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Effect of Plant Botanicals on Growth of Mycoflora Associated with Pigeon Pea Seeds

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A B S T R A C T

A study was conducted to determine the mycoflora associated with pigeon pea cultivar MA6 and effect of botanicals on growth of mycoflora. The dominant fungi observed on pigeon pea cultivar MA6 seed were *Rhizopus stolonifer, Aspergillus niger, A. flavus, Penicillium citrinum, P. rubrum, Alternaria alternata, A. solani, Curvularia lunata, Fusarium oxysporium.* Among the treatments, the sulphate and nitrate treated seeds showed the less number of fungi isolated in comparison to untreated seeds. The nature of fungal flora had changed with prolongation of storage period of pigeonpea seeds. Effect of plant botanicals at 15% concentration on the radial growth of mycoflora found that leaf extract of neem (*Azadiracta indica*) inhibited the growth of *Aspergillus* niger upto a maximum of 54.85% and *Penicillium citrinum* was least inhibited. At the same leaf extract concentration of *O. sanctum* treatment, it was observed to be most inhibitory to *Aspergillus flavus* which inhibited the fungal growth up to 37.93% and leaf extract of *C. longa* was observed to be most inhibitory to *Rhizopus stolonifer* inhibiting the fungal growth up to 31.86%.

Introduction

Pigeon pea (*Cajanus cajan* L.) belonging to the family *Fabaceae* is grown all over the world have played a significant role in the evolution of human civilization. India is the world's largest producer of pigeon pea and generates 67.28% of total global production. The cultivation area in India was 56.02 lakh hectares in 2016-17 and the production level was 32.90 lakh tonnes at 587 kg/ha (Ackermann, 1998, Chakravarthy *at el.*, 2002). Myanmar is the second largest producer of pigeon pea with 11.76% of global production followed by lesser producers Malawi, Kenya, Tanzania and Haiti.

Damages due to pests and diseases and the consequent loses, both in quality and quantity of grains, that always take place in storage are attributable to a number of factor, temperature and relative humidity being of prime importance. Seed carry a wide range of microorganism either externally or internally or both and these organism become active in favourable conditions and cause considerable

damage to the seed and several diseases on the crop rose from them in various ways. Some of the seed borne mycoflora might reduce the germinability of seeds when planted or result in disease in the growing plant. Losses due to storage fungi in the conditions prevailing in India may be as high as 30 per cent of the total harvest (Neeergaard, 1977).

Fungi growing on stored seeds are well known to produce metabolites toxic to other organisms including domestic animals and man (mycotoxins). Aflatoxins may cause serious disorders in the human beings or animals, when consumed, including liver damage and even cancer. Some other fungi producing mycotoxins includes *Pencillium rubrum*, *P. pupurogenum* (rubratoxins), *P. viridicatum, P. citrinum* (citrinin),*P. cyclopium* (penicillic acid);*Aspergillus clavatus, A.terreus, A.patulum, A. palitans*; *Fusarium oxysporum, F. moniliforme, F. roseum* (Zearalenone).

Although, chemical fungicides have been used since long time for treatment of the seeds, they are well known for their non-target effects over the mankind and their hazards have forced the scientists of the era to rethink of their use particularly with the theme of 'sustainable agriculture'. Consequently, people associated with plant protection sciences tried to search a suitable alternative of the agrochemicals, particularly pesticides, in the return of higher plants and their products. Due to high potency of antimicrobial properties, non phytotoxic nature and easy availability with no side effects, the higher plants and herbs have been used to control several diseases of human being and animals. The application of higher plants and herbal products against phytopathogenic microorganism in general and in storage were given much attention now-a-days to control pathogenic microorganism causing severe damage to the crop and food commodities. The volatile and nonvolatiles fraction of higher plants have been given much emphasis due to their high fungi toxic properties against parasitic, saprophytic and pathogenic fungi. The volatiles do not leave any residue, hence there is least chance of residual toxicity in treatment of food commodities (Asthana *et al.*, 1986, Chakravarthy, *et al.*, Singh, 2010, Sinha *et al.*, 1999, Pandey *et al.*, 2012, Devi and Chhetry, 2012). Keeping in view the above gap in research effect of plant botanicals on mycoflora associated with pigeon pea seeds was studied.

Materials and Methods

Isolation of seed fungi

Isolation of fungi associated with pigeon pea cultivar MA6 seeds were carried out for a period of nine months periodically from July 2012 to March 2013 following Agar plate method (Musket and Malone, 1941), Blotter Method (Doyer, 1938) and the techniques recognized by International Seed Testing Association.

Preparation of pure culture and storage of fungi

The pure culture of the target fungi were obtained through hyphal tip culture technique wherein tip of the fungal mycelium was transferred on to 3 % water agar medium poured in petriplate after solidification. The plates were incubated at 25±1˚C for 5-7 days. After development of fungal colony single hyphal tip were cut out and placed on another fresh plate of water agar medium with the help of stereomicroscope and incubated again 25±1˚C for 5-7 days to obtain pure culture and the pure cultures were maintained on nutrient agar slant at 25 ±1^{o}C and stored in deep freezer.

Seed treatment with plant botanicals

Different concentration of three plant botanicals *viz.,* turmeric (*Curcuma longa*), tulsi (*Ocimum sanctum*) and leaves of neem (*Azadiracta indica*) were prepared by methods suggested by Bhat and Sivaprakashan (Bansal and Sobti, 1990) and evaluated their potential to control mycelial growth of dominant seed associated fungi *viz*., *Alternaria alternata, Fusarium roseum, Aspergillus niger, Aspergillus flavus, Curvularia lunata* and *Rhizopus stolonifer.* The percent inhibition in growth due to treatment of various botanicals at different concentration was computed as followed:

Mycelial growth inhibition (%) = dc-dt/dc ×100

Where dc = average diameter of fungal colony in control, and dt = average diameter of fungal colony in treatment group.

Results and Discussion

Isolation of fungi

Isolation of fungi associated with pigeon pea cultivar MA6 seeds were carried out for a period of nine months periodically from July 2012 to March 2013 following Agar plate method and blotter method

Agar plate method

Fungi that were isolated from potassium sulphate, potassium nitrate and untreated control of pigeon pea cultivar MA6 seeds stored at 0 days *i.e.* fresh seed, 90, 180 and 270 days from July 2012 to April 2013 by agar plate method were presented in table 1, 2 and 3 respectively. The dominant fungi that were observed irrespective of storage duration and treatment by this method were *Rhizopus stolonifer, Aspergillus niger, A. flavus, Penicillium citrinum, P. rubrum, Alternaria alternata, A. solani, Curvularia lunata, Fusarium oxysporium* and *white sterile mycelium.*

Blotter Method

Fungi that were isolated from potassium sulphate, potassium nitrate and untreated control of pigeon pea cultivar MA6 seeds stored at 0 days *i.e.* fresh seed, 90, 180 and 270 days from July 2012 to April 2013 by Blotter method were presented in table 4, 5, and 6 respectively. A total 14 to 15 fungal species were isolated from the pigeon pea seeds at different period of storage from July 2012 to April 2013.

The common fungus isolated by blotter paper method were *Rhizopus stolonifer, Aspergillus niger, A. flavus, Penicillium* c*itrinum, P. rubrum*, and other dominant fungi like *Alternaria alternata, A. solani, Curvularia lunata, Fusarium oxysporium, and White sterile mycelium.*

Among the treatment the sulphate and nitrate treated seeds show the less number of fungi isolated in comparison to untreated seeds. Total 15 fungal species were isolated from pigeon pea seeds during different period of storage from July 2012 to April 2013 by Blotter method.

The nature of fungal flora had changed with prolongation of storage period of pigeonpea seeds. The fungi were recorded as *Aspergillus niger, A. flavus, A. terreus, Penicillium citrinum, P. rubrum* which were partially replaced by *Rhizopus spp., Alternaria alternata, A. solani, Curvularia lunata, Trichoderma viride, Fusarium spp., Drechslera sorokinia.* The dominant fungi were recorded from fresh seeds like *Aspergillus niger, A. flavus, Penicillium citrinum, Alternaria alternata* and *Rhizopus stolonifer.*

Newly harvested seed were generally infected with a variety of fungi which were contaminated in field contaminants. The numbers of seed borne fungi increased along with the period of storage found variation in fungus of stored seed in different storage period.

The dominant fungi on fresh seeds were recorded like *Alternaria alternata, A. solani, Rhizopus spp., Curvularia lunata, Trichoderma viride, Fusarium spp.* and *Drechslera spp.* theses fungi were replaced by storage fungi viz. *Aspergillus niger, A. flavus, A. terreus* and *Penicillium citrinum*. It had been observed that the field fungi decreased along with increase the storage time. It was evident from observation that maximum fungi were recorded in rainy season, summer season and lesser number of fungal species was recorded in winter season. The field fungi declined under storage due to development of storage fungi under the ecological condition prevailing during the storage latter can thrive better. Among the species *Aspergillus niger, A. flavus, A. terious, Penicillium spp. and rhizopus spp.* were the most dominant.

Decreasing in number of fungal species during storage had also been reported by other workers (Reddy *et al.,* 1983; Vijay lakshmi *et al.,* 1985; Paul mishra *et al.,* 1992; Singh, 1999; Singh, 2006 and Singh *et al.,* 2011). Fungi infection during storage produces mycotoxins was reported by (Al-Yahya, 1999).

Comparison between Agar plate and Blotter technique

It was observed that more fungi were isolated by blotter technique in comparison to agar plate method. Many workers (Roy, 2010; Tondon, 1977; Upadhyay and Singh, 1978; Singh *et al.,* 1999 and Singh *et al.,* 2011) had reported that more fungi were isolated by blotter technique than agar plate method and this shows that the slow growing fungi and weak competitors could not grow in agar plate due to competition.

Some fungi were observed only on the Blotter technique viz. *Alternaria spp.* and *Drechslera spp*. This indicates that the slow growing fungi could not be grown successfully in culture plates during competition with fast growing fungi. The other possible reasons could be the selective nature of culture media that had not favored the growth of some other fungi.

Comparisons among the treatments and untreated seeds

The present study reveals that more fungi were isolated from nitrate treated seeds in comparison to sulphate treated seeds, untreated seeds in comparison to sulphate treated seed and untreated seed in comparison to nitrate treated seeds.

Effect of plant botanicals on the radial growth of selected mycoflora of seeds *in vitro*

Mycoflora of the seeds treated with three plant botanicals, viz., *Azadiracta indica* (neem), *Ocimum sanctum* (tulsi) and *Curcuma longa* (turmeric).

Azadiracta indica Leaf Extract

The data pertaining to *Azadiracta indica* leaf extract treatment on the extent of mycelia growth inhibition was presented in table (Table 7)

It is evident from table 7 that the fungal species most inhibited by leaf extract of neem (*Azadiracta indica*) include *Aspergillus* niger (54.85*), Aspergillus flavus* (54.02), *A.terreus* (52.10) at 15% concentration.

Table.1 Fungi isolated from fresh *Cajanus cajan* seeds by agar plate method

Treated seed (Potassium sulphate)				
No. of days	0	90	180	270
Fungus spp				
Rhizopus stolonifer	+	+	-	+
Aspergillus niger	+	+	+	+
Aspergillus flavus	+	+	-	-
Aspergillus terreus	-	-	-	-
Aspergillus candidus	+	+	-	+
Alternaria solani	-	-	-	-
Alternaria alternata	+	-	+	+
Fusarium roseum	+	-	+	-
Fusarium oxysporum	-	-	-	-
Penicillium rubrum	+	+	-	-
Penicillium citrinum	+	+	+	+
Curvularia lunata	+	+	+	+
Drechslera sorokinia	-	-	-	-
Trichoderma spp.	-	-	-	-
White sterile mycelium	-	-	+	+

Table.2 Fungi isolated from fresh *Cajanus cajan* seeds by agar plate method

Treated seed (Potassium nitrate)				
No of days	0	90	180	270
Fungus spp				
Rhizopus stolonifer	-	-	-	+
Aspergillus niger	-	+	+	+
Aspergillus flavus	-	+	-	+
Aspergillus terreus	-	-	-	+
Aspergillus candidus	-	+	-	-
Alternaria solani	-	-	-	-
Alternaria alternata	-	+	+	+
Fusarium roseum	-	+	+	-
Fusarium oxysporum	-	-	-	-
Penicillium rubrum	-	+	-	-
Penicillium citrinum	-	+	+	+
Curvularia lunata	-	+	+	-
Drechslera sorokinia	-	-	-	-
Trichoderma spp.	-	-	-	-
White sterile mycelium	-	+	+	+

Table.3 Fungi isolated from fresh *Cajanus cajan* seeds by agar plate method:

Untreated seed(control)				
No of days	0	90	180	270
Fungus spp				
Rhizopus stolonifer	+	+	+	+
Aspergillus niger	+	+	+	+
Aspergillus flavus	+	+	+	+
Aspergillus terreus	-	+	+	-
Aspergillus candidus	+	+	+	+
Alternaria solani	-	-	-	-
Alternaria alternata	+	+	+	+
Fusarium roseum	+	+	+	+
Fusarium oxysporum	+	+	+	+
Penicillium rubrum	+	+	+	+
Penicillium citrinum	+	+	+	+
Curvularia lunata	+	+	+	+
Drechslera sorokinia	-	-	-	-
Trichoderma spp.	-	-	+	+
White sterile mycelium	-	+	+	+

Table.4 Fungi isolated from *Cajanus cajan* seeds by Blotter paper method

Treated seed (Patassium sulphate)				
No of days	0	90	180	270
Fungus spp				
Rhizopus stolonifer	+	-	-	-
Aspergillus niger	+	+	+	+
Aspergillus flavus	-	-	-	+
Aspergillus terreus	+	+	+	+
Aspergillus candidus	-	-	+	-
Alternaria solani	+	+	-	-
Alternaria alternata	+	-	-	-
Fusarium roseum	+	-	-	-
Fusarium oxysporum	-	-	-	-
Penicillium rubrum	-	-	+	-
Penicillium citrinum	+	+	-	-
Curvularia lunata	+	+	+	-
Drechslera sorokinia	-	+	-	+
Trichoderma spp.	-	-	-	-
White sterile mycelium	+	-	+	+

Table.5 Fungi isolated from *Cajanus cajan* seeds by Blotter paper method:

Treated seed (Patassium nitrate)				
No of days	0	90	180	270
Fungus spp				
Rhizopus stolonifer	-	+	+	+
Aspergillus niger	+	+	+	+
Aspergillus flavus	-	-	-	+
Aspergillus terreus	+	+	+	+
Aspergillus candidus	+	-	-	-
Alternaria solani	+	+	+	+
Alternaria alternata	+	-	+	-
Fusarium roseum	+	-	-	-
Fusarium oxysporum	-	-	-	-
Penicillium rubrum	-	-	+	-
Penicillium citrinum	+	+	-	+
Curvularia lunata	+	+	+	+
Drechslera sorokinia	+	+	+	+
Trichoderma spp.	+	-	-	-
White sterile mycelium	-	-	-	+

Table.6 Fungi isolated from fresh *Cajanus cajan* seeds by Blotter paper method

Untreated seed(control)				
No of days	0	90	180	270
Fungus spp				
Rhizopus stolonifer	+	+	+	+
Aspergillus niger	+	+	+	+
Aspergillus flavus	+	+	+	+
Aspergillus terreus	+	-	+	-
Aspergillus candidus	+	+	+	-
Alternaria solani	+	+	+	+
Alternaria alternata	+	+	+	+
Fusarium roseum	+	+	+	+
Fusarium oxysporum	+	+	+	+
Penicillium rubrum	+	+	+	+
Penicillium citrinum	+	+	+	+
Curvularia lunata	+	+	+	+
Drechslera sorokinia	+	-	+	+
Trichoderma spp.	+	+	+	+
White sterile mycelium	-	+	+	-

Table.7 *In vitro* effect of *Azadiracta indica* on the radial growth (percent inhibition) of selected mycoflora of *Cajanus cajan* cultivar MA6

Fungal spp.	Concentration					
	5		10		15	
	Average colony diameter (mm)	Percent growth diameter (mm)	Average colony diameter (mm)	Percent growth diameter (mm)	Average colony diameter (mm)	Percent growth diameter (mm)
Aspergillus flavus	79	9.19	60.05	30.97	40	54.02
Alternaria alternata	69.5	20.11	54	37.83	42.30	51.37
Aspergillus niger	67	22.98	53.1	39.06	39.28	54.85
Aspergillus terreus	65.05	25.22	57.30	34.13	41.67	52.10
Penicillium citrinum	78	10.34	71	18.39	59.28	31.16
Rhizopus stolonifer	81.49	6.33	73	16.09	59	32.18
Control	87		87		87	

*****At the time when colony diameter of fungi in any of the respective treatments including control was 87 mm. The inoculating disc size (i.e. 5 mm) was substracted from the colony diameter of all the treatments.

Table.8 *In vitro* effect of *Ocimum sanctum* on the radial growth (percent inhibition) of selected mycoflora of *Cajanus cajan* variety MA6 (*in vitro*)

Fungal spp.	Concentration					
	5		10		15	
	Average colony diameter (mm)	Percent growth diameter (mm)	Average colony diameter (mm)	Percent growth diameter (mm)	Average colony diameter (mm)	Percent growth diameter (mm)
Aspergillus flavus	80.98	6.91	72.32	16.87	54	37.93
Alternaria alternata	86	1.14	76	12.64	55	36.78
Aspergillus niger	81	6.89	78	10.34	76	12.64
Aspergillus terreus	85	2.29	80	8.04	77	11.49
Penicillium citrinum	84	3.89	79	12.19	65	25.28
Rhizopus stolonifer	84	3.79	76	12.64	67	22.98
Control	87		87		87	

*****At the time when colony diameter of fungi in any of the respective treatments including control was 87 mm.
The inoculating disc size (i.e. 5 mm) was substracted from the colony diameter of all the treatments

Table.9 *In vitro* effect of *Curcuma longa* on the radial growth (percent inhibition) of selected mycoflora of *Cajanus cajan* variety MA6

Fungal spp.	Concentration					
	5		10		15	
	Average colony diameter (mm)	Percent growth diameter (mm)	Average colony diameter (mm)	Percent growth diameter (mm)	Average colony diameter (mm)	Percent growth diameter (mm)
Aspergillus flavus	83.67	3.82	80.39	7.59	79	9.19
Alternaria alternata	81.35	6.49	78.58	9.67	75.85	12.81
Aspergillus niger	79.29	8.86	77.05	11.43	74.98	13.81
Aspergillus terreus	77.20	11.40	76.98	11.51	72.59	16.56
Penicillium citrinum	73.80	16.08	69.29	20.35	63.93	28.51
Rhizopus stolonifer	69.20	20.67	65.09	25.18	59.28	31.86
Control	87		87		87	

*****At the time when colony diameter of fungi in any of the respective treatments including control was 87 mm.
The inoculating disc size (i.e. 5 mm) was subtracted from the colony diameter of all the treatments.

However, *Penicillium citrinum* was least inhibited by leaf extract of *Azadiracta indica* at this concentration (31.16). The inhibitory effect of leaf extract of *A. indica* continued to be maximum on the radial growth of *Aspergillus niger* (39.06) and *A. flavus* (30.97) at 10% concentration. It was least inhibitory to *Penicillium citrinum* (18.39) and *Aspergillus niger* were inhibited to 22.98 at 5% concentration. However fungi not inhibited at this concentration include *Alternaria alternata, Penicillium citrinum* and *Rhizopus stolonifer*. Neem was found most effective in controlling pathogen in pigeon pea stem blight (Upadhyay *et al.*, 2012), neem seed extract reduces flower and pod damage with increased yield of bean (Rouf and Sardar, 2011).

Ocimum sanctum leaf extract

At 15% concentration, the leaf extract of *O.sanctum* was observed to be most inhibitory to *Aspergillus flavus* inhibiting the fungal growth up to 37.93%. At 10% concentration, maximum effect was observed on *Aspergillus flavus* (16.87% inhibition). However, only single fungal species, viz., *Aspergillus flavus* was inhibited by the leaf extract of *O. sanctum* at 5% concentration to a small extent 6.91% (Table-4). *O. sanctum* showed fungicidal property against *Fusarium oxysporum* and *Rhizoctonia solani* (Upadhyay *et al.*, 2012).

Curcuma longa leaf extract

At 15% concentration, the leaf extract of *C. longa* was observed to be most inhibitory to *Rhizopus stolonifer* inhibiting the fungal growth up to 31.86%. At 10% concentration, maximum effect was observed on *Rhizopus stolonifer* (25.18% inhibition). However, only single fungal species, viz., *Rhizopus stolonifer* was inhibited by the leaf extract of *C. longa* at 5% concentration to a small extent to 20.67% (Table 5). The extract of *curcuma longa* was found to inhibit *Aspergillus flavus and Aspergillus niger* in green gram (Swami and Alane, 2013).

In the present study it was found that a total of 15 mycoflora were associated with pigeon pea cultivar MA6. The fugal mycoflora associated was vared with the storage duration of the seed. Among the treatments with botanicals, extract of need inhibited the radial growth of *Aspergillus* niger by 54.85, *Ocimum sanctum* Leaf Extract of *O.sanctum Aspergillus flavus* inhibiting the fungal growth up to 37.93 and *Curcuma longa* leaf extract of *Rhizopus stolonifer* up to 31.86%.

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