Plant Pathology Journal

ISSN 1812-5387

science alert

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Plant Pathology Journal 8 (1): 22-26, 2009
ISSN 1812-5387

Alkaline Seed-Bed: An Innovative Technique for Manifesting *Verticillium dahliae* on Fennel Seeds

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Abstract: *Verticillium dahliae* attacks a wide range of plants including fennel causing a wilt disease. The fungus grows slowly on seeds when tested at the seed health laboratories. This habit character allows saprophytes to impair the fungal growth and interfere the identification on both Moist Blotters (MB) and the Deep-Freezing Blotters (DFB). Since, these two techniques are not efficient enough to detect this fungus, the researchers planned to search for an alternative technique for detecting this fungus. Soaking three layers of blotters used as seed-beds in water solutions alkalined with KOH or NaOH at pH 10 presents the optimum seed-bed condition for manifesting the fungus on seed. This seed-bed condition also suppress the growth of saprophytes, so as the fungus was transparently shown on seeds. The *in vitro* study presents pH 9.5 as the optimum condition for the growth, sporulation and maximum glucose coefficient of the fungus. So far, it is recommended to use the alkalined seed-bed when searching for *V. dahliae* on fennel seed.

Key words: Slow growing fungi, seed borne *Verticillium dahliae*, pH, fennel, alkaline blotter

INTRODUCTION

Verticillium wilt is a worldwide disease caused by the soil and seed-borne fungi, *Verticillium dahliae* and *Verticillium albo-atrum*. *V. dahliae* attacks seeds of more than 120 plant species including parsley and fennel (Blum *et al*., 2006). The fungus survives in forms of mycelium or conidia in or on seed's surface (Xu, 2000; Huang *et al*., 2004).

The Moist Blotter (MB) and Deep-Freezing Blotter (DFB) are recommended techniques by the International Seed Testing Association (ISTA, 1999) for detecting seed-borne fungi. MB technique develops saprophytes which often seriously impaired with the growth of parasitic fungi. DFB technique enhances the development of saprophytic bacteria and yeasts on seeds and inhibits the spore-germination of some important seed-borne fungi as well. Subsequently, it is difficult to isolate and identify the slow growing seed-borne fungi including *Acremonium* sp. and *Verticillium* sp. on seed when using such techniques. These fungi produce poor mycelium and do not show their imperfect stage on seeds (Neergaard, 1979).

The research presents the role of seed-bed pH on manifesting the slow growing seed-bone fungi on fennel seeds and establish an efficient technique for transpiring *V. dahliae* when seed health test is carried out.

MATERIALS AND METHODS

Samples: Twenty fennel seed samples collected from various regions of Egypt including Alexandria, Domitta, Gharbia, Dakhlia, Cario and Assuit, were used in the present study.

Seed health testing: The conventional technique for the detection of seed-borne fungi on fennel seeds was carried out following two techniques recommended by the International Seed Testing Association (ISTA, 1999) i.e., MB and DFB. The proposed method by the researchers was applied. The percentages of the recovered fungi in each method were tabulated.

The alkalined proposed seed-bed: Three layers of blotter (filter paper) were soaked in tap water at pH ranged from 5 to 11. Blotters moistened in sterilized tap water were

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used as check treatment (MB and DFB). An acidic water was prepared by adding drops of HCl to the tap water and adjusted to give different acidic pHs whereas; the alkalined water was prepared by using KOH or NaOH. The soaked blotters in a wide range of pH (4.5-12.5) were placed in 9 cm diameter Petri-dish, where 25 seeds were distributed on each seed-bed as described by ISTA (1999). The plates were incubated at 20±2°C under cool white fluorescent lights with alternating cycles of 12 h light and 12 h darkness.

Seven days latter, the incubated seeds were examined under a stereoscopic microscope at 6-50X magnification to detect fungi on seeds and to study their morphological characteristics. The compound microscope was used to confirm the identification. Hyphal-tip from each fungus was transferred onto Potato Dextrose Agar (PDA) plates using tip of heat-stretched capillary tube. Pure cultures of the isolated fungi were obtained and all isolates were maintained on slants of potato-carrot agar for further studies.

The fungal isolates were identified in consultation with the Commonwealth Mycological Institute description sheets, Danish Government Institute of Seed Pathology publications, Raper and Fennel (1965), Ellis (1971), Chidambaram *et al.* (1973), Domsch *et al.* (1980), Booth (1985), Burrges *et al.* (1988) and Moubasher (1993).

***In vitro* growth, sporulation and glucose utilization of *V. dahliae* at different pHs:** *Verticillium dahliae* was grown on plates of Czapek's Agar medium for 10 days at 25°C in dark. The fungal growth was scraped gently from the medium surface by using a glass rod and suspended in sterile distilled water. The collected spores was regulated to about 4×10^6 spores mL^{-1}, while 0.5 mL adjusted to inoculate 50 mL of Drews liquid medium (Drews, 1983) in 250 mL Erlenmeyer flasks. The growth media presented different pH values ranged from 4.5 to 12.5. The cultures were incubated at 20±2°C for 14 days under cool white fluorescent light with alternating cycles of 12 h light and 12 h darkness.

The final culture pH of *V. dahliae* was measured at the end of incubation period. Spore formation per 1 mL of culture was determined using hemocytometer. Cultures were then, filtered though filter paper (Whatman No. 1) and dried at 80°C till constant weight.

Glucose residue in the cultural supernatant was estimated at the end of the incubation period following *o*-toluidine method (Sasaki *et al.*, 1972).

Statistical analysis: The statistical analysis software CoStat 6.311 was used to compare means using Duncan's multiple range test, as well as to estimate the correlation coefficient (r), at $p\leq0.05$.

RESULTS

Optimum seed-bed pH condition for manifesting *V. dahliae* on fennel seeds: A wide range of pH was used for soaking the blotters to detect the slow growing fungus; *V. dahliae* on fennel seeds. Among the wide range of blotters pH, the alkalined seed-bed condition was suitable for detecting *V. dahliae* in compare with MB and DFB (Table 1). These condition also suppress the growth

Table 1: Effect of blotter pH in detection of lurked seedborne fungi of fennel

	Blotter pH											
	5	6	Standard methods**		8		9		10		11	
Fungi (%)	HCl	HCl	MB	DFB	KOH	NaOH	KOH	NaOH	KOH	NaOH	KOH	NaOH
Acremonium sp.	0.0c*	0.0c	1.6a	1.5a	0.0c	0.0c	1.0b	0.0c	0.0c	0.0c	0.0c	0.0c
Alternaria alternata	23.0b	24.0b	23.0b	24.5b	24.0b	27.0a	27.0a	24.0b	18.0d	18.0d	18.0d	20.0c
Aspergillus flavus	3.0a	3.0a	3.0a	0.0c	3.0a	0.0c	2.0b	2.0b	0.0c	0.0c	0.0c	0.0c
Aspergillus niger	0.0e	1.7d	9.0a	0.0e	5.0b	1.0de	0.0e	3.0c	2.0cd	0.0e	3.0c	0.0e
Aspergillus ochraceus	1.0a	0.3ab	1.0a	0.3ab	0.7ab	0.7ab	0.0b	0.0b	0.7ab	0.0b	0.0b	0.0b
Cladosporium sp.	15.7b	16.3b	11.0d	22.7a	17.0b	23.0a	22.5a	13.0c	13.0c	9.0e	16.7b	10.7d
Curvularia sp.	0.3b	0.0b	0.0b	0.0b	0.8a	0.0b	0.0b	0.0b	0.0b	0.0b	0.0b	0.0b
Drechslera sp.	4.5b	4.5b	2.3de	3.8bc	1.8e	7.5a	1.8e	3.7c	0.8 f	2.8d	0.8 f	3.7c
Epicoccum sp.	5.7b	6.0b	7.3a	2.7c	1.8cd	1.2d	1.5d	0.0e	1.3d	0.0e	1.0d	0.0e
Fusarium oxysporum	0.0b	0.0b	0.0b	0.7a	0.2b	0.0b	0.8a	0.0b	0.0b	0.0b	0.0b	0.7a
F. solani	0.0b	0.0b	0.0b	0.0b	0.0b	1.7a	0.0b	0.0b	0.0b	0.0b	0.0b	0.0b
F. verticillioides	0.0d	0.0d	0.0d	1.3c	1.2c	1.2c	2.2b	1.2c	0.3d	3.2a	0.0d	0.0d
Myrothecium sp.	3.7a	3.2a	2.0b	0.0d	1.3c	1.0c	0.8c	0.0d	0.8c	0.0d	0.0d	0.0d
Rhizopus sp.	1.3d	5.2b	7.2a	0.3e	3.3c	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e
Stemphylium sp.	13.7h	13.0h	27.3b	19.5d	17.3efg	28.5a	18.2e	16.7g	17.2fg	23.7c	19.2d	18.0ef
Verticillium dahliae	3.0g	4.0f	1.0i	2.3h	3.2g	5.0e	6.2d	5.3e	8.2b	9.2a	5.2e	7.0c

*Mean of fungal presence in the tested samples. Three samples each of 400 seeds were investigated. **The pH of standard methods is approximately 7. Values within a raw followed by the same letter(s) are not significantly differed at $p\leq0.05$

Table 2: Comparison of MB and DFB and alkaline blotters (pH 10) for manifesting fungi on fennel seeds

					Alkaline blotter (pH 10)			
	MB		DFB		KOH		NaOH	
Fungus	%*	Mean**	%	Mean	%	Mean	%	Mean
Acremonium sp.	60	0.77b	70	1.11b	70	1.55a	80	1.88a
Alternaria alternata	100	22.00a	100	26.60a	100	22.40a	100	25.6a
Alternaria radicina	10	0.50ab	0	0.00b	20	1.00a	10	0.70a
Aspergillus flavus	80	6.87a	40	1.00b	70	5.12a	80	4.12a
Aspergillus niger	80	4.55a	30	1.44b	60	2.55b	60	2.33b
Aspergillus nidulans	50	3.60a	20	6.80a	50	4.40a	50	4.60a
Aspergillus ochraceus	50	7.12a	20	3.00b	60	7.62a	40	7.00a
Aspergillus tamarii	20	1.00a	0	0.00b	20	0.40b	20	0.60ab
Aspergillus versicolor	40	6.33a	30	8.00a	50	6.00a	30	7.00a
Chaetomium sp.	20	1.00a	0	0.00b	0	0.00b	0	0.00b
Cladosporium sp.	10	9.80a	100	13.20a	90	12.10a	90	12.10a
Curvularia sp.	0	0.00b	10	1.00a	0	0.00b	10	1.00a
Drechslera tetramera	70	3.60a	30	0.71b	50	1.42b	40	1.71b
Epicoccum sp.	20	1.14a	30	0.43a	40	0.71a	40	1.28a
Fusarium equiseti	20	1.50a	0	0.00b	10	0.50b	0	0.00b
F. oxysporum	10	0.33b	10	0.33b	20	1.33a	10	0.33b
F. semitectum	40	1.12a	60	2.62a	40	3.25a	30	1.75a
F. solani	20	0.50b	0	0.00b	20	1.75a	10	0.25b
F. verticillioides	30	0.50b	30	1.00b	50	1.00b	60	2.00a
Humicola sp.	10	0.25b	0	0.00b	40	1.50a	10	0.75ab
Mucor sp.	10	0.20b	0	0.00b	30	0.60a	20	0.40ab
Myrothecium sp.	30	1.00a	20	0.33b	40	1.16a	20	0.66b
Nakataea sp.	20	1.66a	10	0.33b	0	0.00b	10	0.33b
Nigrospora sp.	10	0.66a	10	0.33a	10	0.33a	0	0.00a
Penicillium sp.	90	8.00a	80	7.10a	90	6.50a	90	10.20a
Rhizopus sp.	80	6.12a	20	0.50b	20	0.78b	50	0.62b
Stachybotrys sp.	70	11.14a	60	4.00b	60	10.85a	60	11.85a
Stemphylium sp.	90	13.22a	90	14.00a	90	11.33a	90	13.44a
Trichothecium sp.	10	3.00a	10	3.00a	10	0.50c	10	1.50b
Verticillium dahliae	40	0.40b	50	1.80ab	70	2.20ab	70	2.50a

*Percentage of positive samples for the specific fungus. Twenty samples, each of 400 seeds, were tested for fungal assay. **Mean within the positive samples containing the specific fungus. Values of means within a row followed by the same letter(s) are not significantly different ($p \leq 0.05$)

of the saprophytic fungi. Blotters of pH 10 presented the optimum condition for manifesting *V. dahliae* abundantly on fennel seeds. This alkaline seed-bed at pH 10 condition was studied in details.

Significant differences in detecting seed-born fungi of fennel among MB, DFB and the proposed alkalined seed-bed. A total of 30 species belongs to 20 genera of fungi were isolated from fennel seeds by using the above mentioned techniques (Table 2).

MB enhanced the recovery of the fast growing saprophytes i.e., *Rhizopus* sp. and *Nakataea* sp. as well as the pathogenic *Fusarium equiseti*. On the other hand, DFB showed a significant reduction in the presence of most saprophytes including *Aspergillus* sp., *Chaetomium* sp.; the commonly growing fungi on the non-germinated seeds.

The Alkalined Blotters (AB) technique used in this investigation enhanced the recovery of the slow growing fungi and proved to be a sensitive method in manifesting the slow growing seedborne fungi including *Acremonium* sp. and *V. dahliae*. It also, increased the detection of *Alternaria radicina* and *F. verticillioides* on seeds. Sporulation of *V. dahliae* was also increased.

Optimum culture pH for growth, sporulation and glucose utilization of *V. dahliae*: *V. dahliae* was able to grow in a wide range of pH on drews liquid medium (4.5-12.5) during the tested intervals (6, 10 and 14 day) (Fig. 1). The cultural pH of 9.5 enhanced the fungal growth while it reached 7.59 g L^{-1} after 14 days of incubation at 20±2°C. In all treatments the final culture pH was reduced to the direction of acidic side. After 14 days of incubation, the final culture pH was kept within a narrow pH range (4.5-5.5). The sporulation gradually increased by increasing the initial pH and decreased at the lower pHs. When the fungus was grown at pH 9.5, it recorded the highest glucose coefficient. At the end of incubation period, the statistical analysis reveals a significant positive correlation between the glucose coefficient and both mycylial dry weight ($r = 0.908$, $p \leq 0.01$) and sporulation ($r = 0.938$, $p \leq 0.01$). The increment in growth and sporulation of *V. dahliae* was directly related to the utilized glucose and glucose coefficient.

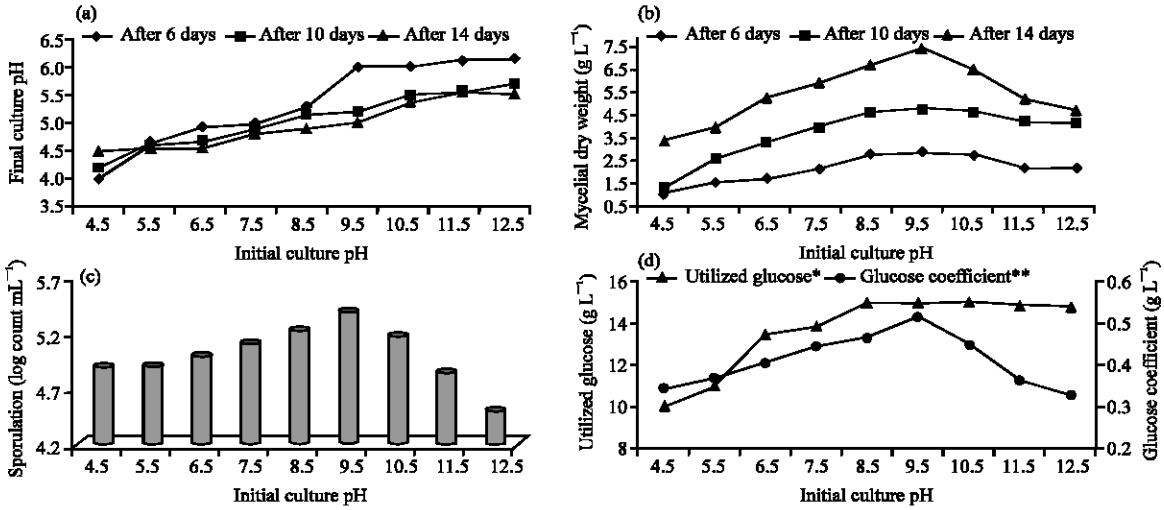

Fig. 1: Effect of initial culture pH on (a) final culture pH, (b) mycelial dry weight during various growth periods of *V. dahlia*, (c) sporulation and (d) glucose utilization and coefficient at the end of incubation period (14 days). *Initial glucose in the medium was 20 g L^{-1}, **Glucose coefficient = Mycelial dry weight (g L^{-1})/utilized glucose (g L^{-1})

DISCUSSION

Fennel seeds used in preparing medicine should be free from toxins which produced by several seed-borne fungi including *V. dahlia*. The fungus produces protein-lipopolysaccharide complexes toxins in nutrient-limited culture filtrates (Meyer and Dubery, 1993). These toxins are reported also to be a cause of wilt and dehydration symptoms on many plants (Pegg and Brady, 2002; Wang *et al*., 2004).

The methods used for seed health testing may be suitable for detecting particular fungus at high percentage; other(s) may be good for detection other pathogens. The recommended seed-health techniques by ISTA (1999) i.e., MB and DFB are not-efficient in detecting the slow growing fungi. They allow the saprophytes to impair the identification of pathogenic fungi and the annalists find difficulties in manifesting the pathogens on seeds under the stereoscopic microscope. This research recorded an innovative technique for manifesting *V. dahliae*, as it is classified as one of the slow growing fungi on fennel seeds.

The results presented here proved that the alkalined seed-bed condition is optimum for detecting the slow growing fungi from fennel seeds including *V. dahliae*. Soaking blotters in alkalined tap water at pH 10 is recommended for the detection of *V. dahliae* (Table 1).

Moistened blotters in alkaline solution of pH 10 show a significant reduction in the saprophyte growth (Table 2). The presence of saprophytes on seed compels the analyst to use high magnification of the stereoscopic microscope (X50) which is distressful to eyes. The lower magnifications (X6 and 10) are not suitable for the detection of slow growing pathogens which are always covered by the saprophytes.

These results were confirmed with that shown in Fig. 1. Although most fungi grow in initial acidic conditions, *V. dahliae* was able to grow abundantly media having initial pH 9.5. However, when the final culture pH was measured, 6, 10 and 14 days after incubation, it turned to be acidic. The acidic final pH of the medium may be the reason of the abundant growth of *V. dahliae* on alkaline pH. It seems likely that the physiological role of this process is to bring the pH of the medium to a range favorable for the fungal growth. The glucose coefficient reached its maximum at pH 9.5 and that reflect the ability of such fungi to survive in alkaline conditions. Abo-Ellil (1999a, b) found a 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 was accomplished with the increase of alkalinity of the medium.

Although, the proposed alkalined technique proved to be more sensitive in up growing the slow growing seed-borne fungus *V. dahlia*, it also, efficient in detecting the pathogenic fungi i.e., *Alternaria radicina* and *F. verticillioides* (Table 2). The presence of the alkaline ions (K^+ or Na^+) replace H^+ in the fungal cell (El-Wakil and Ghoneem, 2002). They increase the uptake of nutrients in the cells of some *Bacillus* strains (Horikoshi and Akiba,

1982). Another explanation is referred only to the pH of the growth media, which may be suitable to the growth and sporulation of such seed-borne fungi (El-Wakil *et al*., 2007).

The over all benefit of the results in this research is recording an innovated technique to help Seed Health Analysts at seed-health laboratories to manifest *V. dahliae* and other slow growing fungi on fennel seeds. More research on the habit character of other slow growing fungi on different seeds under different pH conditions is needed.

ACKNOWLEDGMENT

The authors thank Dr. Conard J. Krass, Primary State Plant Pathologist, California. Department of Food and Agriculture, Sacramento, CA, USA (retired) for critical review of the study.

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