed from solvent system used in TLC) with their activities (diameter of zone of inhibition, including diameter of paper discs 6 mm)

Phenols etc.	Natural Lipids					Alkaloids					Glycosides	Coumarins	Terpenes
	R_f	Ak	Ab	Cl	Rs	R_f	Ak	Ab	Cl	Rs			
-ve	0.939	-	+	-	11	0.64	9	-	7	7	-ve	-ve	-ve
						0.74	7	-	-	+			
						0.80	7	-	-	+			

R_f = distance moved by the solute from the origin or point of application in TLC plate per distance moved by the solvent from the origin; Ak = *Acremonium kiliense*; Ab= *Alternaria brassicicola*; Cl = *Curvularia lunata*; Rs = *Rhizoctonia solani*; -ve = Not present; + = Very small inhibition zones; - = No inhibition zones

potentiality of this extract had not been shown against rest of the pathogens. Among the two pathogens which were inhibited in higher degree by the extract of *Bacopa monnieri* as compare to others, *R. solani* is more devastating crop pathogen than *C. lunata* and that is why *R. solani* was selected for the field trial. The use of biocontrolling agents to combat various diseases caused by *R. solani* has been carried out by several scientist of the world. The extract of *Datura metel*, *Eucalyptus camadulensis*, *E. citridora* and *Calotropis pocera* reduce the growth of soil borne fungi *R. solani* (Yossry *et al*., 1999), whereas the root extract of *Lecus aspera* showed complete inhibition of mycelial growth of *R. solani* at 5-10% concentration in poisoned food technique (Ganesan *et al*., 2004). The extract of *Allium sativum, A. cepa* and *Eucalyptus globules* also showed inhibitory effect against *R. solani* both *in vitro* and *in vivo* on the maize plant (Sharma *et al*., 2005).

The pH value of the extract of *B. monnieri* is 5.9 at the time of treatment, which is moderately acidic and this result indicated that the inhibition zones produced were certainly due to active principles present in this extract, not due to the pH of the plant extract. It was experimentally established that all the test fungi grow well in acidic pH (Sengupta *et al*., 2004). The result of this assay technique may be indicated the synergistic effect of the active principles present in this plant extract.

The Minimum Inhibitory Concentration (MIC) value of the extract is 100 μg mL^{-1} for *A. kiliense*, *C. lunata* and *R. solani* and 200 μg mL^{-1} for *A. brassicicola* (Table 1).

The probable active principles have been separated by Thin Layer Chromatography (TLC), which may be composed of different groups of chemical constituents of the plants like phenols and phenolic acids, natural lipids, alkaloids, glycosides, coumarins, terpenes etc. (Sengupta *et al*., 2004; Saha *et al*., 2005). Literature showed that *B. monnieri* have lipids, amino acids and various alkaloids like brahmine, herpestine etc. (Chatterjee and Pakrashi, 1991). After TLC, it has been revealed that the extract of *B. monnieri* has fractions of alkaloids and natural lipids (Table 2), which may be the important factor for its antifungal properties. From the results of TLC analysis it can also be concluded that the extract of *B. monnieri* showed maximum amount of inhibitory activity against *R. solani* and very little amount against *A. brassicicola* due to the presence of natural lipid with R_f value of 0.939. In case of activity against *A. kiliense* and *R. solani*, all three fractions of alkaloids (with R_f values of 0.64, 0.74 and 0.80) were responsible for the inhibitory activity, whereas in case of *C. lunata* only one fraction

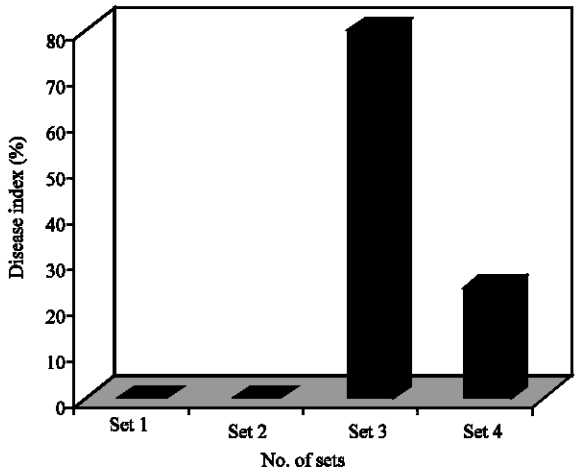

Fig. 1: Effect of extract of *Bacopa monnieri* (L.) Penn. on *Rhizoctonia solani* inoculated seeds of Rice (*Oryza sativa*) in *in vivo* by seed treatment method

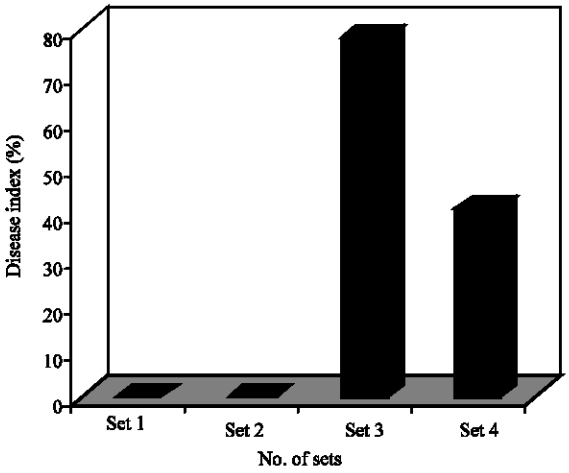

Fig. 2: Effect of extract of *Bacopa monnieri* (L.) Penn. on *Rhizoctonia solani* inoculated seedlings of Rice (*Oryza sativa*) in *in vivo* by foliar spray treatment

of alkaloids (with R_f values of 0.64) was responsible (Table 2). So, the antifungal activities of the plant extract might be due to these active chemical constituents i.e., alkaloids (in *A. kiliense* and *C. lunata*) or lipids (in *A. brassicicola*) or both (in *R. solani*) present in them.

The *in vitro* potentiality of any biocides can be verified by their application in the field trial. Today not only single plant but also mixture of more than one plant has been used for remedy of various soil borne diseases. The garlic extract significantly controlled the disease caused by *R. solani* in the field when applied before (52% PEDC), during (58%) and after (42%) inoculation of the pathogen *R. solani* f. sp. *sasakii* (Kuhn) on the maize plants (Sharma *et al*., 2005). Synergistic application of *Allium sativum* and *Azadirachta indica* extract (1:1) check the sporulation of some fruit rotting fungi (Sharma *et al*., 2006). From the results of the field experiment of this investigating programme (Fig. 1, 2) it can be said that the extract of *B. monnieri* showed 56% reduction in disease index (80 and 24% disease index in set 3 and 4, respectively) (Fig. 1) in case of seed treatment method

whereas it was 36% reduction in disease index (76 and 40% disease index in set 3 and 4, respectively) (Fig. 2) in case of foliar spray treatment method. The results also showed no phytotoxic effect of the plant extract, as in Set 2, the plants were germinated and grown as similar as the control plants of Set 1. In the seed treatment experiment, in Set 3 where the seeds were treated with mycelial suspension of *R. solani*, maximum number of the seeds were died at their germinating stage and whitish mycelium were found in the soil surrounding the seeds. Some of the seeds, which later germinated showed brownish blighted spots or lesions on leaves. The prophylactic treatment by the plant extract gave the seeds a protective barrier in set 4 and hence maximum seeds became viable and germinated as similar as the control set (Set 1). But in case of foliar spray treatment, it has been found that in Set 3, symptoms appeared much more prominently on leaves as compare to seed treatment experiment. In most of the cases the tips of the leaves also affected and ultimately the plants were drooped and died. Here also in Set 4, the rice plants were protected by the extract of *B. monnieri* and so less symptoms appeared in rice plants as compared to set 3, but the intensity of the use of protectant was less as compared to seed treatment experiments. These two parameters help to concluding that seed treatment by the plant extract *Bacopa monnieri* showed more effectiveness in reducing disease in rice plants against the damage caused by *R. solani*, as compared to foliar spray treatment.

Both the results of *in vitro* and field applications signify the potentiality of *B. monnieri* as a source of antimycotic (antifungal) therapies and hence further work is necessary to evaluate its potentiality in *in vivo* on other pathogens as this biofungicidal botanics is environmentally safe and could replace the toxic and hazardous synthetic compounds. Simultaneously investigations are also needed to characterize, formulate and marketwise the active principles of this extract which may provide leads for the discovery of a novel antimycotic compounds from *Bacopa monnieri*.

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