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ORIGINAL ARTICLE

Seed-borne mycoflora of alfalfa (*Medicago sativa* L.) in the Riyadh Region of Saudi Arabia

Abdul-Aziz A. Al-Askar · Khalid M. Ghoneem · Younes M. Rashad

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Abstract Fifteen seed samples of local alfalfa (*Medicago sativa* L.) cultivar (Hegazy) collected from fields at different governorates of the Riyadh region in Saudi Arabia were screened for their seed-borne mycoflora. Standard moist blotter and deep-freezing blotter methods recommended by the International Seed Testing Association, in addition to an innovative alkaline seed-bed method were applied. A total of 24 genera and 35 species of fungi were isolated using the above-mentioned techniques. *Alternaria alternata*, *Cladosporium* sp., *Aspergillus* sp., *Stemphylium* sp. and *Penicillium* sp. were the genera most commonly isolated. Among the various techniques adopted for detection, the alkaline NaOH method was found to be effective and yielded the maximum number of pathogenic fungi. Syndromes of seed discoloration were also investigated. The alkaline seed-bed method using NaOH was used to detect saprophytic and pathogenic seed-borne fungi associated with discolored seeds. A total of 15 genera and 26 species of fungi were isolated. *Cladosporium* sp., *Alternaria alternata* and *Aspergillus* species were the saprophytic species detected most, while *Stemphylium botryosum* and *Fusarium incarnatum* were common pathogenic fungi found on discolored seeds. The average of incidence and occurrence percentages of most detected fungi were higher in discolored seeds than in normal seeds. Seed germination was also affected significantly by discoloration. The data also indicate that seed discoloration decreases seed germination significantly (by 26.3–60%).

Keywords Alfalfa · Germination · Seed-borne fungi and seed discoloration

A.-A. A. Al-Askar · Y. M. Rashad (✉)
Biology Department, Teachers College, King Saud University, Riaydh, Saudi Arabia
e-mail: younesrashad@yahoo.com

A.-A. A. Al-Askar
e-mail: aalaskara@ksu.edu.sa

K. M. Ghoneem
Department of Seed Pathology Research, Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt
e-mail: khalid_ghoneem@yahoo.com

Introduction

Alfalfa (*Medicago sativa* L.) is one of the most important perennial livestock forage crops in the world. In Saudi Arabia, alfalfa is the most important routinely cultivated legume. It ranks first in cultivated forage crops and occupies more than 30% of the cultivated area (Abdel-Aziz et al. 2008). The plant is recognized as a widely adapted agronomic crop, an effective source of biological N_2 fixation, one of the highest sources of protein yield per hectare, and an attractive source of nectar for honey bees (Stuteville and Erwin 1990).

Seeds play a vital role in the production of healthy crops but can carry some important fungi that result in considerable loss of yield. Some seed-borne fungi have been found to be very destructive, causing seed rot, decreased seed germination and/or pre-/post-germination death (Abul-Hayja et al. 1983; Al-Kassim and Monawar 2000). Several reports of seed-borne mycoflora attacking alfalfa seeds have been published worldwide. *Alternaria alternata*, *Aspergillus* spp., *Bipolaris setariae*, *Botrytis cinerea*, *Cephalosporium* spp., *Cladosporium* spp., *Colletotrichum dematium*, *Curvularia lunata*, *Epicoccum purpurascens*, *Fusarium equiseti*, *F. moniliforme*, *F. pallidoroseum*, *Macrophomina phaseolina*, *Microdochium dimerum*, *Penicillium* spp., *Phoma* spp., *Stemphylium* spp., *Trichoderma*

spp., *Trichothecium roseum*, *Ulocladium* spp., *Verticillium albo-atrum* and *Verticillium* spp. have been detected as the most important seed-borne fungi on alfalfa seed samples collected from different countries (Mathur and Manandhar 2003). In Saudi Arabia, there is no literature available on seed-borne fungi attacking alfalfa; however, efforts are being made in the kingdom to develop high resistance alfalfa cultivars.

A number of abiotic and biotic factors cause seed discoloration that may lower seed quality. Abiotic factors, including environmental stress such as a low temperature (Morrison et al. 1998), dampness and high humidity (especially during storage), and the genetic makeup of a cultivar (Gai et al. 2000) can greatly affect seed coat color and lower seed quality. Biotic stress in the form of seed-borne fungi reflects a direct impact on seeds. Many fungi are serious parasites that attack primordial and mature seeds causing quantitative and qualitative yield reduction. Other fungi, including saprophytes and weak parasites, may lower the quality of seeds by causing discoloration, which may seriously depreciate the commercial value of seeds. Many seed-borne fungi infect the seed coat, causing conspicuous necrotic black, or gray-brown discoloration (Neergaard 1979). The present study was undertaken to detect alfalfa seed-borne fungi in Saudi Arabia, and to determine the cause of seed discoloration in alfalfa.

Materials and methods

Samples

Fifteen seed samples of local alfalfa cultivar (Hegazy) collected from growing fields in different governorates of Riyadh region in Saudi Arabia (Al-Deri'yya, Hraymla, Afeef, Al-Kharj, Al-Zulfi, Al-Dawadmy, Rmah, Al-Hareeq, Hotat Bani Tameem, Al-Aflaj, Al-Quway'iyah, Shaqra and Al-Majma'ah beside Riyadh city) during 2010 were used in this study.

Seed health testing

Conventional seed health testing was carried out to detect fungi associated with alfalfa seeds. Standard moist blotter (SMB) and deep-freezing blotter (DFB) methods were used (ISTA 1999). The proposed alkaline seed-bed method was also applied (Elwakil and Ghoneem 2002). A total of 400 seeds from each sample was used and the percentages of fungi recovered from each method were tabulated.

Standard moist blotter method

Twenty-five seeds were plated in a 9-cm diameter Petri-dish containing three layers of blotter paper moistened with sterilized tap water. 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.

Deep freezing blotter method

After plating seeds as described in the SMB method, the dishes were incubated at 20°C for 24 h and then transferred to a −20°C freezer for 24 h. This was followed by a 5-day incubation at 20±2°C under cool white fluorescent light with alternating cycles of 12-h light and 12-h darkness.

Alkaline seed-bed method

Three layers of blotter paper were soaked in tap water alkalinized with KOH or NaOH at pH 10. The blotters were placed in 9-cm diameter Petri-dishes and 25 seeds were distributed on each seed-bed as described (ISTA 1999). The plates were incubated at 20±2°C under cool white fluorescent light with alternating cycles of 12-h light and 12-h darkness.

Seven days later, the incubated seeds were examined under a stereoscopic microscope at 6–50X magnification to determine the presence and morphological characteristics of any seed-borne fungi. Pure cultures of the examined fungi were obtained using single spore and hyphal tip techniques. The isolated fungi were maintained on slants of potato carrot agar for further studies.

Seed-borne fungi associated with discolored alfalfa seeds

Seed samples were subjected to preliminary inspection for seed discoloration either by the naked eye or under a low power stereoscopic microscope. Alfalfa seed samples that exhibited 20% or more of discoloration symptoms were selected for further study. NaOH alkaline treatment was used to determine the presence of fungi on discolored alfalfa seed. After incubation, seeds were examined and seed-borne fungi were identified.

Identification of fungi

Media used for the identification of fungi were: carnation leaf agar (CLA) (Fisher et al. 1982) [pieces of sterilized carnation leaves; agar (10 g); distilled water (1 L)]; cornmeal agar (CMA) [cornmeal (20 g); peptone (20 g); glucose (20 g); agar (15 g); distilled water (1 L); pH 6.5]; Czepek's solution agar (CzA) [$NaNO_3$ (3 g); K_2PO_4 (1 g); KCL (0.5 g); $MgSO_4{\cdot}7H_2O$ (0.5 g); $FeSO_4{\cdot}7H_2O$ (0.01 g); sucrose (30 g); agar (20 g); distilled water (1 L); pH 6.5]; malt extract agar (MEA) [malt extract (20 g); peptone (1 g); dextrose (20 g); agar (20 g); distilled water (1 L); pH 6.5]; oatmeal agar (OA) [oatmeal (20 g); yeast extract (0.5 g);

Table 1 Identification data of the isolated alfalfa seed-borne fungi

Fungus	Colony	Conidiophores	Conidia
Acremonium strictum	Pink, usually moist and smooth, 2.5 cm diam in 10 days at 22°C on MEA[a]	Simple phialides arising from submerged or fasciculated aerial hyphae	Cylindrical, 3.3 x 1.8 µm, no chlamydospores formed
Alternaria alternata	Golden brown, reaching 6 cm diam in 7 days at 25°C on MEA	Simple, straight, septate, to 50 µm long, 3–6 µm wide	Ovoid, short apical beak, brown, one or two longitudinal septa, 18–63 x 7–18 µm
Aspergillus flavipes	Whitish, 3–5 cm diam in 10–14 days at 25°C on CzA and MEA	Smooth-walled, pale yellow to light brown, 2.4-3.2 µm diam	Solitary or botryose aleurioconidia 4–6 µm diam occur
Aspergillus flavus	Yellow-green, 5 cm diam in 10 days at 25°C on CzA and 7 cm on MEA	Hyaline, 0.6 mm long, rough-walled. Conidial heads radiating; a layer of metulae with phialides	Globose, finely roughened, 3–4 µm diam
Aspergillus glaucus	Yellow-green to greenish grey, on CzA reaching 1–2 cm diam in 21 days at 25°C	Smooth-walled, uncolored to pale brown, 200–350 µm long.	Subglobose, densely covered with thick spines, 5.5 µm diam
Aspergillus nidulans	Dark-green to brownish, 6 cm diam in 14 days at 25°C on CzA, on MEA 7 cm in 7 days	Smooth, brown, 60–50 µm, vesicle hemispherical, 8–12 µm diam, metula and phialides on upper part	Globose, rough, 3–4 µm diam
Aspergillus niger	Black powdery, 3 cm diam in 10 days at 25°C on CzA, and 6 cm on MEA	Arising from long, brownish foot cells, 1.5-3.0 mm tall	Large, radiating heads, globose, irregularly roughened, 4–5 µm diam
Aspergillus ochraceus	Light buff, 3 cm diam in 12 days at 25°C on CzA, and 5 cm on MEA	Yellow to light brown, 1.5 mm long, coarsely roughened; vesicles globose, with metulae	Globose, delicately roughened, 2.5-3 µm diam
Aspergillus tamarii	Dark brown, 6 cm diam in 13 days at 25°C on CzA and 7 cm on MEA	Hyaline, 1–2 mm, roughened, vesicles globose, 25 to 50 µm diam, sterigmata in 1 or 2 series	Cylindrical when young, globose at maturity, 5–6 µm diam
Cephalosporium acremonium	Pinkish, centrally tufted, 1.8-2.3 cm diam in 10 days at 22°C on MEA	Simple phialides arising from submerged hyphae, thin walled	Cylindrical conidia, 3.5 x 1.2 µm, chlamydospores with chromophilic walls, 4–8 µm diam after 12 days
Chaetomium sp.	White, gray to olive, cottony, 7 cm diam in 10 days at 20 C on OA	Brown to black perithecia, large, cylindrical asci, unicellular ascospores, brown, lemon-shaped	
Cladosporium sp.	Olivaceous green, velvety to powdery, slow growing on PDA at 25°C	Tall, dark, upright, branched variously near the apex, clustered or single	Dark, 1- or 2-celled, ovoid, irregular, in simple or branched chains
Colletotrichum trifolii	Olive green to gray, slimy, 5 cm in 5 days at 25°C on PDA	Acervulae scattered across the colony, 100–250 mm. Short conidiophores, 8-10 × 2-4 µm	Cylindrical, salmon-apricot, hyaline, straight, nonseptate, rounded at ends, 11–13 x 3–4 µm
Cunninghamella sp.	Mycelium white, extensive in culture, nonseptate on PDA	Simple or branched, with enlarged tips bearing heads of conidia	Hyaline, 1-celled, globose
Curvularia lunata	Dark brown, velvety, 6 cm in 5 days at 25°C on OA	Singly or in groups, simple or branched, straight, 650 x 9 µm	3-septate, curved in the third cell, which is usually larger and darker, smooth-walled, 20 x 10 µm
Drechslera tetramera	Brownish, appearing glassy with sooty powder of conidia, 3 cm diam in 5 days at 25°C on OA	Brownish, unbranched, septate, up to 250 µm long and 4–8 µm wide, single or in clusters of 2–3 Erect	Brown, ellipsoid, straight, rounded ends, lighter towards the terminii, 20.4-35.7 x 8.5-13.6 µm, 3-septate
Drechslera. micropa	Olivaceous-brown but later becoming black, 3.9 cm diam in 5 days at 20°C on OA	Light brown, single, short, slender, straight 10 x 3 µm	Olivaceous brown, ellipsoid, broader at middle, tapered towards both ends, 4–7 septa, 37.4–52.7 x 11.9–13.6 µm
Epicoccum purpurascens	Yellow to brown, 6 cm diam in 10 days at 20°C on CzA and MEA	Hyaline, claviform, 0-2-septate, smooth, 9 x 6 µm, producing a single dark conidium terminally	Globose, 15–25 µm diam, funnel-shaped base, wall golden-brown to dark brown, the septa divide the conidia in up to 15 cells
Fusarium equiseti	White tinged with peach, later beige, finally deep olive buff, 5.8 cm diam in 4 days at 25°C on OA	Branched, monophialides, 12–17 x 3–4 µm, with a single apical opening	Only macroconidia are produced, 3–5 septa, 31–47 x 4–5 µm. Chlamydospores, chains, ochraceous, 7–9 µm diam on CLA
Fusarium incarnatum	Whitish but later becoming buff-brown. 6 cm diam in 4 days at 25°C on OA	Branched; phialides slender, cylindrical, 19–24 x 2–4 µm, one conidium at each one	Macroconidia fusiform, straight, beaked apical cells, 3–5septa, 17–35x3-4 µm. Microconidia absent. Chlamydospores globose, single or chain, 5–10 µm on CLA

Table 1 (continued)

Fungus	Colony	Conidiophores	Conidia
Fusarium solani	White to cream-coloured aerial mycelium, green to bluish-brown when sporodochia are present, 3.2 cm diam in 4 days at 25° C on OA	Elongated, verticillate, 8–16 x 2–4 µm	Microconidia abundant, hyaline, cylindrical, 9–16 x 2–4 µm, Macro-conidia, 3-septate, 28–42 x 4–6 µm. Chlamydospores single or in pairs, hyaline, 6–10 µm diam on CLA
Fusarium verticillioides	Whitish, purplish white or very light orange-white, 4 cm diam in 4 days at 25° C on PDA	Medium length, simple or branched. Conidiogenous cells are monophialides	Macroconidia sparse, nearly straight, 5-septa, 31–58 x 2.7-3.6 µm on CLA. Microconidia abundant, 0 to 1-septate, oval to clavate, 7–10 x 2.5-3.2 µm, Chlamydoconidia absent
Mucor sp.	White and becoming dark grey, 9 cm diam in 4 days at 25°C on MEA	Hyaline, without basal rhizoids, large columella, terminal many-spored sporangia	Sporangiospores were globose to ellipsoidal, hyaline, grayish or brownish, smooth-walled
Myrothecium verrucaria	White to rosy buff, 5.0 cm diam in 14 days at 25°C on PDA	Diffuse olivaceous to black sporodochia; Phialides 3–6 in a whorl, 10.5-14.5 x 1.5-2 µm	Broadly fusiform, pointed apical, basal truncate, bearing an apical, funnel-shaped appendage, 7 x 3 µm
Penicillium sp.	Rapid growing on PDA, velvety, white and became greyish green	Branched near the apex, penicillate, ending in a group of phialides	Hyaline or brightly colored in mass, 1-celled, globose or ovoid, in chains
Phoma trifolii	Grey, gradually darkening, 5.5 cm diam in 7 days at 25°C on OA	Pycnidia usually arising in regularly scattered	Ellipsoidal, 6 x 3 µm, two-celled, dark chla-mydospores, swollen cells, 10–22 µm
Rhizoctonia solani	On PDA, the hyphae of *R. solani* have the following characteristics: some shade of brown, cross wall within the hyphae called a dolipore septum, each cell is multinucleate, branches that are produced at right angles and no asexual spores are formed by the mycelium		
Rhizopus stolonifer	Reddish grey-brown, very fast-growing, over 2 cm high on PDA	Pale to dark brown, straight, 2.5 x 20 µm, Stolons hyaline to brown, branched rhizoids	Sporangia black, 100–200 µm; oval columella. Sporangiospores subglobose, ridged, 7 x 4.5 µm
Sclerotium bataticola	Agar cultures are black and homogenous due to microsclerotia formation	Microsclerotia black, smooth, round to oblong, 50–100 µm diam. Hyphae, 3–6 µm wide, branching at right or acute angles to parent hyphae.	
Stachybotrys chartarum	Covered by dark powdery bloom of conidia, 1.4 cm diam in 5 days on OA at 25°C	Simple, branched, 100–1,000 x 3–6 µm, olivaceous, clusters of 4–10 ellipsoidal, hyaline phialides	Aggregated in slimy masses, ellipsoidal, at first hyaline, at maturity dark olivaceous-grey, 7–12 x 4–6 µm
Stemphylium botryosum	Hyaline to brown hyphae, forming small pulvainate stromata, 6–8 cm diam in 8 days on MEA at 25°C	Straight, simple, 20–72 x 4–6 µm, darker brown, roughened at apex	Ovoid, 2–3 septa, pale to deep olivaceous-brown, 24–38 x 15–26 µm
Trichoderma harzianum	White and became pale green, 9 cm in 4 days at 30°C on PDA	With paired branches, secondary branches at 90° angle, longest ones near the base and the axis	Subglobose, yellow, becoming yellow-green, 2.7 x 2.5 µm, phialides in a whorl or solitary
Trichothecium roseum	Pinkish, zonate in diurnal rhythm, 9 cm in 10 days at 20°C on MEA	Erect, 2 x 4 µm, often with three septa in the lower part	Ellipsoidal, 2-celled, upper cell slightly larger, hyaline, thick-walled, 12 x 8 µm
Ulocladium atrum	Black with white margin on CMA, mycelium pale olivaceous brown, septate, 5 µm diam	Erect, simple or branched, dark brown, 5–8 x 120 µm	Multiform, solitary, dark brown, three or one transverse septa

[a] Media used for identification: *CLA* carnation leaf agar, *CMA* cornmeal agar, *CzA* Czepek's solution agar, *MEA* malt extract agar, *OA* oatmeal agar, *PDA* potato dextrose agar

agar (20 g); distilled water (1 L); pH 6.5]; and potato dextrose agar (PDA) [potato dextrose (20 g); agar (20 g); distilled water (1 L); pH 6.5].

Fungi were identified according to their cultural properties, morphological and microscopical characteristics as described by Raper and Fennel (1965); Ellis (1971); Chidambaram et al. (1973); Domsch et al. (1980); Booth (1977); Burrges et al. (1988). For determination of morphological structures, portions of fungal growth were mounted in lacto-phenol cotton blue stain on clean slides as proposed by Sime and Abbott (2002). The prepared slide was examined under a light microscope using the 40X and 100X objectives for vegetative mycelium; septation, diameters, conidiophores (sporangiophores) and the reproductive structures: conidia, sporangiospores etc. Fungal colonies were examined under the 10X (low power) objective of the microscope. The colonial characteristics of size, texture and color of the colony were investigated.

Germination percentage

A total of ten collected seed samples, showing more than 20% symptoms of seed discoloration was used in this study.

Table 2 Occurrence of alfalfa seed-borne fungi using standard moist blotter (SMB), deep-freezing blotter (DFB) and alkaline seed-bed methods

Fungus	SMB		DFB		Alkaline seed- bed			
					KOH		NaOH	
	F %[a]	I %	F %	I %	F %	I %	F %	I %
Acremonium strictum	6.70	0.033±0.03	20	0.13±0.07 (0.5-1)	26.7	0.23±0.1 (0.5-2)	26.7	0.17±0.079 (0.5-1)
Alternaria alternata	100	10.33±3.65 (0.5-55)	86.70	9.47±2.55 (1–28.5)	93.30	5.76±1.8 (0.5-25.5)	80	4.93±1.68 (1–34.5)
Aspergillus flavipes	80	1.57±0.4 (0.5-5.5)	40	1.3±0.6 (0.5-9)	53.3	0.73±0.25 (0.5-3)	21.8	0.9±0.32 (1–1.5)
Aspergillus flavus	86.67	3.27±0.69 (0.5-8)	46.70	0.7±0.31 (0.5-4)	53.33	1.27±0.48 (0.5-6)	60	1.47±0.41 (1–4.5)
Aspergillus glaucus	26.70	0.27±0.14 (0.5-2)	53.30	0.27±0.067 (0.5-1)	53.30	1.67±0.54 (0.5-5)	53.30	1.63±0.62 (0.5-5.5)
Aspergillus nidulans	80	2.4±0.78 (0.5-10)	33.30	1.53±0.1 (0.5-13)	60	1.87±0.73 (0.5-10)	66.70	1.93±0.69 (0.5-9.5)
Aspergillus niger	66.67	3.7±0.98 (0.5-8)	46.70	0.57±0.21 (0.5-2.5)	66.67	1.9±0.43 (1–5)	60	1.76±0.49 (1–4.5)
Aspergillus ochraceus	33.30	1.97±0.96 (0.5-10)	33.30	1.47±0.78 (0.5-9.5)	60	0.73±0.25 (0.5-4.5)	73.3	0.9±0.32 (0.5-6.5)
Aspergillus tamarii	73.30	1.63±0.39 (0.5-4)	13.30	0.1±0.072	26.70	0.17±0.079 (0.5-1)	26.70	0.27±0.14 (0.5-2)
Cephalosporium acremonium	13.30	0.1±0.07 (0.5-1)	60	1.33±0.076 (1.5-3)	73.30	1.63±0.13 (0.5-6)	60	1.23±0.41 (0.5-4)
Chaetomium sp.	6.70	0.33±0.03	6.70	0.067±0.067	20	0.1±0.053	13.30	0.67±0.045
Cladosporium sp.	100	21.7±5.8 (0.5-63)	53.30	3.97±1.84 (0.5-13.5)	60	6.63±3.04 (0.5-35.5)	40	7.3±3.30 (0.5-37.5)
Colletotrichum trifolii	13.30	0.06±0.04	13.30	0.13±0.35	13.30	0.5±0.035 (3–4.5)	13.30	0.37±0.25 (2.5-3)
Cunninghamella sp.	-	-	13.30	0.1±0.072	-	-	-	-
Curvularia lunata	-	-	-	-	6.70	0.03±0.033	6.70	0.03±0.033
Drechslera tetramera	26.70	0.2±0.095 (0.5-1)	20	0.13±0.08 (0.5-1)	13.30	0.1±0.072 (0.5-1)	26.70	0.13±0.059
Drechslera micropa	-	-	26.70	0.2±0.095	13.30	0.1±0.072	33.30	0.2±0.082
Epicoccum purpurascens	20	0.13±0.076 (0.5-1)	-	-	-	-	6.70	0.03±0.033
Fusarium equiseti	26.70	0.5±0.28 (1–4)	40	0.37±0.13 (0.5-1.5)	33.30	0.2±0.08 (0.5-1)	46.70	0.37±0.13 (0.5-1)
Fusarium incarnatum	60	2.5±1.33 (0.5-20)	60	3.43±1.86 (0.5-25.5)	80	4.6±2.5 (0.5-30.5)	80	4.53±2.5 (0.5-36.5)
Fusarium solani	-	-	-	-	6.70	0.03±0.03	13.30	0.1±0.072 (0.5-1)
Fusarium verticillioides	-	-	-	-	33.30	0.23±0.095 (0.5-1)	33.30	0.23±0.095 (0.5-1)
Mucor sp.	6.70	0.03±0.033	-	-	6.70	0.067±0.067	20	0.2±0.10
Myrothecium verrucaria	20	0.17±0.093 (0.5-1)	13.30	0.1±0.072 (0.5-1)	33.30	0.37±0.18 (0.5-2.5)	46.70	0.47±0.2 (0.5-3)
Penicillium sp.	73.30	0.57±0.14 (0.5-2)	66.70	0.7±0.17 (0.5-1.5)	66.70	0.8±0.23 (0.5-2.5)	66.70	1.17±0.36 (0.5-4)
Phoma medicaginis	-	-	33.30	0.23±0.10 (0.5-1.5)	6.70	0.07±0.07	-	-
Rhizoctonia solani	-	-	13.30	0.1±0.072 (0.5-1)	13.30	0.1±0.072 (0.5-1)	13.30	0.13±0.1 (0.5-1.5)
Rhizopus stolonifer	33.30	0.4±0.24 (0.5-3.5)	13.30	0.067±0.045	13.30	0.23±0.2 (0.5-3)	33.30	0.47±0.29 (0.5-4.5)
Sclerotium bataticola	-	-	13.30	0.43±0.40	20	0.13±0.076	-	-
Stachybotrys chartarum	6.70	0.03±0.033	13.30	0.67±0.045	-	-	20	0.13±0.076 (0.5-1)
Stemphylium botryosum	73.30	1.87±0.5 (0.5-5)	80	1.43±0.45 (0.5-5)	60	1.33±0.5 (0.5-6)	53.30	1.37±0.56 (0.5-6.5)
Stemphylium sp.	66.7	0.8±0.19 (0.5-2)	46.7	0.7±0.28 (0.5-4)	40	0.77±0.37 (0.5-5)	26.7	0.43±0.21 (0.5-2.5)
Trichoderma harzianum	33.30	0.6±0.27 (0.5-3)	20	0.17±0.093 (0.5-1)	13.30	0.1±0.072 (0.5-1)	20	0.13±0.076 (0.5-1)
Trichothecium roseum	-		-		-		6.70	0.03±0.033
Ulocladium atrum	46.7	0.33±0.1 (0.5-1)	33.3	0.3±0.14 (0.5-2)	26.7	0.4±0.15 (0.5-2)	26.7	0.23±0.13 (0.5-2)

[a] F %=frequency percentage, I %=mean intensity percentage of infected seeds±standard error. Numbers in parenthesis indicate infection range

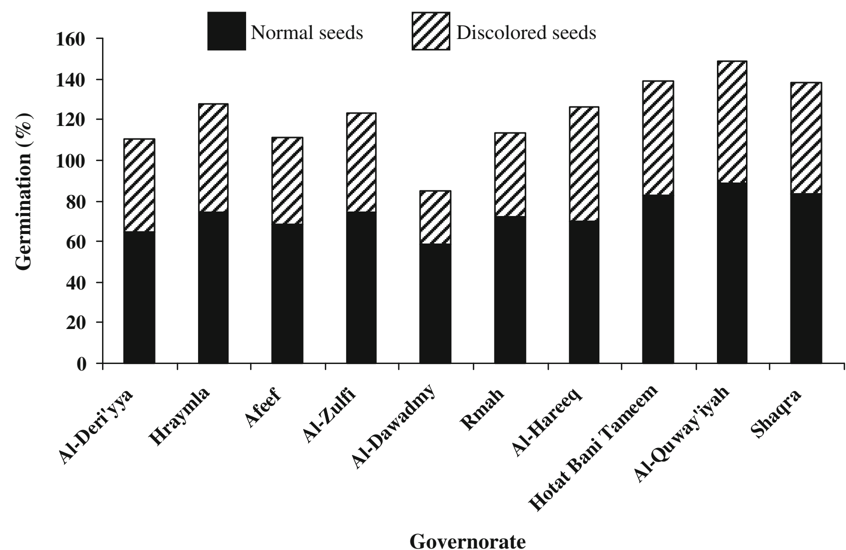

Fig. 1 Germination percentages of normal and discolored seed samples from different governorates. Least significant difference (LSD; P=0.05)=3.41 for normal seeds and 1.84 for discolored seeds

The standard blotter test recommended by the International Seed Testing Association (ISTA 1999) was used to determine the germination percentage of each seed lot. For each sample of normal and discolored seeds, 400 seeds were taken at random, laid out on moistened absorbent paper, which was then wrapped up in a wet cotton towel and placed inside a plastic bag. The paper was kept moist for 10 days at 20±2°C under cool white fluorescent light with alternating cycles of 12-h light and 12-h darkness. The numbers of germinated seeds following incubation for 10 days were counted.

Statistical analysis

Comparison of means was performed using LSD at P=0.05 and the standard error was calculated using the statistical analysis software "CoStat 6.34" (CoStat 2005) .

Results and discussion

Seed health testing methods

A total of 24 genera and 35 species of fungi were isolated from alfalfa seeds using the techniques mentioned in Materials and methods. Data from identification of the alfalfa seed-borne fungi thus isolated are summarized in Table 1. Considerable differences in the frequency of the presence of alfalfa seed-borne fungi among the SMB, DFB and the alkaline seed-bed techniques were recorded (Table 2). Of the three tested methods used, NaOH alkaline treatment yielded the highest number of fungi (21 genera and 32 species) as compared to KOH alkaline treatment, SMB and DFB methods.

Data of incidence percentages and occurrence ranges of the isolated fungi revealed that *Alternaria alternata*,

Fig. 2a,b Alfalfa seeds. **a** Healthy (x5). **b** Discoloration symptoms caused by certain fungi (x5)

Cladosporium sp., *Aspergillus flavipes* , *A. flavus* , *A. niger*, *A. nidulans*, *A. tamarii*, *Stemphylium* sp. and *Penicillium* sp. were the most abundant. *Fusarium incarnatum* was the most frequently isolated dominant *Fusarium* species, followed by *F. equiseti*, while *F. verticillioides* and *F. solani* were detected at low percentages. The data indicated also that the SMB method enhanced the recovery of fast-growing saprophytes as *Cladosporium* sp. (100%), *A. flavipes* (86.7%), *A. flavus* (86.7%), *A. nidulans* (80%) and *A. tamarii* (73.3%), which commonly attack nonviable (dead) seeds, while the DFB method enhanced the recovery of *Stemphylium* sp. (80%) and *Phoma medicaginis* (33.3%).

Fusarium is a highly pathogenic fungus and its different species have been reported to cause seed rot, seedling blight and wilt in a number of legumes crops (Booth 1977). *Phoma medicaginis* var. *medicaginis* and *Stemphylium botryosum* were reported as being responsible for leaf spotting and defoliation, which result in a significant reduction of forage yield and quality (Campbell and Madden 1990; Stuteville and Erwin 1990; Nutter et al. 2002; Guan and Nutter 2002). In addition to quantitative yield losses, some foliar pathogens including *Fusarium* spp. and *Alternaria* spp. can lower forage quality by producing mycotoxins (Scudamore and Livesey 1998). In Australia, foliar diseases have been reported to reduce *Medicago herbage* yield by 16–20%, seed yield by 37% and seed weight by 7–13% (Barbetti 1995).

The alkaline blotter method enhanced the recovery of slow-growing seed-borne fungi beyond that obtained with the SMB and DFB methods. Both treatments of the alkaline seed-bed method were efficient in the detection of *F. incarnatium* (80%) on alfalfa seeds. Treatment with KOH increased the frequency of the detected *Cephalosporium acremonium* (73.3%) on the tested seeds, compared to 13.3%, 60% and 60% in SMB, DFB and alkaline NaOH methods, respectively. Both alkaline treatments showed an

Table 3 Occurrence of fungi on normal and discolored alfalfa seeds using the alkaline seed-bed method (NaOH)

Fungus	Normal seeds		Discolored seeds	
	F %*	I %	F %*	I %
Alternaria alternata	80	6.95±2.97	100	10.95±5.43
Aspergillus flavipes	70	1.05±0.33	90	4.5±0.95
Aspergillus flavus	50	1.6±0.68	100	10.1±2.07
Aspergillus glaucus	10	0.1±0.1	30	0.2±0.1
Aspergillus nidulans	60	1.05±0.41	90	4.25±1.59
Aspergillus niger	60	2.35±0.81	90	7.9±1.57
Aspergillus ochraceus	20	0.5±0.4	30	0.4±0.23
Aspergillus tamarii	60	1.65±0.53	100	6.2±0.71
Cephalosporium acremonium sp. *acremonium*	20	0.25±0.17	30	0.75±0.5
Chaetomium sp.	0	-	10	0.05±0.05
Cladosporium sp.	80	4.3±2.39	100	20.5±7.78
Colletotrichum trifolii	20	0.4±0.3	30	0.95±0.63
Drechslera tetramera	20	0.2±0.13	30	0.4±0.2
Drechslera micropa	20	0.1±0.06	20	0.15±0.1
Fusarium equiseti	40	0.75±0.4	50	1.05±0.67
Fusarium incarnatum	40	1.9±1.21	60	10.6±5.62
Fusarium solani	0	-	10	0.1±0.1
Fusarium verticillioides	20	0.1±0.06	30	0.2±0.11
Myrothecium verrucaria	30	0.2±0.11	30	0.5±0.26
Penicillium sp.	40	0.45±0.21	60	0.5±0.19
Rhizoctonia solani	0	-	20	0.15±0.1
Rhizopus stolonifer	30	0.25±0.13	60	0.8±0.29
Stemphylium botryosum	50	1.4±0.57	70	2.45±0.95
Stemphylium sp.	40	0.85±0.38	60	1.9±0.93
Trichoderma harzianum	50	0.4±0.14	20	0.1±0.06
Ulocladium atrum	20	0.15±0.1	30	0.35±0.21

equal increase in the frequency of *F. verticillioides* and *A. glaucus* found on alfalfa seeds compared with the SMB and DFB methods. On the other hand, no differences were observed among the two alkaline seed-bed techniques and the DFB method in the frequency of the detected *Cephalosporium* sp. on alfalfa seeds, and no differences were observed among all techniques in the frequency of the detected *Rhizoctonia solani* and *Colletotrichum trifolii*. On the other hand, the incidence of saprophyte fungi was lower in the case of the alkaline seed-bed method than with the other techniques.

The results presented here show that the alkaline KOH seed-bed proved an efficient method of detecting slow-growing fungi such as *Cephalosporium* sp on alfalfa seeds. In addition, it was also efficient in detecting other pathogenic fungi, e.g., *F. verticillioides*. These results are in agreement with the findings of Ghoneem et al. (2009), who reported abundant growth of slow-growing *Verticillium dahliae* and *Cephalosporium* sp. on fennel seeds when using alkaline seed-bed techniques at pH 9.5. The ability of such fungi to grow abundantly may be due to the high pH of the medium, which is favorable for the growth of this fungus (Ghoneem et al. 2009). Abo-Ellil (1999a, b) reported that the uptake of sugars in the *V. lateritium* cell increases when the alkalinity of the medium increases. This enhancement may be attributed to the alkaline ions (K^+ or Na^+) that replace H^+ in the fungal cell and increase the uptake of nutrients in the cells (Horikoshi and Akiba 1982; Elwakil and Ghoneem 2002). It was found also that alkalinized seed-beds help analysts at seed-health laboratories to more easily detect slow-growing fungi compared with other methods.

Germination percentages

Germination of ten normal seed samples obtained from different locations varied significantly from 58.3% to 88.7% depending on the location of the sample (Fig. 1). The highest germination was recorded in seed samples obtained from Al-Quway'iyah (88.7%), followed by shaqra, Hotat Bani Tameem and Rmah (83.6, 82.9 and 72.3%, respectively); while the lowest germination was reported in the sample collected from Al-Dawadmy (58.3%). The data indicated that seed discoloration decreased seed germination significantly (by 26.3–60%). Highest germination was recorded in the discolored seed sample obtained from Al-Quway'iyah (60%), followed by AL-Hareeq and Hotat Bani Tameem (55.8%, for both); while the lowest germination was recorded in the sample collected from Al-Dawadmy (26.3%).

Symptoms caused by certain fungi associated with discolored alfalfa seed samples are characteristic and more easily distinguishable by severe infection. The discolored seeds appeared smaller and shriveled, with variable color from light brown to dark brown or black. Infected seeds are usually flattened, elongated and deformed with wrinkled seed coat (Fig. 2).

Seed-borne fungi associated with alfalfa seed discoloration

Data in Table 3 show a total of 15 genera and 26 species of fungi isolated from normal and discolored alfalfa seeds using the NaOH alkaline method. In general, discolored seeds yield a higher number of fungal species than normal seeds. *Cladosporium* sp., *Alternaria alternata, Aspergillus flavus*, *A. niger*, *A. tamarii*, followed by *A. flavipes* and *A. nidulans* were most commonly isolated fungi. *Fusarium incarnatum* was the frequently dominant isolated *Fusarium* species at frequency percentage of 60% and mean intensity percentage of 10.6%. The average frequency and intensity percentage of several fungal species that have pathogenic references were highest on discolored seeds compared to normal seeds. *Stemphylium botryosum* was recorded at a maximum frequency of 70% and intensity percentage of 2.45%, while the lowest intensity percentages were for *F. equiseti* (1.05%), *Colletotrichum trifolii* (0.95%) and *Myrothecium verrucaria* (0.5%).

The presence of *Aspergillus* spp., especially *A. niger* and *A. flavus*, on alfalfa seeds in higher frequencies, and their association with ungerminated seeds confirmed the findings that species of *Aspergillus* occur as saprophytes that may cause low germination in seeds (Christensen 1967; Dawar 1994; Sultana and Ghaffar 2009). Shafie and Webster (1981) reported that these fungi may invade seed pericarp and embryos. Contamination by *Alternaria* and *Cladosporium* sp. is common in many kinds of seeds, which may depreciate their market value heavily because of their sooty appearance (Neergaard 1979). Many seed-borne pathogenic fungi, e.g., *F. equiseti*, *F. semitectum* and *Macrophomina phaseolina* were reported to infect the seed coat causing conspicuous necrotic black, brown-to-gray discolorations in mungbean and *Phaseolus aureus* (Nath et al. 1970). These fungi associated with seeds produce proteolytic enzymes that help in the spread and development of plant pathogens (Aboughania and Faraj 1978). Therefore, discolored seeds are not good for cultivation as this will not only lead to low yields but also contaminate other seeds.

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