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ANTIFUNGAL ACTIVITY OF PLANT EXTRACTS AND *TRICHODERMA* SP. AGAINST SEED FUNGI OF COWPEA

RAZIA K ZAIDI & NEHA PATHAK

Section of Plant Pathology and Nematology, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

ABSTRACT

Seeds of many crops are known to carry various types of pathogenic and non-pathogenic fungi which are commonly known as seed mycoflora or seed-borne fungi. Depending upon the presence of fungi either on seed coat or in the seed it is further called as external seed-borne fungi and internal seed-borne fungi. Among the different disease causing agents, fungi are major agents responsible for seed deterioration. Hence the present investigations were carried out to study the mycoflora associated with cowpea (*Vigna unguiculata*) seeds, deteriorations caused by these pathogens and to find out an effective control by integrated methods. The detection of seed-borne fungi of cowpea was done by using the blotter, agar plate and deep freezing methods as recommended by ISTA. Sample of cowpea seeds collected from the agriculture farm of Aligarh, a total number 10 species belonging to different genera of fungi were isolated and identified on the bases of their mycelium growth and fruiting bodies.

Aspergillus niger and *Alternaria alternata* were quite common in both non sterilized & sterilized seeds with high frequency value and relative abundance. The samples were treated with fungicides, botanical and biocontrol agents to control the seed-borne pathogens. The experiment was conducted in randomize complete block design with five replication. The results were recorded as regards to the highest seed germination (86.3%) reflected the lowest percentage of pathogen (5.0%) in the treated seeds with natural products and biocontrol agents. Furthermore, the use of fungicides has resulted in the buildup of toxic chemicals potentially hazardous to man and environment and also in the buildup of resistance by pathogens. Therefore the development of bio pesticides has been focused as a viable pest control strategy in recent years. A natural product produced by plants is definitely a new source of potential pesticides as different botanicals in the present studies gave encouraging results.

KEYWORDS: Cowpea (*Vigna unguiculata*), Seed-Borne Fungi, Botanicals, Chemicals, Biopesticides

INTRODUCTION

Cowpea is one of the most ancient human food sources and has probably been used as a crop plant since Neolithic times. It is mainly consumed as a favorite foodstuff in the form of dried seeds, either as flour or vegetable. It is a good source of carbohydrates, vitamins, and protein, providing more than half of plant protein in human diets. Cowpea (*Vigna Ungiculata*) is considered as one of the vegetable crops which have an importance for local consumption and exportation purposes in India. Cowpea legumes are affected by various fungal pathogens; though various systemic fungicides are used to control fungal diseases but their indiscriminate use may cause environmental hazards.

Seed health plays an important role for successful cultivation and yield exploitation of crop species. Among various factors that adversely affect seed heath, the most important are the seed borne fungi that not only lower seed germination but also reduce seed vigor resulting in low yield. Seed health testing for the presence of seed borne pathogens is an important step in the management of crop diseases.

The increasing agricultural production became an urgent issue since projections suggest that the global population will reach 9 billion people by the middle of this century (Godfray et al., 2010). According to the estimation, 1 billion people will suffer from hunger because they do not have access to food in terms of quantity (protein deficit) and quality (micronutrient deficit), while the vast majority will be living in the developing countries. Besides the increase of the human population, the world is facing new challenges, such as shrinking cultivable lands, stagnant yields, increasing bio-fuel demands, new emerging pathogens and pests, and salinity and flooding due to climate change.

Chemical control of disease has several disadvantages and it is neither environmentally safe nor friendly. The use of alternative biocontrol option is more preferable whenever possible. The antifungal properties of some plant extracts were investigated with the aim of finding alternatives to the use of chemicals.

However, the average Indian farmer cannot afford the increasing cost of synthetic chemicals. Furthermore, the use of fungicides has of late resulted in the buildup of toxic chemicals potentially hazardous to man and environment and also in the buildup of resistance by pathogens (Sinclair, 1971; Adesiyan, 1983).

Plant extracts are being used to control the diseases since last several years. Extracts of the various plant parts like leaf, stem, root, fruit and seeds are found to be effective against seed-borne pathogenic fungi.

Problems of environmental contamination which have adversely affected the biodiversity in agro ecosystems, as well as health and human safety problems inherent to the production and inadequate use of agrochemicals, have led to the search for and implementation of ecological alternatives. Members of the fungal Genus *Trichoderma* have been studied extensively, particularly because of their ability to act as biocontrol agents. (Melo 1991, Monte 2001.) *Trichoderma harzianum* has been efficient in control of several pathogens. (Adekunle et al., 2001). Therefore the development of biopesticides has been focused as a viable pest control strategy in recent years.

Source of potential new pesticides is natural products produced by plants. Exploitation of plant metabolites in crop protection and prevention of bio-deterioration caused by fungi appear to be promising. In view of these, the author screened some leaf extracts and biopesticide (*Trichoderma viride*) against seed-borne pathogenic fungi and the data has been presented in this paper.

Methods and Treatments for the Detection of Seed-Borne Fungal Pathogens

For the detection of seed borne fungi, the blotter method, PDA, and Deep freezing methods were employed (ISTA, 1996) using 300 seeds per sample.

Blotter Method: Three pieces of blotting paper were placed in each petriplates 9 cm diameter and incubated at 25±2^{o}C. Three replicates were prepared. The percent germination of cowpea seeds and fungal colony development on seeds were calculated after 8 days.

PDA Method: Sterilized petriplates of 9 cm diameter containing the potato dextrose agar media were used. In each petriplate 10 seeds were placed. Three hundred seeds were used for each sample. Petriplates were incubated at

20±2°C for 8 days with cycles of 12 hours light and 12 hours darkness. After 8 days of incubation fungi which developed on the seeds were identified.

Deep Freezing Method: The procedure is similar to the standard blotter method except that after 48 h of incubation under 12/12 h NUV and darkness, petriplates were transferred to deep freeze at 20°C in complete darkness for 12 h alternate cycles of NUV and darkness for 5 days. Seeds were then examined for fungal growth under a stereo microscope. Identification by habit character was supplemented by microscopic examination of spores and fruiting bodies using a compound microscope.

Management Strategy

Collection of Cowpea Seeds: Stored cowpea seeds of previous season were collected from Quarsy agricultural farm.

Collection of Plant Materials: Fresh and healthy leaves of five plants viz., *Azadirachta indica*, *Euphorbia hirta*, *Lantana camara*, *Calotropis procera*, *Eucalyptus globulus* were collected from the Aligarh Muslim University Campus, Aligarh. Leaves were washed thoroughly with detergent to remove any dust. Washed leaves were dried in an electric oven at 30 °C for 72 hours and crushed to make powder.

Preparation of Aqueous Extract: Twenty grams of dried leaf powder of each of the 5 test plants was soaked in 100 ml of sterilized distilled water for 24 hours. Extract was filtered through a double layered muslin cloth followed by Whatman No. 1 filter paper and used in the further experiment immediately.

Aqueous Extract Bioassay: Cowpea seeds were soaked in 20% (W/V) aqueous extracts of test plant species for 10 and 20 minutes. For control, the seeds were soaked in distilled water. For reference a treatment of surface sterilization with 1% Sodium hypochlorite (NaOCl) for 2 min., was also added. Seeds of all the treatments were placed on filter papers in 9 cm diameter Petri plates, moistened with 3ml. of distilled water. Plates were incubated in a growth room at 25 ± 2 °C for 10 days. Each treatment was replicated thrice.

Cup Plate Method: 20 ml of PDA medium was poured in sterilized petridishes (9 cm diameter) and allowed to solidify. Then pure cultures of fungi were streaked out in regular intervals on the media poured in petriplates. In the centre of the medium, a cup cavity of 8 mm diameter was made with sterilized No. 4 cork borer. This cup was filled with 0.1 ml of the leaf extract (Pawar and Papdiwal, 2010).

Antifungul Activity of *T. Harzianum* on Seed Borne Fungi

To study the inhibition (%) effect of *T. harzianum* against all five seed-borne fungi of cowpea, PDA was inoculated with a 6 mm block of *T. harzianum* isolates on one side of the plate and with all five fungus on each separate petriplates, 7 cm away on the other side. Control plates containing only seed borne fungus at the center of the plate were then incubated at 28 °C and observed regularly to assess growth. The growth of all five seed borne fungus was inhibited after it contacted *T. harzianum*.

Seed Treatment with Biopesticide *Trichoderma Harzianum*

For the seed treatment efficacy of the biological agent *T.harzianum* was applied as suspension of ground mycelia mat at the concentration of 2.0×10^8 cfu for 10 and 20 min respectively. Seeds uniformly coated with *Trichoderma* formulations were held for air drying overnight in darkness at room temperature. Treated seeds were placed on filter papers in 9 cm diameter Petri plates, moistened with 3ml. of distilled water. Plates were incubated in a growth room at 25 ± 2 ^{o}C for 10 days. Each treatment was replicated thrice.

Counting Methods for Inhibition Effects of *Trichoderma harzianum* and Plant Extracts

The percent inhibition of radial growth (PIRG) of the pathogenic fungi was calculated as described below.

$$\text{Inhibition } (\%) = \frac{X-Y}{X} \times 100$$

where X = mycelial growth (radial) of the pathogen on the control plate, and Y = mycelial growth (radial) of the pathogen on the dual culture plate.

Treatment with Fungicides

To test the efficacy of fungicides against the incidence of seed mycoflora, seeds were treated with fungicides namely vitavax and benlate. For this treatment, seeds were placed in 150 ml flasks with required doses of fungicides. The flasks were plugged and shaken for 10 and 20 minutes on the mechanical shaker so as to get the uniform distribution of fungicides on seed surface. The seeds after fungicidal treatment were removed from the flasks and placed in sterilized petriplates on moist blotter paper.

After 10 days of incubation, germination percentage of cowpea seeds was recorded. Fungal species found growing on the surface of seeds were identified and their percentage frequency of occurrence was calculated by applying the following formula:

$$\text{Frequency of occurrence } (\%) = \frac{\text{No.of seeds on which fungus appears}}{\text{total No.of seeds}} \times 100$$

Statistical Analysis

Data regarding germination and frequency of occurrence of various

Seed-borne fungi were analyzed statistically by applying SPSS 12.00 software (SPSS Inc. Chicago, IL, USA) wherever considered necessary. The data obtained were analysed statistically and significance was calculated at $p< 0.05$ and $p< 0.01$ levels of probability. Each experiment was replicated thrice.

RESULT AND DISCUSSIONS

Plant pathogens have a worldwide host range covering all groups of plants. The biological control plays an important role as per the modern concept of integrated disease management and for sustainable agriculture. Bio-pesticides, apart from reducing the use of synthetic fungicides also help to avoid damage avoid damage of non-targeted beneficial flora. Plant extract which are ecofriendly offer much important scope for their exploitation as a promising material for use in plant disease control.

The association of different pathogenic and saprophytic mycoflora with food crops reported by various researchers (Fakhrunnisa et al., 2006; Niaz and Dawar, 2009). In the present study, the incidence percentage varies among the three detection methods, *Alternaria alternata* followed by *Fusarium moniliforme* and *Aspergillus niger* recorded at maximum level 54% and 21% both in blotter method and PDA method respectively but at low level 10% to 07% in deep freezing method.

Isolation of the Fungal Flora

Cowpea seeds were found to differ both in quantity and quality of associated seed fungi. From Blotter method 10 fungi were detected. Similarly from PDA method, again 10 fungi were isolated from cowpea seeds. From Deep freezing method 7 fungi were isolated from cowpea seeds (Figure 1).

Fungal species viz *Alternaria alternata*, *Aspergillus niger, A.flavus, Rhizopus oryzae, Rhizopus* spp*, Mucor* spp, *Fusarium moniliforme*, *Drechslera australiensis*, *Penicillium*, *Curvulariya lunata* and *Cladosporium* spp*,* were isolated using the blotter method, PDA method and deep freezing method (Figure 1). Surface sterilization with 1% sodium hypochlorite arrested the growth of many fungal species except *A.alternata* and *Fusarium moniliforme* because of their internally seed-borne habit. However, sodium hypochlorite treatment significantly reduced the frequency of occurrence of these fungi up to 70% (Table 2).

The deep freezing and PDA method were good for the isolation of deep seated pathogenic fungi viz., *Fusarium* and *A.alternata* while blotter method was found suitable for germination and isolation of *Aspergillus*.

Effect of Aqueous Extracts of Plant, Biocontrol Agent and Fungicide on Mycoflora

All the plant extracts and *Trichoderma harzianum* as biocontrol agent effectively reduce the fungal flora of cowpea. When the fungi toxic activity is compared among the plant extract and *Trichoderma harzianum* than it formed that *T.harzianum* gave best results on reduction percentage of seed borne fungi.

The antifungal activity of plant extract and biocontrol agents *T.harzianum* against seed-borne fungus is presented in table 1 as zone of inhibition (in mm). It was observed that out of all plant extract, fungicide and biocontrol treatment, *T.harzianum* showed maximum activity from (mean activity zone 43.33 mm) against most frequently fungus *Alternariya alternata* and minimum activity was observed with leaf extract of *Euphorbia hirta* (mean activity zone 12.22 mm) on the same fungus. (Table 1)

In case of treatment with plant extract *Azadiracta indica* (13.2%,12.6%) exhibited maximum fungi toxicity as compared to control 42%, against the most frequently occurring spp i.e. *A.alternata* in the present study, resulting in significant reduction of fungal incidence and is equals to *T.harzianum* (13.0, 10.6) in 10 and 20 minute treatment respectively. The incidence of other fungal species was also markedly reduced by the extracts (Table 2). *Eucalyptus globules* extract was found to be the second most toxic agent against *A. alternata* resulting up to 60% suppression in fungal incidence. Incidence of other four fungal species was also markedly declined by the extract treatments (Table 2). Aqueous extract of *A. indica* has also been reported to cause significant growth inhibition of other fungi such as *Rhizoctonia solani, Botrytis cinera* and *Fusarium oxysporum* (Alkhail, 2005).

T.harzianum showed good antagonistic effect to the tested fungi. Significant results were obtain in terms of percentage inhibition of *A.alternata* (10.2, 10.6) and *Fusarium moniliforme* (11.1, 11.6). *Trichoderma* spp have been used as biocontrol agents to protect plants against foliar diseases in several crops. The application of *T. harzianum* for 10 and 20 min seed treatment significant reduced the frequency of seed-borne fungi.

In case of fungicide vitavax showed best result in terms of seed germination, Vitavax showed 80% and Bavistin 75% seed germination. Both the fungicides decreased the percent incidence of mycoflora.

Many reports revealed that plant metabolites and plant based pesticides appear to be one of the better alternatives to be used as they are known to have minimal environment impact in contrast to synthetic pesticide. Recently Hasan et al., (2005) reported that leves of *Azadiracta indica*, *Achyranthes aspera*, *Lawsonia alba*, *Adhatoda vestica*, stem of *Cuscuta reflexa*, root of *Vicia rosea* and seeds of *Nigella sativa* significantly reduced the incidence of seed-borne fungi of wheat viz., *Bipolaris sorokiniana*, *Fusarium spp.*, *Aspergillus spp.*, *Penicillium spp.*, and *Rhizopus spp*.

The ability of the extracts to increase seed germination and seedling emergence could be attributed to the suppression of the incidence of the seed borne fungi that could have killed the embryo of the seeds. This result is consistent with that of Parimelazhagan and Francis, (1999) who established that leaf extracts of *Clerodendrum viscosum* increased seed germination and improved seedling development of rice seeds. The bioactivity of neem extracts has been attributed to various compounds found in seeds and leaves such as nimbin, nimbidin, salannin, but the most important of these compounds is azadirachtin (Lale & Abdulrahman, 1999).

Biological control using antagonistic microbes alone or as supplements has been investigated in recent years to minimizing the use of chemicals. (Annone 2005).

Antagonistic fungi are present in substantial quantity in nearly all agriculture environment and their use is now being recognized world-over as an alternative in plant disease control. (Harman et al 2004, Suttan 2005).

When treatments with plant extracts and *T.harzianum* compared with control and fungicidal treatment, plant extracts and *T.harzianum* produce a better control than fungicide treatment. (Table 2).

The continuous use of chemical treatments has resulted in control failure as the pathogen become resistant to the ingredients (Williams and Gisi, 1952).

The plant extract of *Azadirachta indica* showed maximum activity against fungal pathogen while minimum activity was observed with *Euphorbia hirta* against the fungi under investigation. These plant extracts can possibly be exploited in the management of seed-borne pathogenic fungi to prevent bio deterioration of seeds in an eco-friendly way.

Trichoderma based bio-fungicides are a reality in agriculture, with more the 50 formulation currently available as registered products worldwide. (Lorito and woo 2004). The result presents here show that we can used plant extracts and *Trichoderma* based formulation as non chemical alternative treatment against seed-borne pathogen of cowpea.

Plant metabolites and plant based pesticides appear to be one of the better alternative as they are known to have minimal environmental impact and danger to consumers in contrast to synthetic pesticides (Verma and Dubey, 1999).

The present study justifies the claimed uses of leaves in the traditional system of medicine to treat various infectious disease caused by the microbes. However, further studies are needed to evaluate the potential effectiveness of the crude extracts as the antimicrobial agents. The present results will form the basis for selection of plant species for further investigation in the potential discovery of new natural bioactive compounds. Further such studies which aimed at the isolation and structure elucidation of antipathogenic active constituents from the plants and secondary metabolites of plant kingdom.

CONCLUSIONS

From these finding it is concluded that the seed health testing is a primary need to avoid crop failure and it is desirable that seeds of crop plants should invariably be tested for seed health before planting so as to check the introduction of pathogens in new areas and be treated with fungicides to attain maximum yield of crops. Since these pesticides are likely to be hazardous, Latex of different plant parts could be employed as encouraging results have been obtained. Soil amendments and seed treatment with biocontrol agent like *Trichoderma* alone or in combination with have been recommended for the control of soil and seed-borne pathogen.

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APPENDICES

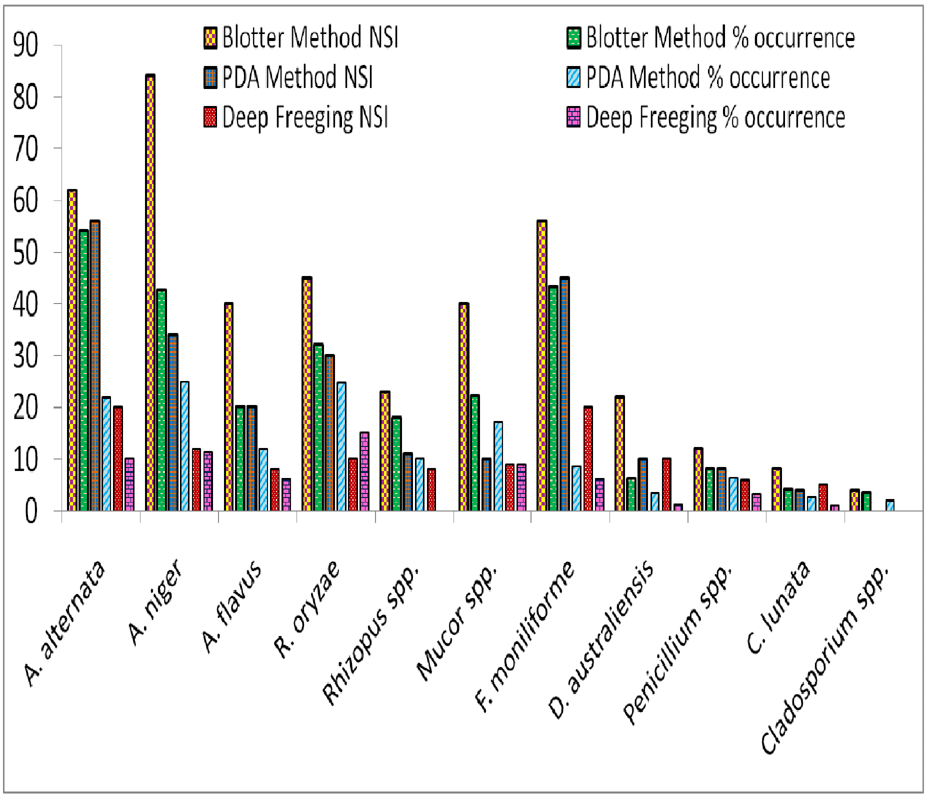

Figure 1: Isolation of Mycoflora of Cowpea by Blotter, PDA and Deep Freezing Methods

Table 1: Mean Percentage Inhibition of Mycelial Growth (Zone of Inhibition (in mm) of Seed- Borne Fungi by Various Leaf Extracts and Biocontrol Treatment by *T.harzianum*

S. No	Name of Fungi	*Alternaria alternata*	*Fusarium moniliforme*	*Aspergillus niger*	*Curvulariya lunata*	*Penicillium spp.*
1	*Trichoderma harzianum*	43.33	39.21	28.35	53.48	55.18
2	*Azadiracta indica*	38.33	32.66	23.33	40.33	49.44
3	*Eucalyptus globulus*	21.00	20.33	19.45	18.06	18.22
4	*Calotropis procera*	18.47	18.33	17.10	16.65	16.00
5	*Lantana camara*	17.24	16.12	16.23	15.14	12.22
6	*Euphorbia hirta*	16.12	16.24	15.23	14.12	12.22

Table 2: Antifungal Activity of Leaf Extracts, Biocontrol and Fungicidal Treatment on Frequency of the Occurrence of Seed- Borne Fungi of Cowpea

Treatments	*Alternaria alternata*	*Fusarium moniliforme*	*Aspergillus niger*	*Curvulariya lunata*	*Penicillium spp.*
Control	42	36.04	30.6	24.7	22.10
NaOCL	35.10	32.05	28.12	25.02	20.10
Azadiracta (10 min)	13.2	12.5	10.3	10.1	6.2
Azadiracta (20 min)	12.6	10.2	9.2	8.0	00.0
Eucalyptus (10 min)	14.8	14.0	11.7	9.0	5.0
Eucalyptus (20 min)	13.4	12.8	11.1	6.0	2.4
Calotropis (10 min)	16.08	15.0	14.2	12.8	10.2
Calotropis (20 min)	14.02	15.3	13.6	12.2	10.4
Lantana (10 min)	15.9	15.0	13.0	12.0	11.8
Lantana (20 min)	14.4	14.9	12.9	12.9	10.9
Euphorbia (10 min)	18.01	16.7	15.7	14.6	13.0
Euphorbia (20 min)	16.9	15.7	13.8	13.7	12.2
T.harzianum (10 min)	13.0	11.1	9.2	6.1	3.1
T.harzianum (20 min)	10.6	11.6	9.1	5.1	0.0
Vitavax (10 min)	16.2	14.6	12.1	10.1	11.1
Vitavax (20 min)	18.1	14.2	11.6	10.2	9.2
Benlate (10 min)	17.2	14.1	10.2	12.4	12.2
Benlate (20 min)	16.1	12.0	9.1	7.6	6.4