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Detection of seed borne myco-flora associated with cowpea (*Vigna unguiculata* L. Walp)

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Abstract
The seed borne myco-flora of cowpea cv. Phule Vithai was examined by blotter method, agar plate method, deep freezing, 2-4 D method and Test tube water agar seedling symptom test as recommended by ISTA. A total of four species of fungi viz. *Alternaria alternata, Aspergillus flavus, Aspergillus niger, Fusarium oxysporum, Penicillium* spp. and *Fusarium moniliforme* were reported. Amongst the methods used for detection of seed borne fungi, the standard blotter paper method is more effective followed by agar plate method, 2, 4-D method, deep freeze blotter paper method and test tube water agar seedling symptom test.

Keywords: Cowpea, seed borne mycoflora, blotter paper method, agar plate method, deep freeze method, 2, 4-D method, test tube water agar seedling symptom test

Introduction
Pulses have been recognized as a major source of proteins (20-35%) with required minerals and vitamins. Among the pulses, cowpea (*Vigna unguiculata* L. Walp) is a large seeded legume grown for its rich green pods, grains and stover by resource-poor farmers of under developed and developing countries of Africa and Asia. It is evident by the survey of literature showed both pathogenic and saprophytic fungi associated with cowpea seeds. Many workers have reported the association of *Fusarium. oxysporium, F. equiseti, F. vertiloides, Aspergillus niger, A. flavus, Penicillium digitatum P. crycogenum, Rhizopus arrhizopus*, and *Rhizoctonia solani* with seeds of cowpea crop (Kritzinger, 2003; Mogle and Maske, 2012; Makun *et al.*, 2012) [7]. Hence the present study on detection of seed-borne mycoflora of cowpea was conducted to know the seed mycoflora associated with the cowpea seed.

Material and Methods
Collection of seed samples of cowpea
The seed sample of cowpea cv. Phule Vithai was collected from Pulses Improvement Project MPKV, Rahuri.

Standard Blotter Paper Method
Standard blotter method was used for the detection of seed borne fungi of cowpea. The 400 seeds were sown on three layers of pre-soaked moist blotter paper having 9 cm diameter. In each plate 10 seed were arranged, 9 seeds in the outer ring and one in the center of plastic plates. Petri plates were incubated at 20± 2 ºC giving alternate cycle of light and darkness (12 hours each) for 7 days. After incubation, the fungal colonies appeared on the seed surface were observed under stereoscopic binocular microscope. Wherever necessary, fungal growth was also be examined by research microscope. Seed mycoflora load in respect of number of colonies and types of fungi were recorded.

Agar Plate Method: Agar plate method is preferred mostly in plant pathological studies as it provides nutrients rich substrate for development of mycelial growth and sporulation of pathogen on seed, particularly for slow growing fungi. Four hundred infected seeds of cowpea were placed at the rate of 10 seeds per petri plate containing 20 ml of two per cent water agar.

Petri plates were incubated at 20± 2 ºC giving alternate cycle of light and darkness (12 hours each) for 7 days. After seven days of incubation, the fungal colony growth was examined under stereo-binocular microscope (Khare, 1996) [5].

Deep Freeze Blotter Paper Method This method was developed by Limonard (1968) to detect slow growing pathogens. This method allows better growth of certain fungi as the imbibed seeds on moist blotters are killed by deep freezing and the enclosed nutrients in seed are utilized by fungi. Four hundred seeds were placed at the rate of 10 seeds per plate on moistened blotters in the way as described under standard blotter method. The petri plates were incubated at 20± 2 ºC for 24 hrs. under alternate cycles of 12 hrs. NUV light and darkness, for next 24 hours the plates were incubated at –20 ºC in darkness then kept back under original conditions for next five days. After eight days of incubation, the seed were examined under stereo-binocular microscope (Khare, 1996) [5].

2, 4 - D blotter paper method: 2,4-D, is a herbicide retards seed germination and seedlings growth due to which the seeds are not displaced and the examination of fungi becomes easy. Four hundred seeds were placed at the rate of 10 seeds per petri plate with moistened blotter paper dipped in 0.2 per cent of sodium salt solution of 2,4 – dichloro phenoxy acetic acid. The petri plates were incubated at 20± 2 ºC giving alternate cycle of light and darkness (12 hours each) for 8 days After seven days of incubation, the fungal growth on seeds was examined by using stereo-binocular microscope (Khare, 1996) [5].

Test tube water agar seedling symptom test

Collected cowpea seed samples were examined for seedling symptom test. Culture tubes (100 x 16 mm) were filled with 10 ml of 2 per cent water agar and solidified to have slight slant. Hundred seeds were placed individually in each tube and incubated at 20± 2 °C with alternate cycles of 12 hrs light and dark periods for 15 days. The cotton plug was removed after seedling reached to rim portion of the tube and observation was taken on symptom expressed in the seedling (Khare, 1996) [5].

Results

Five different methods employed for detection of seed borne fungal infections presented in Table no.1.The results found that among the five methods, Standard blotter test with untreated seeds was found effective for detection of overall pathogens (44.0%) followed by standard blotter test with pre-treated seeds (26%), Standard agar plate method (21.0%), 2,4-D blotter soak method (19.0%),Test tube water agar seedling symptom test method (15.0%) and Standard deep freeze blotter method (10.0%). Standard blotter test with untreated seeds was found effective for detection of almost all the seedborne fungi associated with cowpea seeds except *Fusarium oxysporum* for which Standard deep freeze blotter method was found effective.

Domsch *et al*. (1980) [2] reported that standard blotter method was the best method for the detection of cellulose decomposing fungi like *Chaetomium* and *Fusarium* species. Jovicevic (1980) [3] suggested filter paper (blotting method) best for the routine analysis of seeds health because in agar plate method intrafungal antagonism becomes an issue. Niaz and Dawar, (2009) [10] reported the deep-freezing method was best for the isolation of *F. oxysporum.* Sultana and Ghaffar (2009) [10] found similar results and suggested blotter and deep-freezing methods best for the isolation of fungi.

The seed samples of cowpea cv Phule Vithai recorded six fungi (Table 1) viz., *Aspergillus niger* the infection ranges from 0. 0 to 16.0%, *A. flavus* 0.0 to 8.0%, *Fusarium oxysporum* 3.0 to 16.0%, *Alternaria alternata,* 0.0 to 5.0%, *Penicillium spp.* 0.0 to 4.0%, and *F monoliforme* 0.0 to 3.0% in all methods employed for detection of seed borne fungal infections. Khare *et al*. (2016) [5] reported the

Ahmed *et al.* (2007) [1] reported the association of nine fungal species *i.e. Aspergillus flavus, Aspergillus niger, Alternaria* sp.,*Cladosporium* sp., *Fusarium semitectum, Fusarium solani, Fusarium* sp., *Fusarium oxysporum and Penicillium* sp. with the seeds of cowpea.

Mogle and Maske (2012) [8] reported total 12 fungal species *viz. Rhizoctonia solani, Aspergillus flavus, Cladosporium* sp., *Aspergillus niger, Penicillium* sp., *Fusarium oxysporum, Fusarium solani, Fusarium semitectum, Trichoderma viridie, Curvularia lunata, Mucor* sp., and *Verticillium* sp. were associated with cowpea seeds.

Khare *et al.* (2016) [4] found the association of total eight fungi from seeds of cowpea. These were *Aspergillus flavus, A. niger, Cylindrocarpon* sp., *Fusarium equiseti, F. oxysporum, Penicillium chyrosogenum, Rhizopus oligosporus* and *R. stolonifer*. *Rhizopus* spp. were dominant fungi recovered from seeds, followed by *Penicillium, Aspergillus, Fusarium* and *Cylindrocarpon.*

Table 1: Efficacy of different seed health testing methods for detection of seed-borne mycofloraof cowpea (Cv. Phule Vithai)

S. No.	Detection methods		Pathogens associated (%)						
			Seed borne pathogens of significance						
			Aspergillus niger	***Aspergillus flavus***	***Fusarium oxysporum***	***Alternaria alternata***	***Penicillium* spp.**	***Fusarium moniliforme***	**Total**
1.	Standard blotter test	Un-treated Seeds	16 (23.57)	8 (16.42)	8 (16.42)	5 (12.92)	4 (11.49)	3 (9.90)	44
		Pre-treated seeds	12 (20.26)	4 (11.49)	3 (9.97)	3 (9.90)	2 (8.13)	2 (8.13)	26
2	Standard Agar Plate		0 (4.05)	0 (4.05)	16 (23.57)	4 (11.53)	1 (5.73)	0 (4.05)	21
3	Standard deep freeze blotter		0 (4.05)	0 (4.05)	10 (18.42)	0 (4.05)	0 (4.05)	0 (4.05)	10
4	2,4-D blotter soak		6 (14.17)	4 (11.53)	3 (9.97)	2 (8.13)	2 (8.13)	2 (8.13)	19
5	Test tube water agar seedling symptom test		3 (9.97)	3 (9.97)	5 (12.92)	3 (9.97)	1 (5.73)	0 (4.05)	15
S. E. ±			0.13	0.24	0.15	0.28	0.24	0.28	
CD at 5%			0.38	0.73	0.47	0.84	0.73	0.84	
C.V. (%)			2.05	5.12	2.33	2.03	3.81	3.52	

(Figures in parentheses indicates arc sin transformed value)

Conclusion

Among the five different methods employed for detection of seed borne fungal infections of cowpea cv Phule Vithai Standard blotter test with untreated seeds (44.0%) was found the most effective for detection of overall pathogens. Six fungi species viz. *Alternaria alternata*, *Aspergillus flavus*, *Aspergillus niger*, *Fusarium oxysporum*, *Penicillium* spp. and *Fusarium moniliforme* were reported.

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