Mycoflora associated with Vigna munga (L.) Hepper. and Vigna radiata (L.) R. Wilczek and their bio-control by aqueous medicinal plants extracts

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Abstract

To determine the mycoflora associated with stored *Vignamungo* (L.) Hepper. and *Vignaradiata* (L.) seeds and their control biologically by aqueous medicinal plants extract, this study was aimed to isolate and dentify seed-borne fungi associated with seeds collected from Triprayar market, Kerela, South India, in both agar plate method and moist blotter method. Seven dominant fungal species were isolated from these seeds *viz., Aspergillus niger, A. fumigatus, A. terreus, A. flavus, Rhizopusni gricans, R. stolonifer,* and *R. oryzae*. Percentage of fungal incidents on seeds and their impact on germination rate and growth is also studied using agar and blotter plate method. Spore suspension of two dominant fungi *A.terreus* and *A.niger* were prepared and frequency of fungal infection in seeds was observed to identify the effect of particular fung us on seeds.The seed-borne fungi are capable of reducing the germination rate and further development of the plant. In view of this, the present study has been undertaken to screen some medicinal plant leaf extracts against the seed-borne fungi. Four medicinal plants named *Alliumcepa* L., *Alliusativum* L., *Zingiber officinale* Roscoe. and *Ocimum tenuiflorum* L. were screened for their anit-fungal activities. All seeds were treated with aqueous extracts of fresh samples.*Alliumsativum* L. and *Zingber officinale* Roscoe. exhibited the highest antifungal activity among all the plants tested.

Key words: antifungal activity *Aspergillus, Rhizopus,* medicinal plants, spore suspension.

INTRODUCTION

Almost 90% of all the world's crops are grownfrom seeds. It possesses the qualities necessary for cell division, morphogenesis and r egene ration of species.

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The study of seed itself is a good study of life. Seeds are widely distributed in national and international trade, and germ plasm is also distributed and exchanged in the form of seeds in breeding programs. Due to their high mobility, seeds are highly effective means for disseminating plant pathogens over long distance (Agarwal and Sinclair,1997).

Legume seeds have comparatively higher protein content than non-legume plants. The high protein content makes them desirable cropin agriculture. On a world wide basis, legumes contribute by about one-third to human direct protein intake , while also serving as an important source of fodder and for age for animals and of edible and industrial oils. One of the most important attributes of legumes is their capacity for symbiotic nitrogen fixation.

Seed-borne pathogens cause enormous losses to our crop. Presence or absence of seed-borne fungi on seed surface is one of the important aspects that determines the quality of seeds. Seed-borne fungus cause seed rot, germinationfailure and seedling mortality and then reduce crop production. The infected seeds may fail to germinate, transmit disease from seed to seedlings and from seedling to growing plants (Fakir *et al*., 2002). The legume members are predominately colonized by various species of *Aspergillus.*

This work was designed to identify the seed-borne fungi and their effects on germination and evaluate the efficiency of medicinal plant extracts on seed-borne fungi isolated from some *Vigna mungo* (L.) Hepper. and *Vigna radiata* (L.) R. Wilczek. collected from Thriprayar markets, Kerala, South India, during storage.

MATERIALS AND METHODS

Collection of seed samples:

Some stored seed of economically importantcrops, *viz*., Blackgram [*Vignamungo* (L.) Hepper] and green gram [*Vigna radiate* (L.) R.Wilczek.] were collected from Triprayar market, Thrissur, Kerala.

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Fumigation of culture laboratory:

Safety goggles and hand gloves were used during fumigation and the A/C was put OFF. About 15 gm of Potassium Permanganate was taken in a petridish and kept in the area where fumigation is to be carried out. The petri plate was placed on a polythene bag and about 25ml of 35% formaldehyde solution was added to Potassium Permanganate. The area was immediately closed and labeledas" Area Under Fumigation" so that nobody enters the room. The room was kept under fumigation for a minimum of 8-12 hours and defumigated by putting on the A/C unit.

Detection and identification of seed-born fungi of stored seeds:

The samples of Black gram and green gram were assayed for the presence of seed borne fungi by using (A) Agar plate method(B)Blotter method as recommended by ISTA (1966), De Tempe (1970), Neergaard(1977) and Agarwal (1981).

Sterilization of Laminar air flow chamber and glasswares:

Sterilization of Laminar air flow chamber: The laminar air flow chamber is a device which isused for sterilization. This reduces the possibility of contamination whileworkingwith cultures of fungi inside a laminar air flow chamber. The working area was cleaned by using 100% alcohol and cotton. After this, UV radiation is providing for 15 minutes for sterilizing the environment.

Sterilization of glasswares: Glasswares such as test tubes, petri-dishes etc. were sterilized using hot air oven. Electric hot air sterilizing oven was turned on and pre-heatedto160^{0}C. Usingasoft, nonabrasive cloth, antibacterial dish so a pand warm water,the petri-dishes were cleaned and dried with a soft non-abrasive dry cloth. Then the petri-dishes were wrapped using clean papers and they were kept in the oven at 160^{0}C for two hours for sterilization.

Surfacesterilizationofseeds: Collected seeds were surface sterilized using 0.1% HgCl2 (Mercurychloride) for four minutes, rinsed with distilled water for 3-4 times and dried between sterilized filter papers.

Preparation of Potato Dextrose Agar(PDA):

Type: General purpose medium Composition
Potato - 250g/l

Agar - 15g/l

Dextrose - 20 g/lDistilled water-1000ml

The potatoes were pealed, cut in to small pieces and boiled in 250ml water. The boiled potatoes were smashed to form a thick paste and strained through cheesecloth. Dextrose,agarandwater were added to make upto a liter. A pinch of Ampicilin (an antibacterial medicine) was added to the medium to prevent the bacterial growth and to make this medium devoid of contamination.Then prepared standard PDA was poured in two 500ml conical flasks and sterilized (121^{0}C,15psi for 15min.) in pressure cooker. (Kanika Sharma, 2007).

Agar plate method (PDAmethod):

Twenty five ml of sterilized PDA medium was poured in pre-sterilized borosil glass Petri-plates. The Petri–plates were allowed to cool at room temperature 33±^{0}C. Then 1gm of sterilized seeds were plated at equal distances in triplicates and unsterilized seeds were kept as control under aseptic condition. Plates wereincubated at room temperature for seven days.On eighth day the seeds were examined undermicroscope for the preliminary determinationof seed mycoflora. The seed-born fungi found one a chand every s eed were isolated and identified, brought into pure culture and maintained on PDA plates.

Moistblotterplatemethod (BPT):

In moist blotter plate method, the seeds were placed on water soaked three layered white blotter papers of 8.0cm diameter and placed in presterilized borosil glass petri-dishes. One gm sterilized seeds were placed at equal distance in triplicates and unsterilized seeds were kept as control in moist blotter paper under aseptic condition. The triplicates were incubated at room temperature 33±^{0}C for seven days. On the eighth day the seeds were examined under microscope for the preliminary determination of seed mycoflora. The seed-borne fungi found on each and every seed were isolated and identified, brought into pure culture and maintained on PDA plates.

In both Agar plate method and Moist blotter method, the frequency of seed-born fungi during incubation were examined.The result was expressed in percentage.

Frequency of germination% =

Isolation and purification of seed–born fungal pathogen of stored seeds:

Isolation of causal pathogenic fungi was carried out on PDA medium. PDA plates were inoculated by taking a bit of mycelia from theseed surface and transferred on PDA plates byusing sterilized inoculation needle. The fungi were isolated, purification was done by recapture.

Preparation ofmoist chamber:

Asterile Petri-plate was lined with a sterile filter paper. A sterilized glass slide was placed on a 'U'shaped

glass rod. Sterilized PDA media was poured in a Petri-plate and allowed to solidify. The agar was cut into small squares with a scalpel and in accordance with cover slips were placed on the glass slide. By using sterilized inoculation needle, the fungal hyphae were inoculated at the middle of each side of the agar and a cover slip was placed on the agar piece. A Moistened filter paper was used to cover the Petri-plates and incubated insterilized condition. The filter paper was kept moist by adding distilled water to it every day. When fungal growth appears, the cover slip was removed with the help of asterile forceps and a small amount of growth was stained with lactophenol cotton blue and observed under microscope for identification is based on the; 1) Colonycolor, and 2) Morphological features.

Preparation of spore suspension:

Spore suspension of dominant seed-borne fungi of pulse were prepared separately by adding 10ml of distilled water in to the sporulating pureculture of seed-borne fungi maintained on PDA slants for seven days at room temperature 33±^{0}C. The slants were shaken and content filtered through muslin cloth to separate mycelium and spore. The filtrate thus obtainedwas used as spore suspension.

Sporesuspensionculture:

One gm of seeds were dipped in the spore suspension prepared from the dominant seed-born fungi for few minutes and placed on thewater soaked three layered blotter paper of 8.0cm diameter, placed in a pre-sterilized Petri–dishes. The plates were incubated at room temperature 33±^{0}C for seven days and the affected number of seeds and their growth were observed.

Preparation of Medicinal plant extracts: Four fresh samples were used in this study. Samples were Onion bulb [*Allium cepa* L.], Garlic Bulb [*Allium sativum* L.], Ginger rhizome [*Zingiber officinale* Roscoe.] and Thulasi Leaves[*Ocimum tenuiflorum* L.] Water extract of fresh samples were prepared as follows :Ten gm of each plant material were cut into small pieces using a sharp knife. Thecut plant material was ground with 25 ml of distilled water by using pestle and rotor. The homogenate was placed in a closed container and was left for 48 hours (Dababneh and Al-Delaimy, 1984).

Effect of medicinal plant extracts on incidence of fungi: After soaking of seeds in medicinal plant extracts for half an hour, they were placed in sterilized Petri–plates on three layer of blotting paper water soaked in distilled water. Each dish was containing 1 gm of seed.Then the Petri-dishes were kept in the sterilized incubation chamber at 33±^{0}c and data were recorded seven days after sowing. The percentage of fungal infection and their effects on growth were observed.

Seed germination method: In seed germination method, evaluate the impact of seed-borne fungi on percentage in seed germination was evaluated. Effects of seed-born fungi on rate of seed germination were observed in all the following methods;

Agar plate method:

In this method, one gm of surface sterilized seeds were placed at equal distance in triplicate and unsterilized seed as control in PDA containing Petri-plate. The effects of seed-borne fungi on the percentage of seed germination of both sterilized and unsterilized seeds were observed for sevendays andthedetails were recorded.

Moist blotter method:

In order to evaluating the effect of seed born-fungi on the processes of seed germination in BPT method, the seeds were incubated in themoist blotters at room temperature for sevendays. After incubation period the rate of seed germination of sterilized and unsterilized seeds were recorded.

Spore suspension culture method:

In order to evaluate the effect of seed-bornefungi on the processes of seed germination, the seeds of cereals and pulses were infested separately with spore suspension of dominant seed-borne fungi. These seeds were incubated in sterilized moist blotters at room temperature for seven days. After incubation period the rate of seed germination of each seed were recorded.

Medicinal plant extract method:

Seeds were treated in all four medicinal plant extracts for half an hour and they were placed in sterilized Petri-dishes on three-layer blotting paper socked in distilled water. ThePetri-plates were kept in sterilized incubation, observed and recorded the data after the seven days of incubation. The percentages of seed germination of each seed were recorded.

In all these cases, the germination rate after incidents of fungi during incubation were examined. The results were expressed in percentage.

RESULTSANDDISCUSSION

Many pathogenic fungi are seed transmitted,often reduce the germination ability or killthe infected plants or substantially reduce theproductivity. Therefore, control of seed-bornefungi is extremely important and the damaging effects can be revealed through integrated approaches (Diaz *et.al.,* 1998).

This study was conducted to determine the prevalence

of seed borne fungi of stored seeds that were collected from the Triprayar market and forthe experiment, different methods were carriedout simultaneously employing BPT and PDA method .Seed health test in two methods yielded seven different fungi *Aspergillus niger, A. fumigatus, A. terreus, A. flavus, Rhizopus nigricans, R.stolonifer, R.oryzae.* A considerable number

Table 1. Fungal isolates of stored seeds of the present study.

Sl. No	Seed Mycoflora	Isolated Seed Mycoflora						
		A.niger	*A.fumigatus*	*A.terreus*	*A.flavus*	*R.nigricans*	*R.stolonifer*	*R.oryzae*
1	Blackgram	✓	✓	✓	×	✓	✓	✓
2	Greengram	✓	✓	✓	✓	✓	✓	✓

Table 2: Comparative incidents of seed mycoflora in PDA and BPT culture method in percentages

Sl.No	Seed Sample	Incident of Seed Borne Fungi(%)			
		Agar plate		Blotter method	
		Sterilized Seed	Control Seed	Sterilized Seed	Control Seed
1	Blackgram	38.33	100	32.5	50
2	Greengram	11.66	30	10	12.33

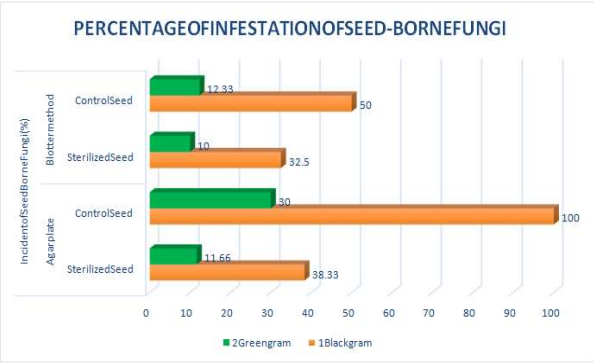

Fig. 1. Comparative incident of seed borne fungi on different seed in PDA and BPT culture method in percentage.

Table 3. Comparative rate of germination in test seeds by two different culture methods in percentage.

Sl. No.	Seed Sample	Rate of Germination of Seeds (%)			
		Agarplate		Blotter method	
		Sterilized Seed	Control Seed	Sterilized Seed	Control Seed
1	Blackgram	100	100	100	100
2	Greengram	100	100	100	100

Fig. 2. Comparative rate of germination in test seeds by two different culture methods in percentage.

Table 4. Frequency of fungal infection and germination rate in spore suspension method in percentage

Sl. No.	Seed Sample	*A.terreus*		*A.niger*	
		Fungal Incidents (%)	Germination Rate (%)	Fungal Incidents (%)	Germination Rate (%)
1	Blackgram	40	10	20	10
2	Greengram	50	100	100	100

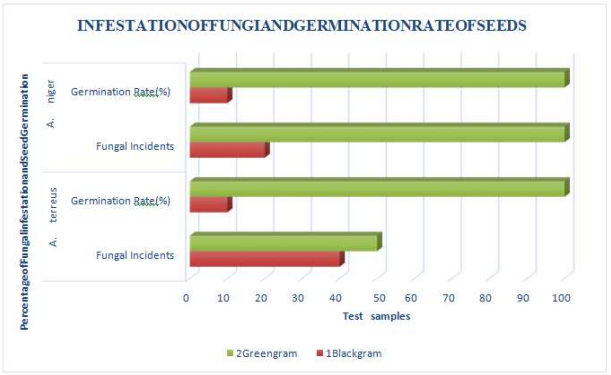

Fig 3. Frequency of fungal infection and germination rate in spore suspension method in percentage

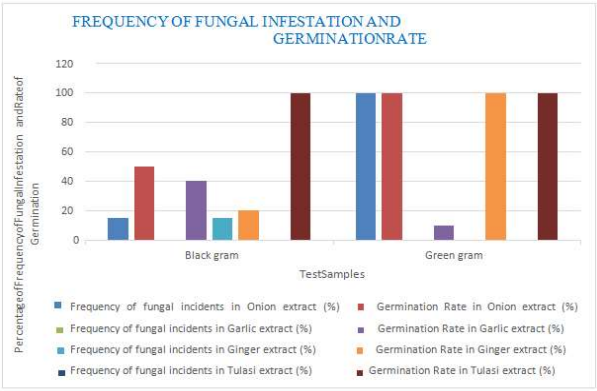

Fig. 4. Anti-fungalactivity of medicinal plant extract Frequency of fungal incidents after medicinal plant extract treatment.

Table 5: Anti-fungal activity of medicinal plant extract Frequency of fungal incidents after medicinal plant extract treatments.

SL. No	Test Sample	Medicinal Plant Extracts							
		Onion		Garlic		Ginger		Thulasi	
		Frequency of fungal incidents (%)	Germination Rate (%)	Frequency of fungal incidents (%)	Germination Rate (%)	Frequency of fungal incidents (%)	Germination Rate (%)	Frequency of fungal incidents (%)	Germination Rate (%)
1	Blackgram	15	50	-	40	15	20	-	100
2	Greengram	100	100	-	10	-	100	-	100

of seed borne fungi belonged to the two genera *Aspergillus* and *Rhizopus* (Tripti *et.al.,* 2011)(Table-1)

Comparative incidents of seed mycoflora by two different culture methods reveals seven dominant fungal species were present in both the BPT and PDA method.

Percentageofincidentsoffungiismoreinblack gram than green gram in both the methods in sterilized condition. Incidents of fungi is comparatively lower in green gram even in controlled seeds. Present study revealed the incidents of more seed borne fungi both quantitatively and qualitatively in PDA culture than BPT culture (Mari., 2015.)(Table–2, Fig - 1). Because both methods depend on two factors, namely, moisture and temperature, when moisture increases with a decrease in temperature, which was the most suitable condition for fungal growth. In PDA culture availabilityof nutrients continuous along with moisture may be there as on for increased fungal growth.

In the present work seven genera of fungi were detected in both the methods under sterilized and unsterilized seed slots tested. Similar study conducted earlier in all varieties of green gram and found four genera of fungi which included *Curvularia sp.,Macrophomina sp., Aspergillus sp., and Alternaria sp.* were detected in both the methods under sterilized and unsterilized seed lots tested (Tandel., 2015.).

In this study Rate of germination was not thatmuch affected after the incidents of fungi in both methods. Both seeds exhibit 100% germination insterilized and unsterilized condition. The number of leaves and length of root and shoot are more in PDA than BPT in both seeds because of the nutrient availability being more in PDA media. It revealed that after incidents of fungus, seedling can survive in adverse conditions. But in BPT method inhibition of plant growth may occur and further growth and elongation of root and shoot does not take place. Generally, in blackgram and greengram root-shoot elongation was inhibited after germination in both the cases (Table–3,Fig-2). Related studies were carried out with other crops (Imolehin, 1983).

The present work revealed that after treatment in spore suspension of *A.terreusand A.niger* possessed moderate level of infection in both seeds except green gram. In black grammoderate level of infectionalso showed highreduction in germination rate. The black gram *A.niger*showed 100% infection but the germination was not affected (Table – 4, Fig - 3). The results revealed these fungi have inhibitory effect onthe growthan delongation process.The predominant fungi of the present study can cause deterioration in stored seeds and reduce the seed quality.

Aqueous medicinal plant extracts prepared from fresh materials of Onion bulb, Ginger rhizome, Garlic bulb and Ocimum leaves have shown anti-fungal activities. The medicinal plant extracts treatment showed reduction of seed borne fungi. Earlier, similar studies were conducted in various ways to control seed borne fungi in biological method. (Zakaria, 2014 and Dauda, 2015).

This study revealed that Thulasi and garlic aremost suitable plants to make extract to controlfungi pathogen. In this study thulasi is most suitable for blackgram and it exhibited 100% germination rate but in greengram ginger and thulasi are equally good. Garlic was also good for greengram showed lower germination rate.(Table– 5,Fig- 4).

According to Zakaria.,(2014). from all samples extracted from fresh samples, only Garlic bulb exhibited the highest anti-fungal activity against isolated fungi. Lawson (1998) reported that the most abundant sulfur compound in Garlic is allicin (S-allylcysteinesulfoxide), which is present at 10 mg/g fresh Garlic or 30 mg/g dry weight. Water extract of Ginger also suitable one and exhibited anti-fungal activity against all tested fungi. Ficker *et.al.*, (2003) reported that the gingerols and gingerdiol are the main

anti-fungal activity against all tested fungi.

Seed-borne fungi appeared in sterilized and unsterilized conditions. They cause toxicity in human consumption. Fungal growth was established in a moisture rich environment easily. Their occurrence on stored seed adversely affected the germination rate and more severely affected seedling elongation and also inhibited the plant growth. Infestation of seed-borne fungi affected the plant growth and there by severely affected the productivity of the crop. High seed quality is essential in any crop production venture to attain higher yield and good quality products. Present work revealed that aqueous medicinal plant extracts had antifungal property and aqueous extracts obtained from Thulasi, Garlic and Ginger plants were more suitable alternative stominimize the seed-borne fungus of the stored seeds. It is a less expensive and an eco-friendly approach to reduce the severity of seed-borne fungus in agriculture and human consumption. This study recommends the use of natural plant extract because they are safer than chemical fungicides. The use of these extracts can be helpful in increasing seed germination during agriculture. Thulasi, Garlic and Ginger extract are recommended as anti-fungal agents for preserving seeds.

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