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An Innovative Method for Detecting Slow Growing Seed-Borne Fungi of Peanut

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Abstract: A method suitable for detecting the slow growing seed-borne fungi of peanut was developed. Twenty-five seed samples collected from commercial markets in Egypt were used in this investigation. With the Standard Moistened Blotter Method (SBM) and Deep-Freezing Blotter method (DFB) recommended by the International Seed Testing Association, saprophytes developed quickly and often impaired the detection of parasitic fungi and inhibited the germination of some important seed-borne fungi. Moistening the blotter disks used for seed germination with an alkaline solution at pH 12.5 using NaOH (0.8%) or KOH (0.4%) enhanced the growth and recovery of the slow growing seed-borne pathogens *Cephalosporium* sp. and *Verticillium* sp. The treatments also were effective in suppressing the growth of saprophytes which impair the detection of pathogenic fungi on seed. We recommend using the alkaline blotter method for seed health testing when searching for slow growing seed-borne fungi.

Key words: Slow growing fungi, seed-borne fungi, detection, peanut

INTRODUCTION

Seeds, pods and seedlings of peanut are susceptible to the attack of several soil-borne fungi, including *Rhizoctonia* sp., *Fusarium* sp., *Pythium* sp., *Rhizopus* sp., *Penicillium* sp., *Aspergillus* sp., *Trichothecium* sp., *Macrophomina phaseolina*, *Alternaria* sp., *Botrytis cinerea*, *Helminthosporium* sp., *Mucor* sp., *Curvularia* sp., *Cladosporium* sp., *Botryodiplodia theobromae*, *Chaetomium* sp. (Porter *et al*., 1990; Richardson, 1990; Baird *et al*., 1993a, b; Dharmaputra and Retnowati, 1996; Shim *et al*., 1996), *Sclerotium rolfsii* and *S. bataticola* (Porter *et al*., 1990; Hollowell *et al*., 1998). *Verticillium* sp., the cause of floury rot disease, was isolated from peanut pods at a later stage of maturity (Mathur *et al*., 1975; Melouk *et al*., 1983). *Rhizoctonia solani*, the major causal organism of seedling damping off and *Fusarium* sp. were isolated from seeds.

An improved method of seed health testing was developed by Elwakil and Ghoneem (2002) to detect lurked pathogens on fenugreek, which coexist at a low percentage. Blotting papers were soaked in solutions of NaOH and KOH, 0.3 and 0.2 Mole, respectively, instead of tap water, which is used in the standard blotter and deep-freezing methods. KOH and NaOH treatments detected *Verticillium dahliae* at 6.5 and 7.5%, respectively, compared to 0.3% in the standard blotter and 1% in the deep-freezing method. KOH treatment also detected *Fusarium moniliforme* and *F. solani* at 5.4 and 0.7%, respectively, compared to 0.5 and 0.2% in the standard blotter method and 2.9 and 0.4%, in the deep-freezing method. They also found that sodium hydroxide stimulated the growth of *F. semitectum* and *Curvularia* sp. Since little information about methods for detecting the slow-growing seed-borne fungi of peanut is available, the present research was planned to create a seed health testing method to detect the slow growing seed-borne fungi of peanut and suppress the growth of saprophytes which impair the microscopic examination of seeds.

MATERIALS AND METHODS

Twenty-five seed samples of mixed peanut cultivars (Giza 4 and 5) representing peanut producing areas in Egypt were used in this study.

The conventional technique for the detection of seed-borne mycoflora was carried out following the procedures published by ISTA (1996). Two hundred seeds of each sample were tested using the standard blotter and deep freezing methods.

The innovative method (Alkaline blotter method): Several trials were carried out by soaking blotters in one of two alkaline solutions, potassium hydroxide solution (KOH) or sodium hydroxide solution (NaOH). The blotters were placed in a Petri-dish, where 10 seeds were distributed. NaOH (0.4, 0.8 and 1%) and KOH (0.2, 0.4 and 0.8%) were

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used. The plates were incubated at 20±2°C for 7 days under cool white fluorescent light with alternating cycles of 12 h light and 12 h darkness. Seeds were then ready for examination under a stereoscopic binocular microscope (6-50 X) for the presence of seed-borne fungi and to study their growth characteristics. When necessary, a compound microscope was used for confirming the identifications after having examined the morphology of conidia and conidiophores. The fungi were micro-photographed.

Fungi present on seeds were identified by utilizing the description sheets of the Commonwealth Mycological Institute (CMI) Kew, Surrey, England, Danish Government Institute of Seed Pathology (DGISP) publications and publications of (Raper and Fennel, 1965; Ellis, 1971; Chidambaram *et al*., 1973; Moubasher *et al*., 1977; Booth, 1985; Burrges *et al*., 1988; Singh *et al*., 1991).

Measuring the linear growth and determining fungal sporulation: The following pathogenic fungi were investigated; *Cephalosporium* sp., *Fusarium verticillioides*, *F. oxysporum*, *F. solani* and *Verticillium* sp. NaOH or KOH were dissolved in 10 mL distilled water. The solutions were added to Czapek's agar medium in 9 cm Petri-dishes. The final concentrations of NaOH and KOH in the medium reached 0.8 and 0.4%, respectively, while the pH was 12.5 in both cases.

A mycelial disk (0.4 cm diameter) was cut from 7 day-old cultures grown on PDA and placed in the center of the agar plate. Three replicates were used per treatment. All cultures were incubated at 25±2°C for 7 days in the dark. The linear growth and number of spores produced by each fungus was recorded 7 days after incubation. The means of three replicates were calculated.

To quantify spore production, the fungus was gently scraped from the Petri-dish with a sharp spatula and washed several times with a total volume of 100 mL of sterilized water. The fungal suspension was filtered through a plastic sieve to separate the spores from the mycelium. The number of spores mL^{-1} was determined using a haemocytometer slide.

RESULTS

Detection of the slow growing seed-borne fungi

Alkaline blotter method: The blotters soaked in a solution of NaOH (0.4, 0.8 and 1%) or KOH (0.2, 0.4 and 0.8%) instead of water showed a decrease in the growth of fast growing saprophytes, viz: *A. flavus*, *A. niger*, *Rhizopus* sp. and *Penicillium* sp. and an increase in the growth of the slow growing seed-borne fungi, viz; *F. solani* (15, 30 and 10% in NaOH and 11.7, 25 and 20% in KOH). *F. oxysporum* (13.3, 21.7 and 10% in NaOH and 10, 15 and 11.7% in KOH) and *Cephalosporium* sp. (3.3, 11.7 and 3.3% in NaOH and 3.3, 6.7 and 0% in KOH). *F. verticillioides* was detected at a rate of 3.3, 10 and 6.7% in NaOH and 3.3, 3.3 and 0% in KOH. *Verticillium* sp. was detected at a rate of 6.7, 8.3 and 0% in NaOH and 0, 6.7 and 0% in KOH as shown in (Table 1).

Table 1: Incidence of seed-borne fungi of peanut using the innovative method of NaOH or KOH solutions

		Blotter method*					
		Detected fungi					
		NaOH (%)			KOH (%)		
Fungus	Check	0.4	0.8	1	0.2	0.4	0.8
Alternaria alternata	15.0ab	15.0ab	8.0d	8.0d	16.0a	14.0b	10.0c
Aspergillus carneus	0.0b	0.0b	0.0b	0.0b	0.0b	0.0b	0.0b
Aspergillus flavus	93.3a	75.0a-d	73.3a-d	73.3a-d	88.3ab	83.3a-c	81.7a-c
Aspergillus nidulans	4.0b	0.0c	0.0c	0.0c	0.0c	0.0c	0.0c
Aspergillus niger	73.3a	73.3a	63.3a-c	63.3a-c	73.3a	68.3ab	66.7a-c
Aspergillus ochraceous	20.0a	10.0a-c	6.7bc	3.3bc	11.7ab	10.0a-c	5.0bc
Aspergillus oryzae	13.3a	10.0ab	8.3a-c	6.7a-c	13.3a	10.0ab	6.7a-c
Aspergillus tamarii	2.0b	0.0c	0.0c	0.0c	0.0c	0.0c	0.0c
Aspergillus versicolor	3.0b	0.0c	0.0c	0.0c	0.0c	0.0c	0.0c
Botrytis cinerea	0.0b	0.0b	1.7b	0.0b	0.0b	1.7b	0.0b
Cephalosporium sp.	3.3g-i	3.3g-i	11.7c-f	3.3g-i	3.3g-i	6.7e-i	0.0i
Drechslera sp.	10.0a	2.0c	0.0d	0.0d	0.0d	0.0d	0.0d
Fusarium verticillioides	1.7b	3.3ab	10.0ab	6.67ab	3.3ab	3.3ab	0.0b
Fusarium oxysporum	8.3g	13.3fg	21.7d-g	10.0fg	10.0fg	15.0fg	11.7fg
Fusarium semitectum	6.7a	2.0bc	0.0d	0.0d	1.0cd	0.0d	0.0d
Fusarium solani	3.0j	15.0f-j	30.0c-f	10.0ij	11.7h-j	25.0d-i	20.0f-j
Penicillium sp.	45.0a	33.3a-c	30.0a-d	26.7a-e	43.3a	36.7ab	31.7a-d
Rhizoctonia solani	8.3a	0.0d	0.0d	0.0d	0.0d	0.0d	0.0d
Rhizopus sp.	78.3a	73.3a	68.3a	53.3b	78.3a	73.3a	68.3a
Sclerotiumbataticola	8.3a	1.7b	0.0b	0.0b	0.0b	0.0b	0.0b
Stemphylium sp.	5.0a	0.0b	0.0b	0.0b	0.0b	0.0b	0.0b
Trichothecium sp.	6.0b	0.0c	0.0c	0.0c	0.0c	0.0c	0.0c
Verticillium sp.	0.0g	6.7d-f	8.3d-f	0.0g	0.0g	6.7d-f	0.0g

*: The method represents 25 seed samples of peanut; Values having different letter(s) are significantly different at $p<0.05$

Table 2: Incidence of seed-borne fungi of peanut using the innovative method of NaOH or KOH solutions

		Deep freezing method*					
		Detected fungi					
		NaOH (%)			KOH (%)		
Fungus	Check	0.4	0.8	1	0.2	0.4	0.8
Alternariaalternata	6.0e	5.0ef	1.0h	0.0h	8.0d	4.0fg	0.0h
Aspergillus carneus	6.0a	0.0b	0.0b	0.0b	0.0b	0.0b	0.0b
Aspergillus flavus	68.3b-e	66.7c-e	50.0ef	50.0ef	68.3b-e	56.7ed	55.0ed
Aspergillus nidulans	10.0a	0.0c	0.0c	0.0c	0.0c	0.0c	0.0c
Aspergillus niger	71.7a	55.0a-d	51.7a-d	45.0a-d	40.0b-e	55.0a-d	38.3bc
Aspergillus ochraceous	10.0a-c	10.0a-c	3.3bc	1.7bc	11.7ab	6.7bc	6.7bc
Aspergillus oryzae	10.0ab	5.0a-c	3.3bc	3.3bc	10.0ab	8.3a-c	6.7a-c
Aspergillus tamarii	12.0a	0.0c	0.0c	0.0c	0.0c	0.0c	0.0c
Aspergillus versicolor	6.0a	0.0c	0.0c	0.0c	0.0c	0.0c	0.0c
Botrytis cinerea	0.0b	0.0b	0.0b	0.0b	0.0b	0.0b	0.0b
Cephalosporium sp.	5.0f-i	6.7e-i	16.7bc	1.7hi	5.0f-i	8.3d-h	3.3g-i
Drechslera sp.	6.0b	0.0d	0.0d	0.0d	0.0d	0.0d	0.0d
Fusarium verticillioides	3.3ab	6.7ab	13.3a	10.0ab	10.0ab	13.3a	10.0ab
Fusarium oxysporum	15.0fg	25.0c-g	33.3b-d	18.3e-g	20.0e-g	26.7c-f	11.7fg
Fusarium semitectum	3.0b	0.0d	0.0d	0.0d	0.0d	0.0d	0.0d
Fusarium solani	15.0f-j	20.0fj	50.0ab	28.3c-g	23.3e-j	40.0b-d	15.0f-j
Penicillium sp.	23.3a-f	16.7b-f	13.3b-f	13.3b-f	23.3a-f	20.0a-f	16.7b-f
Rhizoctonia solani	3.3c	0.0d	0.0d	0.0d	0.0d	0.0d	0.0d
Rhizopus sp.	51.7b	23.3de	20.0ef	20.0ef	38.3c	35.0cd	26.7c-e
Sclerotium bataticola	0.0b	0.0b	0.0b	0.0b	0.0b	0.0b	1.7b
Stemphylium sp.	0.0b	0.0b	0.0b	0.0b	0.0b	0.0b	0.0b
Trichothecium sp.	10.0a	0.0c	0.0c	0.0c	0.0c	0.0c	0.0c
Verticillium sp.	5.0e-g	5.0e-g	10.0de	3.3fg	3.3fg	10.0de	0.0g

*: The method represents 25 seed samples of peanut; Values having different letter(s) are significantly different at $p<0.05$

Table 3: Effect of alkaline treatments on the linear growth and sporulation of five fungi isolated from peanut seeds 7 days after incubation under 25±2°C

		Cephalosporium sp.		*Fusarium verticillioides*		*Fusarium oxysporum*		*Fusarium solani*		*Verticillium* sp.	
Treatments	pH	Growth diameter	Sporulation	Growth diameter	Sporulation	Growth diameter	Sporulation	Growth diameter	Sporulation	Growth diameter	Sporulation
Check	7.2	2.8e	14.1f	7.4c	54.0e	6.5c	134.2c	7.0d	74.2c	5.4d	13.4f
NaOH (%)											
0.4	12.0	2.6f	15.6c	6.0d	59.2d	6.9b	129.5d	8.0b	58.8e	6.9b	27.0c
0.8	12.5	4.5a	21.9b	9.0a	169.7a	9.0a	207.3a	9.0a	80.6b	8.0a	39.4a
1.0	13.2	3.0d	15.3d	5.0e	56.4de	6.6c	125.5e	5.8f	46.7f	4.9e	14.4e
KOH (%)											
0.2	11.8	3.2c	15.1e	6.0d	111.6c	5.7d	64.3f	7.5c	69.6d	6.7c	24.7d
0.4	12.5	4.4a	22.5a	8.0b	162.0b	9.0a	191.3b	9.0a	81.3a	7.9a	35.9b
0.8	13.0	4.0b	12.9g	4.0f	42.6f	5.8d	45.1g	6.0e	42.9g	3.9f	10.7g

Diameter of the colony (cm) grown on Czapek's`gar supplemented with the test compound. Number of spores $\times 10^6$ mL^{-1}. Statistical analysis for data are the means of 3 replicates. Values of means within a column followed by the same letter(s) are not significantly different (p = 0.05) according to Duncan (1995) multiple range test

Alkaline treatment followed by deep-freezing treatment: In this method, the application was carried out as applied in the standard Deep-Freezing Method (DFM) but the blotters were soaked in solutions of NaOH (0.4, 0.8 and 1%) or KOH (0.2, 0.4 and 0.8%) instead of water. Results showed a decrease in the growth of fast growing saprophytes, including *A. flavus*, *A. niger*, *Rhizopus* sp. and *Penicillium* sp. and an increase in the slow growing seed-borne ones. *F. solani* was detected at rates of 20, 50 and 28.3% in NaOH, while 23.3, 40 and 15%, respectively, in KOH. *F. oxysporum* was detected at 25, 33.3 and 18.3% in NaOH and 20, 26.7 and 11.7%, respectively, in KOH. *F. verticillioides* was detected at 6.7, 13.3 and 10% in NaOH and 10, 13.3 and 10%, respectively, in KOH. *Cephalosporium* sp. was detected at 6.7, 16.7 and 1.7% in NaOH and 5, 8.3 and 3.3%, respectively, in KOH. *Verticillium* sp. was detected at 5, 10 and 3.3% in NaOH and 3.3, 10 and 0%, respectively, when KOH was used as sown in (Table 2).

Effect of alkaline solution on the linear growth and sporulation: To verify the effect of alkaline media on the enhancement of slow growing fungal pathogens, a number of seed-borne fungi attacking peanut seeds were grown on Czapek's alkaline medium and the linear growth and sporulation were recorded.

Alkaline treatments enhanced the linear growth and sporulation of *Cephalosporium* sp., *Fusarium verticillioides*, *F. oxysporum*, *F. solani* and *Verticillium* sp. as shown in Table 3 and Fig. 1.

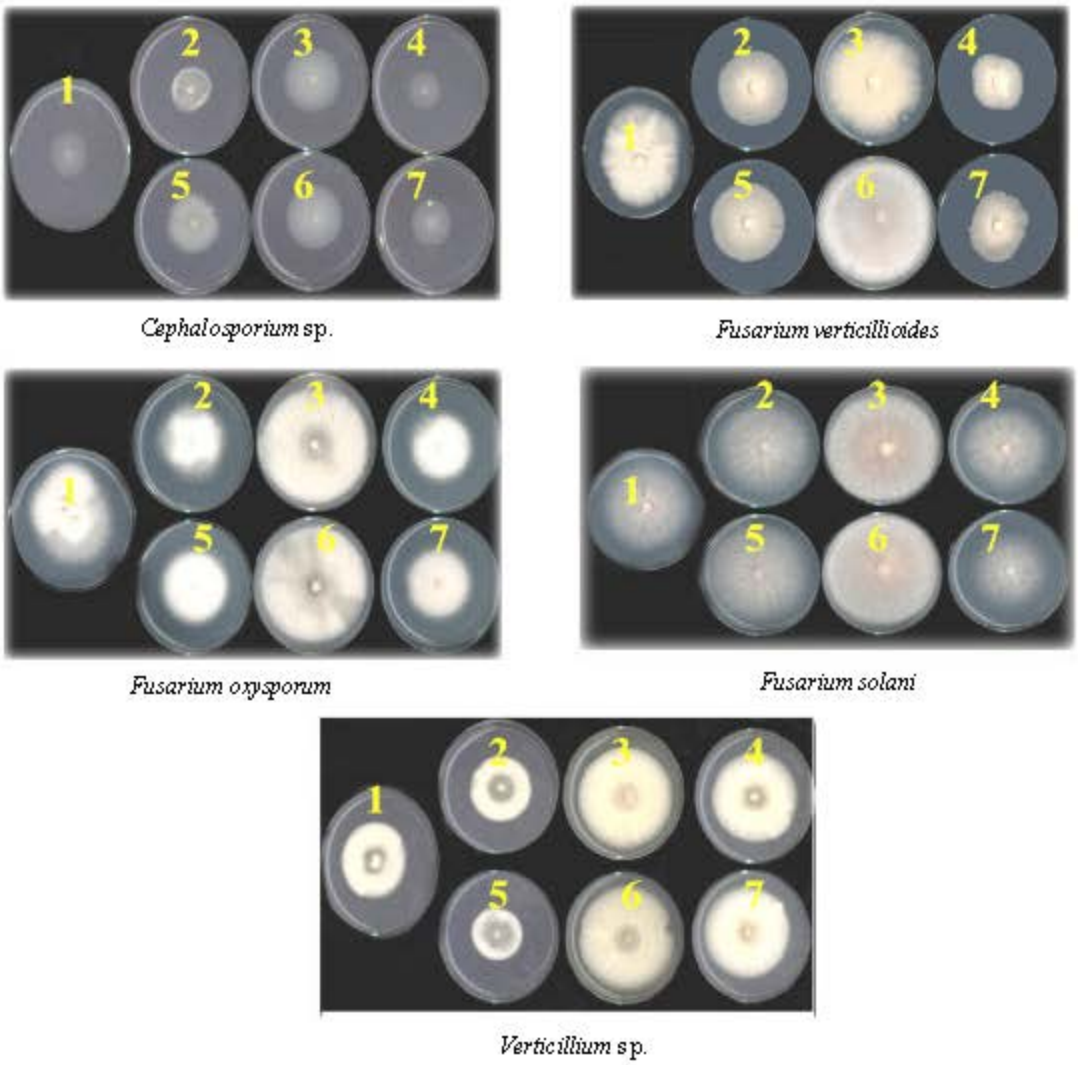

Fig. 1: Effect of NaOH or KOH on the linear growth of seed-borne fungi of peanut; 1: Check; 2: NaOH 0.4%; 3: NaOH 0.8%; 4: NaOH 1.0%; 5: KOH 0.2%; 6: KOH 0.4%; 7: KOH 0.8%

Cephalosporium sp. grew 4.5 cm and produced 21.9×10^6 spores in NaOH 0.8% and 4.4 cm and 22.5×10^6 spores in KOH 0.4%. *Fusarium verticillioides* grew 9 cm and produced 169.7×10^6 spores in NaOH 0.8% and 8 cm and 162×10^6 spores in KOH 0.4%. *F. oxysporum* grew 9 cm and produced 207.3×10^6 spores in NaOH 0.8% and 9 cm and 191.3×10^6 spores in KOH 0.4%. *F. solani* grew 9 cm and produced 80.6×10^6 spores in NaOH 0.8% and 9 cm and 81.3×10^6 spores in KOH 0.4%. *Verticillium* sp. grew 8 cm and produced 39.4×10^6 spores in NaOH 0.8% and 7.9 cm and 35.9×10^6 spores in KOH 0.4%. The check grew (2.8 cm and 14.1×10^6 spores), (7.4 cm and 54×10^6 spores) (6.5 cm and 134.2×10^6 spores), (7 cm and 74.2×10^6 spores) and (5.4 cm and 13.4×10^6 spores), respectively.

Both NaOH (0.8%) and KOH (0.4%) had a pH of 12.5 and gave almost the same results for linear growth and sporulation.

DISCUSSION

A number of standard methods for seed health testing are utilized to test seeds for the presence of seed-borne fungi. The (SBM) and (DFB) are recommended by the ISTA (1996). It is known that with SBM saprophytes develop quickly and often impair the detection of parasitic fungi, while with DFB the growth of saprophytic bacteria and yeasts is enhanced, which may inhibit spore germination of some important seed-borne fungi (Neergaard, 1979).

With these methods, it is difficult to detect some seed-borne pathogens due to their slow growth rates, e.g., *Cephalosporim* and *Verticillium*. This research was designed to develop a method suitable for detecting the slow growing seed-borne fungi. The results presented here show that moistening the blotter disks with an alkaline solution enhanced the growth of the slow growing seed pathogens *Cephalosporim* sp. and *Verticillium* sp.

The alkali treatments also proved to be an effective means to suppress the growth of saprophytes which impair the detection of pathogenic fungi on seed. Examination of seed-testing blotters overgrown by saprophytic fungi required the use of high magnification (X50) and additional time. Lower magnifications (X6 and 10) were not suitable to detect slow growing pathogens overgrown by saprophytes.

On conclusion, the alkaline blotter method is an effective method for the detection of some important seed-borne pathogenic fungi (*Cephalosporium* sp., *Fusarium verticillioides*, *F. oxysporum*, *F. solani* and *Verticillium* sp.) on peanut seeds. The two alkaline treatments were effective for the recovery of lurked *Verticillium* sp. from peanuts.

Elwakil and Ghoneem (2002) stated that the effect of alkaline media on the recovery of lurked seed-borne fungi may be due to the presence of the alkaline ions K^+ or Na^+, which replace H^+ in the fungal cell. This explanation agrees with the findings of Horikoshi and Akiba (1982), who indicated that Na^+ increases the uptake of nutrients in the cells of some *Bacillus* strains. Abo-Ellil (1999a, b) found that the positive relationship between Na^+ ion in the medium and the production of α-amylase in *Verticillium lateritium* and the uptake of sugars in the fungal cell increased with the alkalinity of the medium. Since alkaline chemicals have previously shown their competence in detecting the lurked *Verticillium* sp., the results presented here verify that the alkaline blotter method is a sensitive method for detecting the slow-growing seed-borne fungi of peanut.

Intensive seed health testing research has shown that pH plays an important role in the recovery of seed-borne fungi from seed.

The results of this research clearly indicate that the regular seed-testing methods recommended by ISTA are less effective for detecting both *Cephalosporium* spp. and *Verticillium* spp. than our innovative alkaline blotter method.

Based on present research, it is expected that seeds grown in alkaline soils may be affected by the above fungi, as the soil environment is ideal for their growth. For that reason, we recommend using the alkaline blotter method for seed testing in regions where peanut is grown in alkaline soil.

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