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Evaluation of the effect of plant extracts on soil mycoflora and seedling disease of *Gossypium hirsutum*

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Abstract
A study was conducted to control *Rhozoctonia solani* causing damping off disease in seedlings of *Gossypium hirsutum* with aqueous root extracts of *Rubia cordifolia*. In this study the *R. cordifolia* aqueous root extracts were also tested for sporicidal activity, shelf life period and biocontrol activity of soil mycoflora against cotton phytopathogens *Rhizoctonia solani* and *Alternaria alternata* causing damping off disease in seedlings and leaf spot diseases respectively. The results show that aqueous root extracts of *R. cordifolia* shows good- shelf life stability, sporicidal activity and significant biocontrol against soil mycoflora causing damping off disease in seedlings of *G. hirsutum*. Results of this study suggest that aqueous root extract of *R. cordifolia* can be used to reduce the effect of soil mycoflora on seedling of *G. hirsutum*.

Keywords: *Gossypium hirsutum***,** shelf life stability**,** sporicidal activity**,** damping off disease**,** soil mycoflora

1. Introduction
Cotton (*Gossypium* spp) originated as a tropical/ subtropical perennial plant, but is produced as an annual crop in many temperate regions around the world. The epicotyl of the cotton plant is poorly developed and thus the first true leaves may not expand for a period of several weeks. As a result planting cotton under marginal environmental conditions (i.e, temperate regions) and crops slow physiological development, the plant remains in the seedling stage for an extended period. Seedling diseases on cotton are a worldwide problem [1]. An estimated 3.6% loss in production was associated with seedling diseases over the last 10 years. The primary pathogens in seedling disease complex of cotton include *R. solani*, *Pythium spp*, *Thielaviopsis basicola* and *Fusarium spp* [2, 3]. *R. solani* is a prevalent and important pathogen in the seedling disease complex of cotton wherever cotton is grown [4, 15]. *R. solani* has been associated with all stages of seedling disease including seed decay, pre emergence damping off and post emergence damping off. In addition to seedling disease, it was found that *R. solani* is one of the primary agents in boll rot in *Lousiana* [16, 18]. Neal (1944) reported a leaf spot caused by *R. solani*.
In addition *Aspergillus flavus* is a saprophytic and pathogenic fungus with a cosmopolitan in distribution. It causes boll rot and lint rot in *Gossypium*. It's specific name flavus derives from the latin meaning yellow, a reference to the frequently observed colour of the spores. Spores are more difficult to eradicate compared to its vegetative cells, due to their ability to withstand adverse environmental stress, such as heat and chemical treatments [19]. Now a days there is interest in using natural products such as plant extracts for controlling diseases or pathogenic microorganisms and spores due to their cost effective and ecofriendly nature. Keeping in view the problem of chemical management of plant diseases, the use of plant extracts and biocontrol agents were evaluated both *invitro* and *in vivo* to find out their efficacies for eco-friendly in the management of plant diseases.

2. Material and Methods
Preparation of plant extracts
For *invitro* evaluation 10 plant species were collected and predicting to posses' bioactive compounds, plant species were collected based on the information available from literature [20, 22] folklore [23] and through field observations. The plant materials were collected in and around Visakhapatnam district, Andhra Pradesh, India. The collected plant materials were washed thoroughly with running tap water and finally with distilled water the material was chopped into small pieces and then air dried on a sterile blotter under shade for 20- 30 days.
The completely shade dried plant materials were coarsely powdered and allowed to Soxhlet extraction with solvents (methanol for methanol extract and water for aqueous extract) 5-6 hours at temperature not exceeding the boiling point of the solvent and then filtered through Whatman no-1 filter paper. The extracted liquid obtained was subjected to rotary evaporator and subsequently concentrated under reduced pressure (in vaccum at 40c). The residues obtained were designated as crude extracts, were labelled and stored in refrigerator for further study [24]. The dried plant extract residues obtained were redissolved in 0.1% Dimethyl Sulfoxide (DMSO)/ water to get necessary concentration of crude extracts and filtration through a 0.45µm membrane filter and stored in sterile brown bottles in a freezer at 20^0c until bio- assayed.

Microorganisms
Based on the disease index of *Gossypium*, *Fusarium oxysporum*, *Aspergillus flavus*, *Rhizoctonia sola*ni and *Alternaria alternata* (Table-1) were selected to screen for

antimicrobial activity against 10 plant extracts. The microorganisms tested were purchased from microbial type culture collection and gene bank (MTCC) Chandigarh, India. All the pure cultures were obtained in lyophilized or freeze-dried form are reconstituted in sterile water and produced a suspension of the microbial cells, inoculation was done with sterile inoculating loop to liquid broth medium. Liquid cultures are then incubated to allow cell replication and adequate growth of the culture, for use in bio assays. Following incubation, liquid cultures are refrigerated to store for further use. Typically, 24hrs will provide sufficient growth to allow visibly thick spread of the microbes for bio assay. The microbial strains are maintained and tested on potato dextrose agar medium.

Table 1: Common pathogen index of *Gossypium hirsutum* L. crop.

S. No	Pathogen	MTCC Code	Disease
1.	*Fusarium oxysporum*	F 1755	Fusarium wilt, boll rot and seedling disease
2.	*Aspergillus flavus*	F 1884	Lint contamination
3.	*Rhizoctonia solani*	F 4633	Leaf spot, boll rot and seedling disease
4.	*Alternaria alternata*	F 2723	Leaf spot

***In vitro* antimicrobial assays**

The development of simple *invitro* prescreens could offer initial idea of the biological activity of plant extracts and its compounds. The antimicrobial activity of microbial strains listed in Table-1was performed by agar ditch/ well/ cup diffusion method [25, 27] at desired concentration with DMSO solvent which did not affect the growth of microbes. The antimicrobial activity was evaluated by measuring zone of inhibition of the organisms against plant extracts.

***In vitro* sporicidal activity**

The effect of root extracts on the *invitro* germination of conidia of *A. flavus* was quantified. Hundred microlitres of each extract 2%, 1% and 0.5% (V/V) of *R. cordifolia* and *G. glabra* with sterile distilled water as control were mixed with equal volume (100µl) of conidial suspension on a cavity slide. The slides were placed in a humid chamber and incubated in dark at 24±1^{0}C and observed for germination of conidia after 24hours of incubation [28]. In each treatment 30 spores were observed and the experiment was repeated twice with 3 replicates. Sporicidal activity of each extract was expressed as percentage inhibition of conidial germination with respect to control. The experiment was repeated with three different concentrations of each tested samples of 2 plant species and the results were presented in Table-2, Figure-1.

Biocontrol of damping off-seedling disease caused by *Rhizoctonia solani* on *Gossypium* with plant extracts

Soil was collected from green house and was double sterilized at 20lb pressure with 48hours time interval. Soil was distributed into sterile polycups. Uninfested soil was used as a control. The seeds were sown in polycups containing sterilized soil served as control and also with 100µl of *R. solani* inoculums was placed in 3 cups of treatment.

The polycups were kept under acrylic domes in proper sterile conditions. The seedlings were grown well for first eight days. From the 8th day onwards there was a falling of seedlings observed in the untreated polycups with pathogen. The pathogen infested polycups soil was treated with 5ml 10% *R. cordifolia* aqueous root extract for 2times at time interval of 2days had no symptom of seedling rot. The treated and control polycups with seedlings were maintained upto 3months, while observing whether there was any negative effect on healthy growth of plants.

Control of aqueous plant extracts on soil borne pathogens

2% and 5% *R. cordifolia* and *G. glabra* aqueous root extracts were prepared with distilled water. Green house soil was collected and autoclaved according to the standard protocol. Soil was distributed into sterile petriplates and was infested with *Fusarium oxysporum*, *Alternaria alternata* and *Rhizoctonia solani* pathogenic strains and were also treated by incorporating 5ml of the *R. cordifolia* and *G. glabra* aqueous extracts into each of three petriplates and are incubated at 25^{0}C in BOD. Uninfested soil was used as control.

Experimental treatments includes- control soil without pathogens, control soil with pathogens only, soil infested with pathogens and treated with aqueous extracts of *R. cordifolia* and *G. glabra* at 5% and 2% concentrations respectively. Population densities were determined using dilution plate technique at 'O' before soil treatment and 1, 3, 5, 7 and 10 days after soil treatment and results were recorded.

Determination of shelf life or stability of the activity of plant extracts

To measure the stability of the bioactive plant extracts different formulated samples to be investigated were prepared with DMSO, water and methanol, the dry samples were stored at room temperature in small vails. They were tested for *invitro* antimicrobial activity as done earlier at 30days interval upto 20months of this experimental study.

3. Results and Discussion

***In vitro* antimicrobial activity**

Out of 10 plant extracts screened 2 plant extracts (*R. cordifolia* and *G. glabra*) showed significant antimicrobial activity as evidenced by a zone of inhibition (Table-2). Of all *R. cordifolia and G. glabra* produced the largest zone of inhibition against all the phytopathogens tested (Figure-1). Root extracts of *R. cordifolia* and *G. glabra* upto 5mg-l (W/V) concentrations showed significant activity against all tested pathogens. This is in agreement with the earlier studies of antimicrobial activity [29, 31] on *R. cordifolia* [32, 35] *G. glabra*. Both aqueous and methanolic root extracts of *R. cordifolia* and *G. glabra* had potent antimicrobial activity. The solvent control of methanol, DMSO and water did not show any effect on microbial growth.

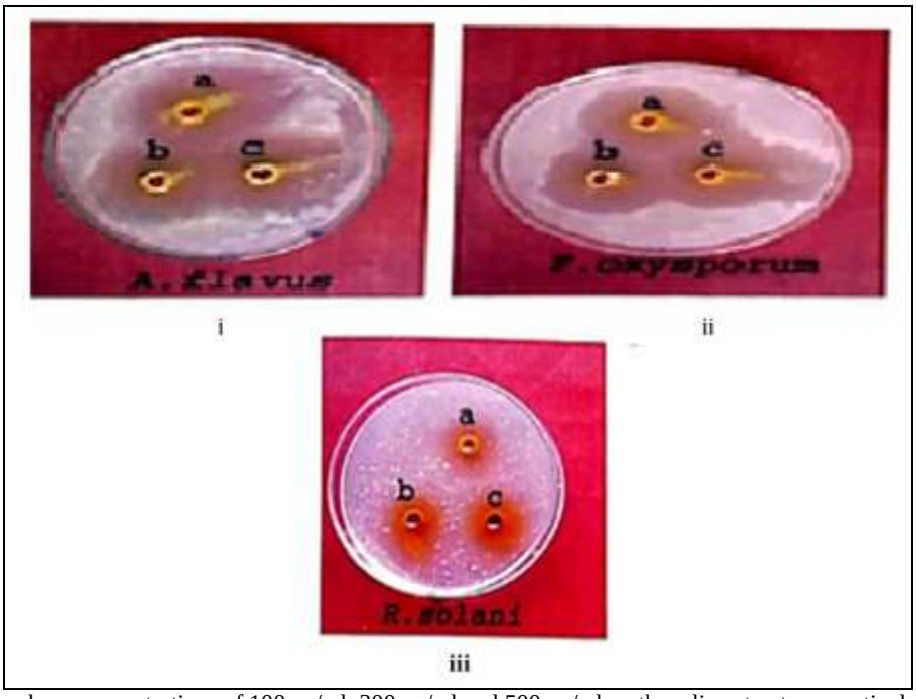
Boerhaavia diffusa and *Croton bonplandianum* extracts did

not show any markable antimicrobial activity towards the tested phytopathogens. This is in agreement with the report of antimicrobial activity reported by Salvat *et al*. [36] against some human pathogens. On the contrary aqueous extracts of *A. marmelos*, *M. kenigii*, *P. guajava*, *T. cordifolia* and *T. terrestris* did not show any remarkable activity [37]. *L. inermis*, *S. cumini*, *P. pterocarpum* have recorded significant antifungal activity. *Fusarium oxysporum*, *Aspergillus flavus*, *Rhizoctonia solan*i and *Alternaria alternata* are resistant to the plant extracts of *M. koenigii* and *D. metel*. This is in accordance with the report made by Saligrama Adavigowda Deepak *et al*. [38].

Table 2: Minimum Inhibition Concentrations of Some Plant Extracts against phytopathogens.

S.no	Plant extracts	*F. oxysporum* A B C	*A. flavus* A B C	*R. solani* A B C	*A. Alternata* A B C
1.	*R. cordifolia*	25 26 27	20 24 26	19 22 24	21 23 24
2.	*G. glabra*	34 35 37	30 31 32	18 20 21	20 22 23
3.	*B. diffusa*	10 10 10	9 10 10	10 10 10	10 12 12
4.	*A. marmelos*	10 10 11	15 17 20	6 6 7	9 9 9
5.	*L. inermis*	8 8 8	10 12 14	9 9 9	13 15 16
6.	*S. cumini*	10 10 11	10 13 13	0 0 0	10 13 14
7.	*M. kenigii*	10 14 15	7 9 10	9 9 9	11 12 13
8.	*T. cordifolia*	10 14 14	7 8 9	8 11 12	9 10 12
9.	*P. pterocarpum*	0 0 0	15 17 19	15 18 19	22 24 25
10.	*L. inermis*	8 8 8	10 12 14	9 9 9	13 15 16

A, B, C- Concentrations of plant methanolic extracts at 100mg/ml, 300mg/ml, 500g/ml respectively. Diameter of zone of inhibition in mm includes well diameter 6mm.



a, b, c- concentrations of 100mg/ml, 300mg/ml and 500mg/ml methanolic extracts respectively.

Fig 1: antimicrobial activity of *G. glabra* (I & ii) and *R. cordifolia* (iii) methanolic extracts.

Shelf life stability of all the active extracts was same and stable for both dry and solvent state at room temperature during the period of testing i.e., up to 20 months revealing the retention of original antimicrobial activity even with prolonged shelf life, when proper care was taken.

Sporicidal effect of *R. cordifolia* and *G. glabra* aqueous root extract against *Aspergillus flavus* spores; The data obtained from the mean of all the three consecutive sets of experiments represent the percentage of spore germination in individual treatment with respect to control. From the data the percentage of conidial germination in water control was 79%. Aqueous root extract of *R. cordifolia* showed 74.58%, 69.24%, 54.84% of inhibition at concentrations of 2%, 1%, 0.5% and *R. cordifolia* and *G. glabra* showed 58.63%, 40.99%, 30.44% of inhibition at concentrations of 2%, 1% and 0.5% respectively (Table-3).

Table 3: Sporicidal effect of *R. cordifolia* and *G. glabra* aqueous root extracts against *Aspergillus flavus.*

	Percentage concentration of sample (W/V)	Percentage of germination	Percentage of inhibition
R. cordifolia	2	15(25.42)	74.58
	1	20(30.76)	69.24
	0.5	28(45.16)	54.84
G. glabra	2	24(41.37)	58.63
	1	36(59.01)	40.99
	0.5	48(69.50)	30.44
Water	control	79(100)	-

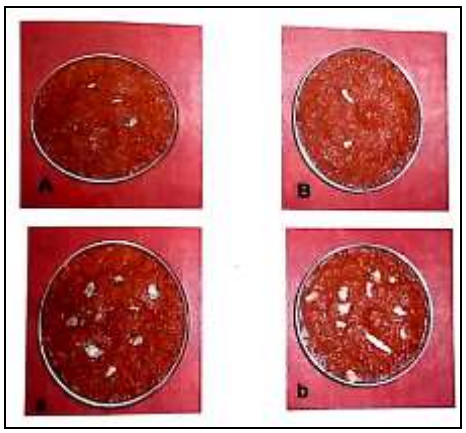
The values are the mean of three sets of experiments. The values presented in parenthesis represent the percentage of spore germination in individual treatment with respect to control.

Field experiments

In comparison with the untreated soil control *invitro* experiments (Figure-2) aqueous root extracts of 5% *R. cordifolia* and *G. glabra* showed significant reduction in the population density of soil borne pathogens *Fusarium oxysporum*, *Alternaria alternata* and *Rhizoctonia solani*. In the green house experimental observations, the formulated aqueous root extracts concentration of *R. cordifolia* and *G. glabra* significantly reduced the severity of seedling falling due to seedling disease caused by *R. solani* event at 10% W/V concentrations and have no adverse effects on seedling emergence and plant growth (Figure-3). The effect of extracts on the seedling germination and plant growth in the green house experiments reveals that *R. cordifolia* and *G. glabra* extracts did not affect the water absorption capacity of the seeds since the results obtained from the tested seeds were similar to those of the control seeds. So, it does not show adverse effect on seed germination of *Gossypium*. This finding supports the earlier report of [39].

Mycoflora biocontrol experiments

As compared with the untreated soil, growth inhibition of soil borne phytopathogenic fungi namely *Alternaria alternata* and *Rhizoctonia solani* was observed (Figure-2). Results reveal that these plant root extracts have potential and ecofriendly, cheap and safe alternatives for pre-plant fumigation with methyl bromide which is being used to control the soil mycoflora. However, the United States Environmental Agency (USEA) and the Clean Air Act have banned the use of methyl bromide [40].



A, B- Soils infested with *R. solani* and *A. alternata* respectively.
a, b- Respective control with *R. cordifolia* and *G. glabra.*

Fig 2: Biocontrol of soil mycoflora by *R. cordifolia* and *G. glabra* aqeous root extracts.

A. Seedlings of *G. hirsutum*, soil infested with R. solani.
B, C- Seedlings showing damping off disease.
D- Disease controlled by *R. cordifolia* aqueous root extract.

Fig 3: Biocontrol of seedling disease on *Gossypium hirsutum* by *R. cordifolia* root aqueous extracts.

4. Conclusion

The control of soil borne phytopathogens is accomplished with MeBr fumigation which contaminates the environment, affects the ozone layer, destroys the soil mycoflora and must be applied every season because of its null residual activity and rapid recolonization of soils by phytopathogens. Considering the difficulty to generate suppressive soils similar to the natural ones, the plant extracts provide an alternative to the use of chemical fungicides.

Based on the results aqueous root extracts of *R. cordifolia* and *G. glabra* showed significant affect against *A. flavus* and reduction in soil mycoflora both *invitro* and *invivo* suggests that these extracts may have potential in management of diseases like damping off and as possible alternatives to synthetic fungicides. The results of the present studies may be helpful in formulating the plant based natural fungicides in controlling common destructive diseases of *Gossypium hirsutum*.

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