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An Improved Method of Seed Health Testing for Detecting the Lurked Seed-borne Fungi of Fenugreek

Elwakil, Mohamed and K. M. Ghoneem
Plant Pathology Department, Faculty of Agriculture, Mansoura University, El Mansoura, Egypt

Abstract: An improved method of seed health testing was developed to detect lurked pathogens on fenugreek which coexist in low percentage. Blotting papers were soaked in solutions of NaOH and KOH 0.3 and 0.2 M, respectively instead of tap water which is used in standard blotter and deep freezing method. Thirty two samples of fenugreek seed collected from Egyptian commercial market were examined. Using 400 seeds of each sample, 25 seeds were distributed on 3 layers of blotting paper soaked in 0.3 M NaOH or 0.2 M KOH in 9 cm diameter sterilized Petri dishes. A comparable set was kept in which sterile water was used as control. The dishes were incubated at 20 ± 2 for 7 days under cool white fluorescent light with alternating cycles of 12 h light and 12 h darkness as recommended by ISTA. KOH and NaOH treatments respectively showed the presence of 6.5 and 7.5% of *Verticillium dahliae* as compared to 0.3% in the standard blotter and 1% in the deep-freezing method. KOH treatment also showed the presence of *Fusarium moniliforme* and *F. solani* which were detected at 5.4 and 0.7% compared to 0.5 and 0.2% in the standard blotter method and 2.9, 0.4%, in the deep freezing method, respectively. Sodium hydroxide stimulated the growth of *F. semitectum* and *Curvularia* sp., which were detected at 1.4 and 0.7%, respectively as compared to SBM and DFB which showed the presence of 0.3 and 0% in case of SBM and 0.5 and 0.3% in case of DFB. No difference was observed in different treatments for detecting *Cercospora traversiana*.

Key words: Fenugreek seed health, lurked seed-borne

Introduction

Fenugreek (*Trigonella foenum-graecum* Linn.) is an indigenous annual herb of southern Mediterranean shores of Egypt and Morocco. It is widely cultivated in India (Wallis, 1967) and has an economic nutritive value to human as well as to livestock as cattle-fodder for increasing breast milk (Tomar *et al.*, 1996). It is used in China as a medicinal treatment of peptic ulcers (Al-Meshal *et al.*, 1985). Moreover, its alkaloid contents of trigonelline, cholesterin, lecithin and choline are known as tonic and digestive substances (Wallis, 1967; Pandey, 1996).

Several reports of seed-borne mycoflora attacking fenugreek-seeds have been given (Richardson, 1990). *Cercospora traversiana* is considered the major seed-borne pathogen of fenugreek. (Leppik, 1960; Nagy *et al.*, 1972; Zimmer, 1984; Ryley, 1989; Bobev *et al.*, 1999). It affects leaves, stems, young-buds causing blights and damping off. Hashmi (1988) studied the seed-borne mycoflora in fenugreek samples collected from 7 different countries by using standard moist blotter method. *Alternaria alternata* and *Fusarium moniliforme* were found preponderant with low occurrence of *Botrytis cinerea*, *Curvularia inaequalis*, *C. lunata*, *Drechslera tetramera*, *Epicoccum purpurascens*, *F. oxysporum*, *F. semitectum*, *F. solani*, *Phoma* sp. *Stemphylium botryosum*, *Ulocladium* sp. and *Verticllium albo-atrum*. He also reported that *F. moniliforme*, *F. oxysporum* and *F. solani* causes seed rot and wilting in tested seedlings. Sinha and Prasad (1989) using standard blotter and agar plate methods recorded a different number of seed mycoflora on fenugreek seeds viz., *Aspergillus niger*, *A. flavus*, *A. tamarii*, *Rhizopus arrhizus*, *F. equiseti*, *Macrophomina phaseolina*, *Alternaria alternata* and *Curvularia lunata*. Mushtaq *et al.* (1996) reported some new seed-borne mycoflora of fenugreek seeds using standard blotter and deep freezing methods viz., *Aspergillus candidus*, *A. fumigatus*, *A. parasiticus*, *A. versicolor*, *Cephalosporium acremonium*, *Chaetomium bostrychodes*, *C. globosum*, *Cladosporium* sp., *Fusarium proliferatum*, *Paecilomyces* sp. and *Scopulariopsis* sp.

Of the standard moist blotter (SBM) and deep-freezing blotter (DFB) as recommended by the International Seed Testing Association (ISTA) for detecting the seed-borne fungi, the SBM develops saprophytes which often seriously impaired recording the growth of parasitic fungi while deep-freezing blotter enhances the growth and development of saprophytic bacteria and yeasts on seeds (Neergaard, 1979) and also inhibit the spore-germination of some important seed-borne fungi. For the above reasons it is difficult to isolate and identify some important seed-borne pathogens from seed. Some of these important pathogens have slow growth rate during cultivation on blotter e.g., *Cephalosporium* and *Verticillium* produce poor mycelial growth and have never been observed in their imperfect state on seeds (Neergaard, 1979). In order to detect the slow growing seed-borne fungi, experiments were carried out to evaluate the ability of alkaline treatments on the recovery of seed-borne fungi of fenugreek.

Materials and Methods

Source of seed samples: Thirty-two seed samples of fenugreek collected from commercial markets in different regions of Egypt were used in the present study.

Seed health testing (SHT): Detection of seed-borne mycoflora was carried out following the rules stated by International Seed Testing Association (ISTA, 1996). Four hundred seeds of each sample were tested using the standard blotter, deep freezing methods, as well as the alkaline treatment method as proposed in the present study.

Standard blotter method (SBM): Twenty-five seeds were plated in a 9-cm diameter Pertri dish containing three layers of moist blotter paper using sterilized tap water (pH 7.2). The plates were incubated at 20 ± 2°C for 7 days under cool white fluorescent light with alternating cycles of 12 hours light and 12 hours darkness.

Deep freezing blotter method (DFB): After plating seeds as described in the blotter method, the dishes were incubated at 20°C for 24 hours and then transferred to a −20°C freezer for 24 hours followed by 5 days incubation at 20 ± 2°C under cool white fluorescent light with alternating cycles of 12 hours light and 12 hours darkness.

Alkali treatments: A simple modification in standard blotter method was applied by soaking the 3 layers of blotters in alkaline solutions viz., 0.2 M potassium hydroxide (KOH) or 0.3 M sodium hydroxide (NaOH) and then placed in the Petri-dish where 25 seeds were distributed according to the described system of ISTA. Both conc. of the alkaline solution showed the maximum value on the pH meter scale (pH 14). The plates were incubated at 20 ± 2°C for 7 days under cool white fluorescent lights with alternating cycles of 12 hours light and 12 hours darkness.

The incubated seeds were examined after 7 days under stereoscopic microscope (6-50X magnification) for the presence of

Corresponding Author: Mohamed A. Elwakil, Plant Pathology Department, Faculty of Agriculture, Mansoura University, El-Mansoura, Egypt

the seed-borne fungi and their morphological characteristics. Whenever necessary, a compound microscope was used to confirm the identification by examining the morphology of conidia and conidiophores. The seed-borne fungi were identified using the Commonwealth Mycological Institute, Kew, Surrey, England (CMI) description sheets, Danish Government Institute of Seed Pathology (DGISP) publications, and after reference to Booth (1985), Burrges *et al.* (1988), Chidambaram *et al.* (1973), Ellis (1971), Raper and Fennel (1965), Singh *et al.* (1991), Domsch *et al.* (1980) and Moubasher (1993).

By the aid of stereoscopic microscope, the hyphal-tips of the fungi were transferred on potato dextrose agar (PDA) using tips of heat-stretched capillary tubes. Pure cultures of the fungi were obtained and all isolates were maintained on potato-carrot agar (PCA) slants for further studies.

Data was analyzed with the statistical analysis system (SAS Institute, 1988). All multiple comparisons were first subjected to analysis of variance (ANOVA). Comparisons among means were made using least significant differences (LSD) at $p=0.05$ and Duncan's multiple range test.

Results

Thirty five species of fungi belonging to 22 genera were identified from fenugreek seeds (Table 1). *Alternaria alternata, Aspergillus flavus, A. niger, Cladosporium* sp. and *Penicillium* sp., were found to be preponderant. A significant difference was observed between standard moist blotter (SBM), deep-freezing blotter (DFB) and alkaline soaking method (0.2 M KOH or 0.3 M NaOH). A higher number of fungi were recovered with deep freezing method (DFB) than with blotter method (BM) or the two alkaline treatments.

Standard moist blotter method (SBM) significantly enhanced the saprophytes viz., *A. niger* (35.2%) and *Rhizopus* sp., (4.7%) while DFB method enhanced *Aternaria alternata* (10.3%), *Aspergillus carnus* (1.2%), *A. clavatus* (1.5%), *A. nidulans* (6.9%), *Cladosporium*, (18%), *Drechslera tetramera* (1%), *Mucor* (1.2%), *Myrothecium* sp. (1.3%), *Nigrospora* sp. (1%) and *Penicillium* sp. (31.5%).

Sodium hydroxide (NaOH) and potassium hydroxide (KOH) treatments significantly showed their potential to enhance the recovery of some important seed-borne fungi as compared to SBM or DFM. Blotters soaked in (0.2 M) KOH enhanced the recovery of *Fusarium moniliforme* (5.4%), *F. solani* (0.7%) as well as *Epicoccum* spp. (0.5%) as compared to 0.5 , 0.2 and 0.3% in the standard method and 2.9, 0.4 and 0.3%, in the deep freezing method respectively. On the other hand, 0.3 M NaOH treatment showed the recovery of both *F. semitectum* (1.4%) and *Curvularia* sp. (0.7%) as compared to 0.3 and 0.0% in the SBM and 0.5, 0.3%, respectively in DFB methods.

Percentage of recovery of *Verticillium dahliae* associated with fenugreek seeds was 6.5% in KOH and 7.5% NaOH as compared to 0.3% in the SBM and 1% in the DFB. The method proved to be more sensitive as being capable of revealing even minor infections. The sporulation was heavy, particularly in *Fusarium moniliforme* and *Verticillium dahliae*.

Table 1: Efficiency of three detection methods for detecting lurked seed-borne mycoflora of fenugreek seeds

	Percentage infection of seeds determined by three different methods											
							Alkaline treatment method					
	Standard blotter method			Deep freezing method			0.215 M KOH			0.3 M NaOH		
Fungi	Total	Range (L-M)	Mean	Total	Range (L-M)	Mean	Total	Range (L-M)	Mean	Total	Range (L-M)	Mean
Alternaria alternata	66	(0-23)	2.68b	91	(0-47)	10.3a	84	(0-41)	5.81b	81	(0-24)	3.28b
Aspergillus carneus	3	(0-3)	0.23b	28	(0-3)	1.15a	6	(0-2)	0.23b	3	(0-1)	0.07b
Aspergillus clavatus	0	-	0.00b	13	(0-3)	1.50a	0	-	0.00b	0	-	0.00b
Aspergillus flavus	100	(1-48)	14.21a	88	(0-68)	14.1a	81	(0-56)	15.0a	75	(0-56)	7.09b
Aspergillus nidulans	38	(0-26)	3.00b	53	(1-33)	6.94a	28	(0-5)	1.12b	31	(0-11)	1.41b
Aspergillus niger	100	(2-90)	35.15a	88	(0-28)	6.90b	81	(0-58)	12.7b	75	(0-42)	5.84b
Aspergillus ochraceus	53	(0-48)	4.59a	47	(0-79)	5.37a	75	(0-72)	9.92a	41	(0-34)	6.70a
Aspergillus tamarii	31	(0-12)	1.40a	25	(0-9)	0.85a	22	(0-12)	1.50a	34	(0-10)	1.40a
Aspergillus versicolor	25	(0-48)	3.59b	56	(0-79)	12.4a	66	(0-72)	11.5 a	69	(1-55)	9.45ab
Aspergillus spp.	6	(0-4)	0.83b	13	(0-15)	4.16a	13	(0-4)	1.16b	6	(0-9)	2.16b
Cephalosporium sp.	0	-	0.00b	50	(0-6)	1.29a	44	(0-3)	1.04a	44	(0-6)	1.54a
Cercospora traversiana	22	(0-5)	1.44a	13	(0-2)	0.66a	9	(0-13)	1.77a	16	(0-8)	1.44a
Cladosporium sp.	56	(0-44)	7.75b	91	(0-62)	18.1a	91	(0-59)	15.4ab	88	(0-48)	12.4ab
Curvularia sp.	0	-	0.00c	3	(0-1)	0.33b	3	(0-1)	0.16bc	9	(0-2)	0.66a
Drechslera sp.	3	(0-1)	0.20b	6	(0-1)	0.60a	6	(0-2)	0.60a	0	-	0.00b
Drechslera tetramera	13	(0-3)	0.75ab	13	(0-3)	1.00a	6	(0-1)	0.50ab	3	(0-3)	0.38b
Epicoccum sp.	3	(0-1)	0.25ab	3	(0-1)	0.25ab	6	(0-1)	0.50a	0	-	0.00b
Fusarium equiseti	0	-	0.00b	3	(0-2)	0.66a	6	(0-1)	0.66a	0	-	0.00b
Fusarium moniliforme	28	(0-2)	0.54c	56	(0-15)	2.88b	63	(0-26)	5.38a	66	(0-9)	3.04b
Fusarum oxysporum	13	(0-2)	0.25b	41	(0-7)	1.65a	50	(0-13)	2.45a	44	(0-7)	1.60a
Fusarium semitectum	13	(0-2)	0.29b	16	(0-2)	0.47b	28	(0-2)	0.71b	31	(0-8)	1.35a
Fusarium solani	6	(0-1)	0.22b	9	(0-2)	0.44ab	19	(0-1)	0.66a	6	(0-1)	0.22b
Mucor sp.	3	(0-1)	0.16c	9	(0-3)	1.16a	13	(0-1)	0.66b	3	(0-1)	0.16c
Myrothecium sp.	0	-	0.00c	9	(1-2)	1.33a	6	(0-2)	1.00b	0	-	0.00c
Nigrospora sp.	0	-	0.00c	6	(0-1)	1.00a	3	(0-1)	0.50b	0	-	0.00c
Penicillium sp.	88	(1-43)	13.89b	81	(2-76)	31.46a	84	(0-83)	20.60b	88	(0-7)	15.42b
Phoma sp.	1	(0-1)	1.00	0	-	0.0	0 0	-	0.0	0	-	0.00
Rhizoctonia solani	0	-	0.0	0 0	-	0.0	0 1	(0-1)	1.00	0	-	0.00
Rhizopus sp.	78	(0-10)	4.73a	59	(0-13)	3.15b	22	(0-4)	0.53c	6	(0-2)	0.07c
Sclerotinia sclerotiorum	0	-	0.00	1	(0-1)	1.00	0	-	0.0	0	-	0.00
Stachybotrys sp.	3	(0-2)	0.22b	22	(0-2)	1.11a	13	(0-7)	1.88a	13	(0-5)	1.22a
Stemphylium sp.	44	(0-23)	1.48a	88	(0-40)	4.58a	66	(0-31)	2.93a	47	(0-43)	2.20a
Trichoderma sp.	0	-	0.000	4	(0-1)	1.00	0	-	0.00	0	-	0.00
Trichothecium sp.	3	(0-1)	0.13b	16	(0-1)	0.63a	6	(0-2)	0.50a	13	(0-3)	0.63a
Verticillium dahliae	16	(0-3)	0.25b	34	(0-6)	1.11b	72	(0-48)	6.48a	56	(0-79)	7.51a

Thirty-two samples were tested for fungal assay by 3 different methods. Values between brackets represent the range of infection percentage from lower (L) to maximum (M). Statistical analysis for data is represented by means of 32 replicates of samples. Values of means within a row followed by the same letter(s) are not significantly different ($P=0.05$) according to Duncan's multiple range test.

Both alkali-methods and DFB were equally effective in detecting the seed-borne *Cephalosporium, F. oxysporum, Stachybotrytis* and *Trichothecium* spp. (Table 1). On the other hand, KOH and DFB-methods showed an equal sensitivity in detection of *F. equiseti, Drechslera* spp. and *A. versicolor*. No difference between SBM, DFB and KOH was observed in the detection of *Aspergillus flavus*. All the treatments showed no differences in the recovery of *Aspergillus ochraceus, A. tamarii, Cercospora traversiana* and *Stemphylium* sp. In the meanwhile, the presence of saprophytes in most samples impaired the identification of important seed-borne fungi. Their incidences were significantly lower when using the alkaline treatments, especially in detection of *Rhizopus* sp. Since the presence of *Phoma* sp., *Rhizoctonia solani, Sclerotinia scleotiorum* and *Trichoderma* sp., in the tested samples were extremely low, they were neglected in this investigation.

Discussion

A significant difference between the standard blotter, deep freezing and the alkaline treatment methods was obsreved. Of these KOH showed a potential to enhance the growth of *F. moniliforme, F. solani* and *Epicoccum* sp. whereas NaOH showed an enhanced growth of *F. semitectum* and *Curvularia* sp. At the same time, the treatments provide an effective control of the saprophytes, which impair the detection of the colonized pathogenic fungi in seeds.

It is interesting to note that the presence of saprophytes during the course of seed examination compel the analyst to use high magnification of the stereoscopic microscope (X 50) which is distressful to the eyes. The lower magnifications are not befitting to the detection of slow growing pathogens, which always are covered by the saprophytes. Alakline treatment could therefore help avoiding such complications.

The effect of alkaline media in the recovery of lurked seed-borne fungi may probably be due to the effect of the alkaline ions (K^+ or Na^+) which replace H^+ in the fungal cell. This explanation is in support with the finding of Horikoshi and Akiba (1982) who indicated that Na^+ increase the uptake of nutrients in the cells of some Bacillus strains. Abo-Ellil (1999a-b) found a positive relation between Na^+ ion in the medium and the production of α-amalyse in Verticillium lateritium and that the uptake of sugars in the fungal cell was accomplished with the increase of alkalinty of the medium. Since alkaline chemicals showed there competence in coming out with the lurked Verticillium dahliae, the results presented there suggested that the alkaline seed-bed technique is a sensitive method for detecting such fungi in fenugreek.

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