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Invitro Studies on Antagonistic Potential of Trichoderma Species and Plant Extracts against Various Plant Pathogens

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Abstract

The current study was conducted out with the aim of isolating disease-causing fungi from infected plant parts and to find an effective biological method for controlling these pathogens. Four pathogenic fungi were isolated from diseased plant parts, viz, *Colletotrichum gloeosporioides*, *Alternaria solani, Fusarium oxysporum* and *Rhizoctonia solani*. These isolated plant pathogens were tested under *in vitro* conditions with different species of fungal biocontrol agent *Trichoderma*, extracts of commonly available plants with known antimicrobial activity and chemical fungicides, to find out a suitable environmentally friendly agent for controlling and managing the plant diseases under study. Amongst the fungal agent for biocontrol, *Trichoderma harzianum* proved to be the most effective. Herbal fungicides are becoming popular due to the fact that they are cost effective as well as eco-friendly. If this antifungal property resides in weeds, it will be a great advantage. In this study, of the 8 plant extracts utilized, *Lantana camara* proved to be most effective in controlling all the four pathogenic fungi. This was followed by *Murraya koenigii*. The biocontrol potential of Lantana is of great importance because it is an obnoxious weed and the use of Lantana will solve the dual purpose of controlling the weed as well as pathogenic fungi. The results achieved with plant extracts are comparable to those observed with the chemical fungicides. It is therefore suggested that a suitable strategy combining all the methods of control can be worked out for these pathogens in a future course of study.

Keywords: *Trichoderma; Biocontrol; Plant Extracts; Fungicide; Ecofriendly*

Introduction

Fungal diseases cause severe losses to agricultural and horticultural crops every year. These diseases result in decreased food supplies, low quality agricultural products and biproducts, low returns per capita for the growers and the processors. All this ultimately leads to higher prices for the consumer [1]. Multiple measures may be taken to prevent, mitigate or control such severe plant diseases. Besides good horticulture and agronomic practices, chemical fertilizers and pesticides are also considered important in the control of plant

diseases. But these are often not economical nor are they very effective under field conditions. The harmful effects of such agents include resistance and biological magnification. These are also dangerous with regards to t human health as well as the entire food web. Many such agents are proven to be either mutagenic, carcinogenic or tetratogenic [2]. Therefore, researchers are looking for viable alternatives to synthetic chemicals the control of pests and diseases. Focus is now building around the development of Biological Control Agents (BCAs) to control plant diseases. These offer environmentally friendly approaches for managing of plant disease and the practice of control of diseases can be strengthened with cultural and physical control measures [3,4]. Biological control therefore offers a way forward in the development of sustainable agriculture systems. An excellent example is the fungus *Trichoderma*. *Trichoderma* species have been considered possible biological control agents since the last 70 years and some strains, recently, have also been made available commercially. Genus *Trichoderma* as potential bioagents was first conveyed in 1932 by Weindling. More research is now being conducted on elucidation of the mechanism of disease control which includes developing an understanding the mode of action of *Trichoderma* [5].

Perusal of literature has shown that in the last few years, plant extracts are gaining importance in plant disease management [2] and may offer ground breaking alternatives to the current harmful and inefficient practices. Therefore, the present study planned to undertake *invitro* studies on various *Trichoderma* species and their potential to antagonize isolated plant pathogens. An attempt to determine by *invitro* studies if plant extracts as potential bio-control agents against the isolated pathogens was also made.

Materials and Methods

Collection, isolation, purification and maintenance of fungal isolates from plant samples with disease

Various infected plant parts were collected from Khrishi Vigyan Kendra (KVK) Dhaula Kuan, Paonta Sahib, Himachal Pradesh and Forest Research Institute (FRI), Dehradun, Uttarakhand. A large machete (surface sterilized) was used to cut large or woody stems, to dig out whole plants specimens and to collect soil samples. A small machete was used to cut small hard stems. Clean water was used to wash all cutting implements Diseased plants were carefully removed from the soil and examined for symptoms. Wilting, stunting and leaf yellowing usually indicated a disease of the roots or stem. The infected plants parts or tubers were individually cut into small pieces and were then surface sterilized. This was done by dipping them in 0.1% mercuric chloride ($HgCl_2$) (20 - 25 seconds) and later washing in sterile distilled water five times. Extensive washing with sterile distilled water followed. The samples were then allowed to air dry and placed on Petri plates containing Potato Dextrose Agar (PDA) and incubated at 27° ± 1°C for five days. The fungal colonies were sub cultured on individual PDA plates. Incubation at 27° ± 1°C for five days followed. Pure cultures were identified and maintained at 4°C.

Screening and evaluation of fungal biocontrol agents

Four species of *Trichoderma* viz *T. longibranchi, T. viride, T. harzianum and T. koningii* were acquired from National Type Culture Collection of the Plant Pathology Laboratory, FRI, Dehradun. All the four species were tested for antagonism using dual culture against plant pathogens [6]. After a 7 day incubation period, mycelia growth inhibition was found by the formula: PG%= (dc - dt)/dt x 100 where PG%=percentage of growth inhibition. Dc is the fungal colony diameter in control sets and dt is the fungal colony diameter in treatment sets.

Invitro evaluation of chemical fungi-toxicants

The poisoned food technique by Nene and Thapliyal [7] was employed for evaluating the fungi-toxicant activity of commercially available fungicides. The experiment was laid out with two different treatments each with three replicates. The experimental design employed was factorial completely randomized design (CRD). Copper oxychloride 50%WP (Treatment 1) and Carbendazin 50% WP (Treatment 2) fungi-toxicant used in lab assay. The tradenames of the agents used were BLITOX 50 and BAVISTIN respectively. Effects of these fungicides on the linear mycelia growth of fungal isolates were evaluated by in *invitro* tests at 500 ppm concentrations.

Invitro interaction of fungicides

Petri plates (9 cm diameter) containing 14 ml fungicide (500 ppm) amended Potato Dextrose Agar (PDA) medium were used. Petri plates containing fungicides free PDA medium were used as control. Three replicates were used for each treatment.

Each petri plate was inoculated with 1 cm disc of 7-day old fungal culture in center. These were incubated at 27° ± 1°C for 7 days. Linear mycelia growth reduction percentage of the pathogenic fungi was calculated the formula: (C-T)/C Where C is the mycelia diameter in the control. T is the mycelia diameter in each treatment.

Invitro efficacy of plant extracts against fungal pathogens

Eight common and readily available plants viz *Lantana camara, Murraya koenigii, Catharanthus roses, Solanum nigrum, Azadirachta indica, Tagetes patula, Eucalyptus* spp. and *Ageratum conyzoides* were used for early screening of their action against phyto-pathogenic fungi. Poisoned food technique as described by Nene and Thapliyal [7] was used.

Preparation of plant extracts and pathogens

50 ml extract was prepared by adding sterilized distilled water to 25 grams of material and crushed by using grinder/pestle and mortar. Whatman filter paper no.1 was used for filtration of the extract. The filtrate was treated as 50% solution. To obtain concentration of 5%, 10 ml of 50% stock extract, was added to conical flasks of 250 ml containing 45 ml of sterilized PDA in laminar air flow. Petri dishes (9 cm diameter) containing potato dextrose agar medium amended with plant extracts were used for *in vitro* interaction of pathogens with plant extracts: Three replicates of each treatment as well as control were used. Each Petri plate was inoculated with 1 cm disc of 7-day old fungal culture in the Centre. All inoculated plates were incubated at 27 ± 2°C, until the mycelia growth reached to edge of the control plate. The percentages of the linear mycelia growth reduction of pathogenic fungi were calculated using the following formula: (C-T)/T x 100. Where I is the percent inhibition, C is the growth in control and T is the growth in treatment.

Results

A diagnosis of the plant diseases was done on the basis of careful study of symptoms and then the isolation and identification of the disease-causing plant pathogens. Four phytopathogenic fungi were isolated from the diseased host plants. The details of the diseases and the isolated fungi are summarized in table 1.

Table 1: *Diseases studied and pathogens isolated.*

Host	Part Infected	Disease	Pathogen
Tomato Lycopersicon esculentum	Shoots	*Fusarium* wilt	*Fusarium oxysporum*
Mango *Mangifera indica*	Fruits	Anthracnose	*Colletotrichum gloeosporioides*
Ginger *Zingiber officinale*	Rhizome	Rhizome rot	*Rhizoctonia solani*
Tomato *Lycopersicon esculentum*	Shoot and Fruits	Early blight	*Alternaria solani*

Control of isolated plant pathogens

Both the fungicides used in the study were found to efficiently inhibited mycelial growth of the isolated pathogenic fungi, viz Colletotrichum *gloeosporioides, Alternaria solani, Fusarium oxysporum* and *Rhizoctonia solani. Colletotrichum gloeosporioides* was the most responsive to the two test fungicides. There was 100% growth inhibition of *Colletotrichum* with both the fungicides used. *Alternaria solani* and *Rhizoctonia solani* were inhibited completely by Blitox, but showed 92.5% and 81.5% inhibition respectively with Bavistin. For *Fusarium oxysporum* 90% growth inhibition was observed with both the fungicides. Table 2 shows the interaction between the isolated pathogenic fungi and four species of *Trichoderma.* It was seen that all the species of *Trichoderma* efficiently inhibit all the four pathogens.

Table 2: *Inhibition of plant pathogens by fungal antagonists dual culture method [6].*

Antagonist	***Plant pathogen (% growth inhibition)***			
	Colletotrichum gloeosporioides	***Alternaria solani***	***Fusarium oxysporum***	***Rhizoctonia solani***
T. harzianum	84.5	54.7	63.6	61.1
T. koningii	81.7	46.8	63.6	48.1
T. longibranchi	85.4	58.7	59.4	61.1
T. viride	83	47.2	60.6	44.4

In case of *Colletotrichum gloeosporioides* all the *Trichoderma* species completely overgrows the test fungus and covers the entire surface of the medium (Figure 1c-1f).

Similar results are also seen in the interaction of the four selected *Trichoderma* species with *Fusarium oxysporum* (Figure 2-4, c-e). For the other two pathogens antagonistic fungi grew in more than two thirds of the media. The percent inhibition of soil borne fungal pathogens by the four species of *Trichoderma* under study are given in table 2. Amongst the fungal antagonists *T. harzianum* proved to be best inhibitor causing more than 55% inhibition of all the pathogens (Table 2). Dry leaf extracts of eight plant species under study were tried as likely biological control agents against the isolated pathogens of plants (Table 3 and figure 1-4).

Table 3: *Inhibition of plant pathogens at different concentrations of crude plant extracts.*

Plant Extract (5% concentration)	**Percent Inhibition of pathogenic fungi**			
	Colletotrichum gloeosporioides	***Alternaria solani***	***Fusarium oxysporum***	***Rhizoctonia solani***
Solanum nigrum	56.3	21.9	-16.4	46
Lantana camara	90.1	78.6	66.8	59.3
Ageratum conyzoides	63.6	40	33.9	16.6
Murraya koenigii	85	61.1	50.9	53.7
Catharanthus roseus	65.2	27.8	8.5	25.9
Azadirachta indica	51.17	10.4	-11.5	42.6
Tagetes patula	82.1	35.8	44.8	24
Eucalyptus spp	71.8	33.8	39.7	24

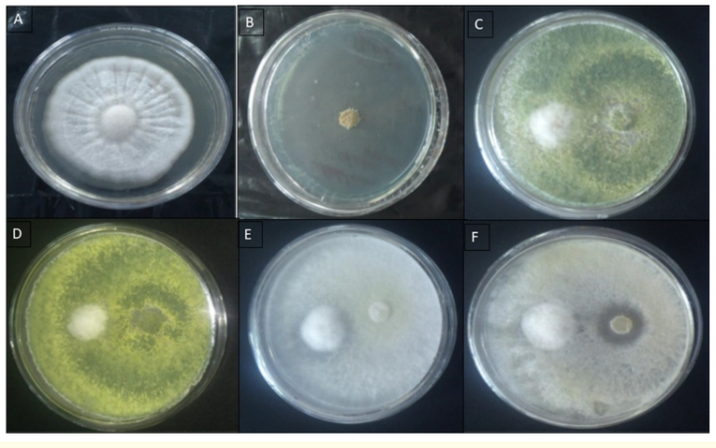

Figure 1: *Interaction of Colletotrichum gloeosporioides with biological and chemical control agents.*
a). Control plate of C. gloeosporioids b). Colletotrichum treated with fungicides Blitox
c). Colletotrichum grown with T. harzianum d). Colletotrichum grown with T. longibranchi
e). Inhibition of Colletotrichum by T. viridi f). Colletotrichum growing with T. koningii

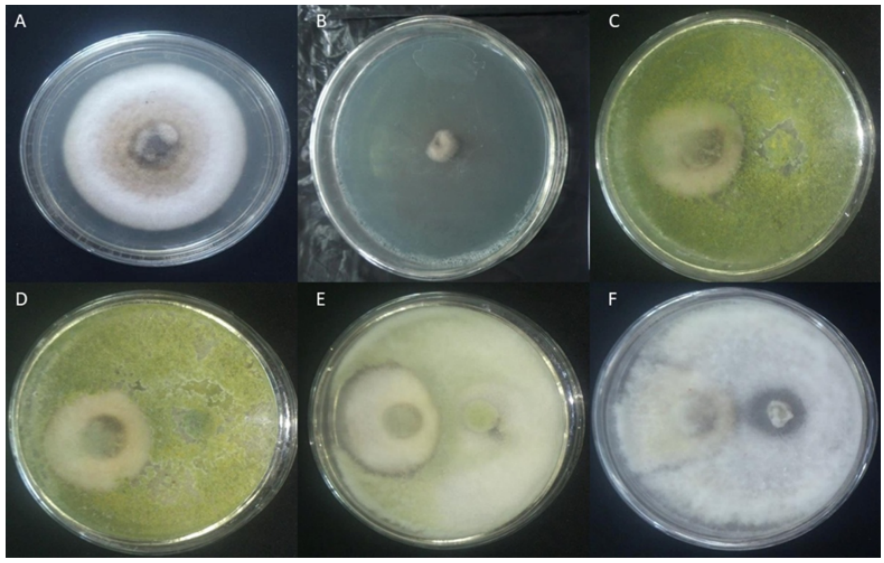

Figure 2: *Interaction of Alternaria solani with biological and chemical control agents a). Control plate of Alternaria solani b). Alternaria in media poisoned with fungicide Blitox c). Alternaria growing with T. harzianum d). Interaction of Alternaria with T. longibranchi e). Dual Culture of Alternaria and T. viridi f). Antagonism of T. koningii with Alternaria*

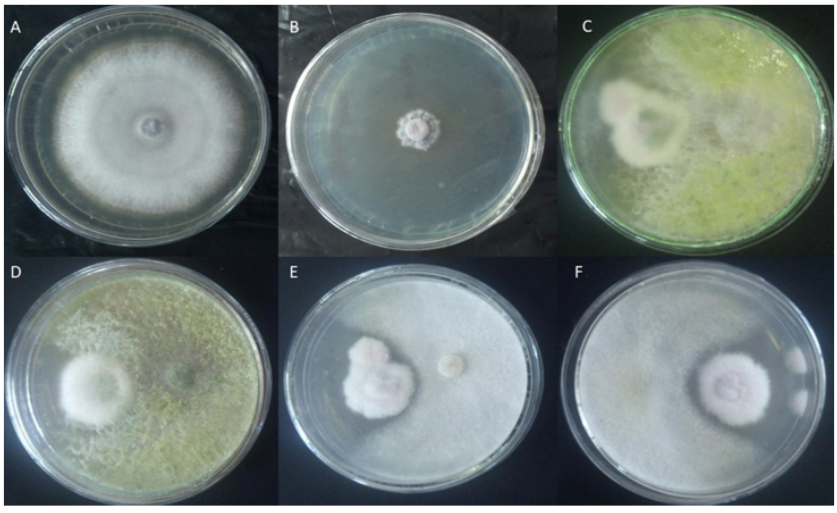

Figure 3: *Interaction of Fusarium oxysporium with Trichoderma spp and chemical control agents a). Control plate of Fusarium oxysporium b). Fusarium in Blitox poisoned media c). Fusarium and T. harziaum dual culture d). Inhibition of Fusarium byT. Longibranchi e). Fusarium growingwith T. viridi f). Fusarium growing with T.koningii*

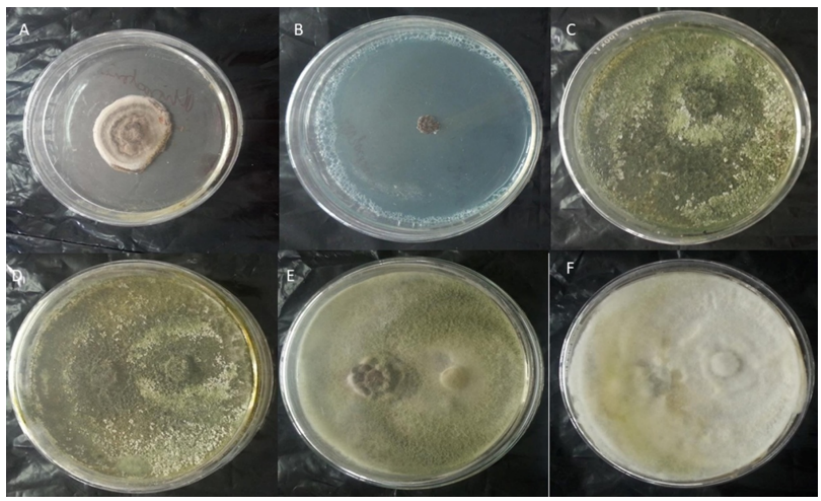

Figure 4: *Interaction of Rhizoctonia solani with Trichoderma and fungicide a). Control plate of Rhizoctonia solani b). Rhizoctonia treated with fungicides Blitox b). Rhizoctonia growing with T. harzianum d). Rhizoctonia growing with T. longibranchi e). Rhizoctonia growing with T. viridi f). Rhizoctonia growing with T. koningii*

Plant pathogens inhibition at different concentrations of crude plant extracts

A comparison of all the control treatments viz., fungal antagonist, plant extract and fungicidal chemicals, was done for each plant pathogen under study (Table 4). The aim was to find a suitable alternative biological agent which could control the disease-causing pathogen to the same or comparable degree as chemical fungicides (Figure 5-8).

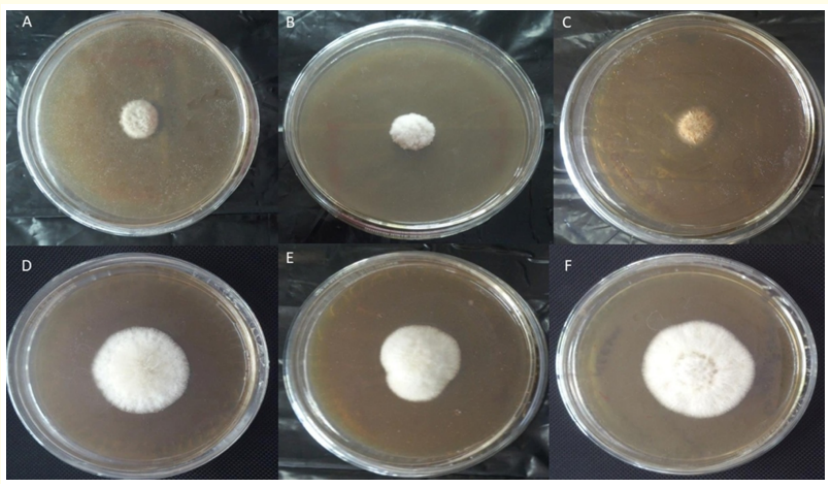

Figure 5: *Colletotrichum gloeosporioides growing in media poisoned with plant extracts a). Colletotrichum in Murraya extract b). Colletotrichum in Lantana extract c). Colletotrichum in Tagetes extract d). Colletotrichum in solanum extract e). Colletotrichum in Catharunthus extract f). Colletotrichum in Neeem extract*

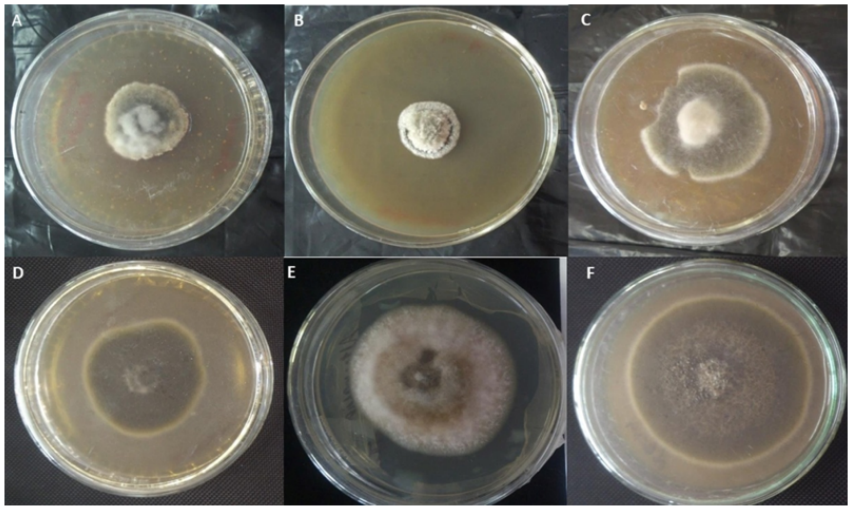

Figure 6: *Growth of Alternaria alternata in different plant extracts*
a). Alternaria in Murraya extract b). Alternaria in Lantana extract
c). Alternaria in Tagetes extract d). Treated Alternaria in solanum extract
e). Alternaria in Catharunthus extract f). Alternaria in Neem extract

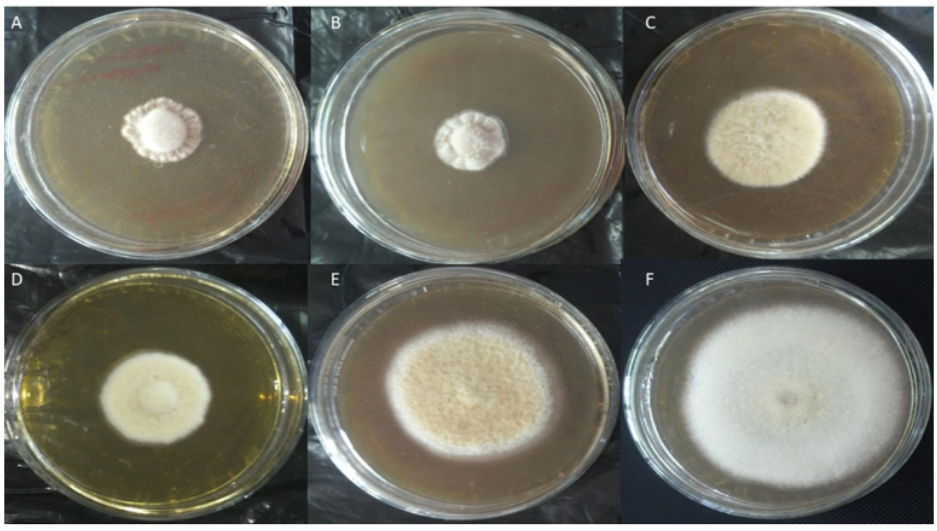

Figure 7: *Fusarium oxysporium growing in media poisoned with plant extracts*
a). F. oxysporium in Murraya extract b). F. oxiysporium in Lantana extract
c). F. oxysporium in Tagetes extract d). F. oxysporium in Ageratum extract
e). F. oxysporium in Catharunthus extract f). F. oxysporium in Neem extract

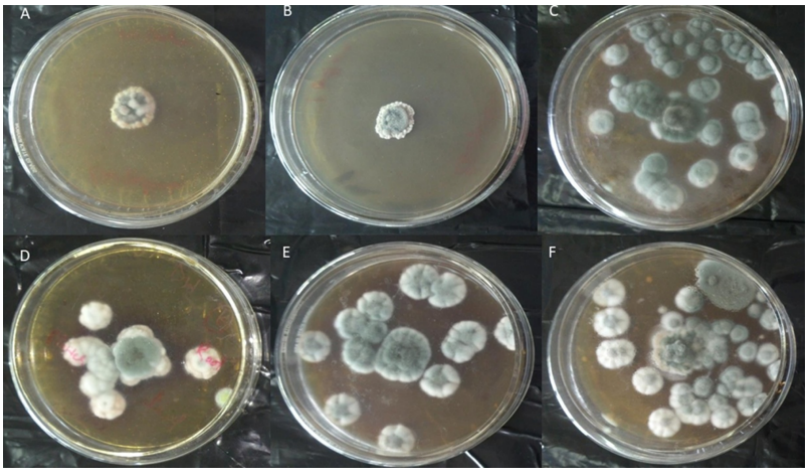

Figure 8: *Rhizoctonia solani in media amended with plant extracts a). Rhizoctonia in Murraya extract b). Rhizoctonia in Lantana extract c). Rhizoctonia in Tagetes extract d). Rhizoctonia inAgeratum extract e). Rhizoctonia in Catharunthus extract f). Rhizoctonia in Eucalyptusextract*

Discussion

The fungicides used in the present study were found to inhibit the test fungi but to variable extents. The two fungicides completely inhibited the growth of *C. gloeosporioides*. lesser inhibitions were seen for the other three pathogens. Comparable studies have been conducted by several earlier researchers. Carbendazim was reported as highly effective [8-11]. Davidse [12] reported that carbendazim induces nuclear instability by disturbing the mitosis and meiosis of the pathogenic agents.

The genus *Trichoderma* has long been recognized for its ability as biocontrol agent against plant pathogens. *Trichoderma* species are saprophytic fungi and are commonly found in a myriad of soil and rhizosphere micro flora. These are seen as potential biocontrol agents due to of their ability to decrease the incidence of disease caused by plant pathogenic fungi. This is especially true for many common soil borne pathogens [13-15]. Imperative to mention that some workers have also occasionally reported it as a plant pathogen. However, the ability of *Trichoderma* isolates in regulatory pathogens of plant is well documented [14] and this is in consonance with the results of our study. Biocontrol with *T. harzianum* under commercial conditions has also been extensively studied, and in this regard, some significant achievements have been reported in greenhouse crops and in vineyards [13]. The first BCA registered, commercialized, and used in greenhouse crops and vineyards was the isolate T-39 of T. harzianum (TRICHODEX). This was found to effectively control *Botrytis cinerea*, *Sclerotinia sclerotiorum* and *Cladosporium fulvum* diseases in greenhouse-grown tomatoes and cucumbers, and also worked well in vineyards [13,14].

The *Trichoderma* spp. antagonism against many fungi may mainly be due to the production of acetaldehyde compound [16,17]. Godtfredsen and Vagedal (1965) [18] reported the production of a compound called Trichodermin and found dermadin, a volatile antibiotic produced by *Trichoderma* spp., which they suggested, suppressed several plant pathogens. *Trichoderma* BCAs are found to exert some other positive effects on plants like increase in plant growth (biofertilization) and stimulation of plant's own defense mechanism [19].

Among the eight plant extracts tested against *C. gloeosporioides, Lantana camara* at the concentration of 5% showed 90.1% inhibition. The inhibition by *Lantana camara* was found to be superior to all the other plant extracts. Similar inhibition pattern of the pathogenic fungi by *Lantana* was also been reported by [19] Tasiwal., *et al* (2009).

Some plant extracts used in the present investigation produced negative inhibition or growth enhancement of the fungal pathogen. However, earlier researchers have demonstrated positive inhibition of fungal growth by these two plant extracts [19]. The results of present study, therefore, present a deviation from the known earlier works and these need to be investigate further. Several published reports were traced on the antifungal activities of medicinal plants, herbs and cultivated plants. However, few reports on the exploitation of antifungal property of weeds plants could be traced and the data regarding use of weeds as antifungal agents is scarce [20]. Therefore, the current study may prove to be a baseline work for the conduct of further research in the area and be of benefit for the health and well being of the man as well as the environment.

Conclusion

We conclude from our study that weeds may potentially be useful for the development new biofungicide. In the Indian scenario Lantana is considered to be one of the most difficult weeds to eradicate. The positive inhibition of the pathogenic fungi by Lantana can be a way forward towards achieving dual benefits viz the ecofriendly control of pathogenic fungi and the eradication of an invasive weed species. Biological control could be the best alternatives to chemical pesticides and may prove to be an eco-friendly, viable solutions against soil borne pathogens.

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