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Management of some seed-borne pathogens attacking alfalfa plants in Saudi Arabia

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Management and transmission of four seed-borne pathogenic fungi namely, *Colletotrichum trifolii*, *Rhizoctonia solani*, *Fusarium equiseti* and *F. incarnatum* were investigated on alfalfa plants. The pathogenicity test on alfalf plant showed that *R. solani* and *C. trifolii* caused high percentages of rotted seeds and seedlings mortality (26.45, 26.1% and 31.6, 21.1% , respectively). However, no significant differences were observed between the treatments with *F. equiseti* and *F. incarnatum* when compared with the control treatment. Transmission of the pathogenic fungi from seed to mature plant of alfalfa was investigated in this study. Results indicate that the recovery percentages of the tested pathogens gradually decreased from root apex up to the first internodes below the shoot tip, but did not reach to the shoot apex. In order to control these fungi, different concentrations of sodium metabisulphite (SM), sodium salicylate (SS) and hydroquinone (HQ) were tested *in vitro*. Treatment with SM at 10 mM completely inhibited the growth of all isolated fungi. Under greenhouse conditions, soaking alfalfa seeds in a water solution of hydroquinone at 12mM showed to be the most effective treatment in reducing seed rot and seedling mortality percentages and increasing seedling survival percentages. Application of the tested treatments presented significant increases in growth parameters, photosynthetic pigments (chlorophyll *a* and *b* and carotenoids) in leaves and total phenol in alfalfa plants. This study therefore recomended the use of HQ and SM as potential and promising antifungal agents in the protection of alfalfa plants against the tested seed-borne fungi.

Key words: Pathogenicity, *Colletotrichum trifolii*, *Rhizoctonia solani*, *Fusarium equiseti*, *F. incarnatum,* transmission, hydroquinone, sodium metabisulphite and sodium salicylate.

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is the oldest and most important forage crop in the world. In Saudi Arabia, it is widely cultivated and comes to be the first cultivated forage crop and occupies more than 30% of the cultivated area (Al-Askar et al., 2012). In 2012, the cultivated area under alfalfa in Saudi Arabia was 122,563 hectares, which produced 2,528441 tons. Cultivation is mainly in Riyadh (62879 ha) area of the Kingdom with an average production of 1,264579 tons (Agriculture

Statistical Yearbook, 2012).

Seed-borne disease refers to the particular plant diseases that are transmitted by seed. In some cases the transmission on seed is insignificant compared to the population of disease organisms that exist in soil or on weed species. In other cases, the transmission on seed is the primary means by which a disease spreads. Seed transmission studies help to confirm the seed to plant transmission to prove the pathogenicity and to design the

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suitable management strategy (Neergaard, 1979). No extensive work on the prevalence of seed-borne fungi and their transmission from seed to seedling of alfalfa seeds has been done in Saudi Arabia. Moreover, every year seed-borne fungi cause heavy yield loss of the crop and some fungi act as a primary source of infection.

Various fungal soil-borne pathogens attack alfalfa plants causing a reduction in the stand establishment, yield, forage quality and longevity. Anthracnose disease caused by the fungus *Colletotrichum trifolii* (Bain and Essary), is a common problem in alfalfa. The disease affects leaves, stems, and crowns, but crown rot is the most important symptom of the disease. The fungus persists in alfalfa debris and crowns. During the growing season (late summer and early fall), the produced spores in stem lesions transmit by splashing rain and irrigation wither onto growing stems and petioles (Vasić et al., 2010). In this respect, O`Neill and Saunders (1994) reported that *C. trifolii* can infect susceptible alfalfa cotyledons by directly penetrating the epidermis *via* a penetration peg that form the appressorium. Appressoria and primary hyphae maturate within 20 h on cotyledons, while the secondary spores are produced on susceptible tissues after 96 h.

Rhizoctonia solani Kühn, is the causative of seedling damping-off and crown and root rot diseases. In winter, the fungus persists (inactive) in infected root sunken lesions, which will turn black. If roots are girdled during summer, the plant will die. The fungus can also infect the crown at the location where new buds emerge and move into the crown, killing tissue (Frate and Davis, 2007).

Fusarium rot disease of alfalfa is caused by *Fusarium* species complex. *F. incarnatum* Berk. & Rav. and *F. equiseti* (Corda) Sacc. are reported as the causal agents of crown and root rot diseases. Except rot, symptoms of the disease are manifested in the form of chlorosis of leaves and lower plants (Couture et al., 2002; Krnjaja et al., 2003; Zaccardelli et al., 2006). Affected seedlings of alfalfa show brownish discoloration and water-soaking symptoms as well as rotting of seeds. Diseased tissues from affected alfalfa plants was found to contain the mycotoxins, nivalenol and (or) zearalenone (Goswami et al., 2008).

Spores, mycellium particles and/or sclerotia of the *C. trifolii* and *R. solani* may also be spread by seed contaminated during the threshing process (Neergaard, 1979; Frate and Davies, 2007). *Fusarium* species were isolated also from alfalfa seed (Krnjaja et al., 2003; Mathur and Manandhar, 2003), which could be a source of further spreading of pathogens on vegetative parts of the plant and cause problem in establishing of alfalfa crops. Recently, *C. trifolii*, *R. solani*, *F. incarnatum* and *F. equiseti* have been reported as important potential seed-borne pathogens on alfalfa in Saudi Arabia (Al-Askar et al., 2012).

Control of seed-borne fungi is currently limited to the use of protecting fungicides (Thomas and Sweetingham,

2003). Increasing knowledge and concerns about the environmental consequences of repeated fungicide applications have prompted the industry and research scientists to explore the potential of alternative strategies, including the use of environmentally alternatives for controlling the diseases without toxicity on plants, animals or human. Many investigators reported the effectiveness of some antifungal substances for seed, soil and foliar treatment (Shabana et al., 2008; Abd El-Hai et al., 2009). Hydroquinone, which synthesized naturally in the leaves, bark and fruit of a number of plants, especially the ericaceous shrubs such as cranberry, cowberry, bearberry and blueberry is reported to be a potential inhibitor for some seed-borne pathogenic fungi of peanut (El-Wakil and El-Metwally, 2000). Sodium salicylate is a sodium salt of salicylic acid, and involved in reducing the sour rot caused by Geotrichum candidum in the mandarin fruit (Talibi et al., 2011). Sodium metabisulfite which is known as medical antiseptic is widely used as a multi-functional antimicrobial gent and is a common preservative used in relishes, fresh and dried fruits and vegetables, tomato paste and mincemeat (Ash and Ash, 2009). Several reports presented their effectiveness in inhibiting mycelial growth and sporulation of many fungal pathogens at different concentrations (Hervieux et al., 2002; Mills et al., 2004).

Considering the seriousness and common occurrence of seed rot of alfalfa in Saudi Arabia and inadequate information regarding the seed-borne fungi and their transmission from seeds to seedlings, this study was undertaken to study the nature of the isolated fungi and elucidate the mode of transmission of some pathogenic fungi (*C. trifolii*, *F. equiseti*, *F. incarnatum* and *R. solani*) from seed to mature plant. The effect of hydroquinone (HQ), sodium salicylate (SS), and sodium metabisulfite (SM) on the isolated seed-borne pathogens of alfalfa was also investigated.

MATERIALS AND METHODS

Chemicals and alfalfa seeds

The three tested chemicals were obtained from Sigma Chemicals Co., USA. Rhizolex-T 50 W.P was obtained from Somatco Chemicals, Saudi Arabia. Seeds of local alfalfa cultivar (Hegazy) obtained from growing fields of Riyadh region, Saudi Arabia during 2011 were used in this study.

Pathogenic fungi

Four fungal species that is *C. trifolii*, *R. solani*, *F. equiseti* and *F. incarnatum* were isolated from seeds surfaces of local alfalfa cultivar (Hegazy) collected from growing fields of Riyadh region in Saudi Arabia (Al-Askar et al., 2012). Conventional seed health testing was carried out to detect fungi associated with alfalfa seeds (ISTA, 1999). The isolated fungi were grown on potato dextrose agar (PDA) (Difco, USA) plates supplemented with antibacterial agent (L-chloramphenicol 5mg/L and streptomycin sulphate 5mg/L) and incubated at 28°C for 4 to 6 days. Purification of the resulting

isolates was done using the hyphal tip or single spore techniques to obtain pure cultures; the detected isolates were then transferred into slant of PDA and kept at 4°C for further studies. Pure cultures of the isolated fungi were identified according to their cultural properties, morphological and microscopical characteristics as described by Booth (1977) and Domsch et al. (1980).

Transmission of seed-borne fungi in alfalfa

Preparation of inocula

Flasks contaning 50 ml of potato dextrose broth were inoculated singly with disks (7 mm in diameter) taken from the growing edge of 5-day-old colony of each fungus. The flasks were then incubated in dark (without shaking) for 10 days at 25 ± 2°C. Fifty grams of each mycelial mat were harvested and blended in 500 ml of sterile distilled water to produce fungal suspensions.

Pathogenicity test

Healthy alfalfa seeds were surface sterilized in 1% sodium hypo-chlorite solution. The disinfected seeds were then soaked in the fungal suspensions containing 2% Arabic gum for 15 min and left to dry at room temperature. Control treatment was carried out by soaking the disinfected seeds in tap water. Ten seeds per pot of alfalfa were planted in 20 cm diameter plastic pots containing sterile soil (2 kg soil/pot). The seeds were allowed to grow under green-house conditions. Ten replicates were used for each treatment. Daily observation for germination and symptoms of pre- and post-emergence damping off were recorded for two months.

Transmission study

Alfalfa plants surviving the challenge with the selected seed borne fungi in the pathogenicity test were left to grow until maturity. After 60 days of planting, 20 plants were pulled from their pots, washed, and surface sterilized. Under sterile conditions, alfalfa plant were cut into different parts including, root, crown, basal stem (from soil surface up to 10 cm), middle stem (from 10-15 cm) and upper stem (from 15-20 cm) and stem apex. The plant parts were then trans-ferred into PDA plates supplemented with antibacterial agent (L-chloramphenicol 5mg/L and streptomycin sulphate 5 mg/L). The plates were then incubated for 4 days at 24±2°C under cool white fluorescent light with alternating cycles of 12 h light and 12 h darkness. Fungi recovered from each treatment were identified and the transmission percentage was recorded.

Evaluation of the antifungal activity of tested substances *in vitro*

The antifungal activity of the tested substances was evaluated on the linear growth of the pathogenic fungi. Seven concentrations (0, 4, 6, 8, 10, 12 and 14 mM) of each substance were incorporated in PDA plates by adding the appropriate amount of each substance aseptically to the melted medium just before solidification. The chemical fungicide Rhizolex-T 50 W.P. at rate of 3.0 g/1000ml was used as positive control. Plates without any additions were used as a negative control. Disks (7 mm in diameter) taken from the growing edge of 5-day-old colony of each fungus were used to singly inoculate the prepared plates. The plates were incubated at 25 ± 2°C for 6 days. Five replicate were used per treatment. The fungal growth was measured after 3 and 5 days from incubation and the average growth diameter was calculated.

Greenhouse evaluation of the antifungal activity of tested substances

Inocula of the isolated pathogens were grown on sterilized medium of sorghum: coarse sand: water (2:1:2 v/v). Plastic pots (40 cm diameter) filled up with 4 kg autoclaved clay soil and singly infested with the previously prepared inocula at the rate of 0.5% by weight. Pot soil were mixed thoroughly with inoculum then, watered and left for one week to ensure even distribution of the inoculated fungi. Ten pots were used as replicates for each treatment as well as control treatment (uninfested soil).

Apparently healthy alfalfa seeds were surface sterilized in 1% sodium hypochloride followed by soaking them in solutions of HQ (10 and 12 mM), SM (8 and 10 mM) and Rhizolex-T 50 (2.5 g/1000 ml) for three hours and then dried using sterilized paper. The negative control treatment was prepared by soaking healthy seeds in sterilized water. Ten seeds were planted in each pot (one week after inoculation with the fungi) and replicated ten times for each treatment. Pots were watered regularly to near field capacity with tap water. All pots were arranged in a completely randomized design and kept in a greenhouse under natural conditions (day temperature 25°C, night temperature 20°C, 16 h photoperiod). The disease incidence was determined visually by recording the percentage of unemerged seeds 10 days after sowing as well as percentage of dead seedlings 45 days after sowing.

After 45 days of sowing, ten plants were randomly selected from each treatment for growth evaluation. Plant height, root and shoot length, number of branches, shoot fresh and dry weight, and leaf area were determined. The total phenolic content was determined in the shoots according to the study of Malik and Singh (1980). Photosynthetic pigments (Chlorophyll *a*, Chlorophyll *b* and Carotenoids) were estimated in the leaves using the spectropho-tometric method as described by Harborne (1984).

Statistical analysis

All data were subjected to analysis of variance using the statistical analysis software "CoStat 6.4" (CoStat 2005). Comparisons among means were made using Duncan's multiple range test at *P*≤0.05 (Duncan, 1955).

RESULTS

Pathogenicity test

Growing-on-test showed that the disease symptoms in the treatments of the two *Fusarium* species were similar in forming of rotted seeds, yellow seedlings and diseased but healthy looking plants. Infection with *R. solani* and *C. trifolii* presented leaf blight, seed rot and seedling damping-off. Following-up the growing-on-test two months later, results indicated that, most tested fungi caused mild to severe infection in alfalfa plants.*C. trifolii* and *R. solani* showed the highest percentages of rotted alfalfa seeds (31.6 and 26.45%, respectively), followed by *F. equiseti* (17.45%) and *F. incarnatum* (15.7%), which is not signi-ficant as compared to the check (1.6%). In case of *Fusarium* spp., it produced whitish fluffy colony on the seeds and around the base of seedlings. On the other hand, symptoms of seedling motality caused by *R. solani* developed the highest infection percentage (26.1%), while *C. trifolii* developed 21.1% infection, followed by *F.*

Table 1. Pathogenicity of isolated fungi from alfalfa seeds and the type of symptoms occurred when seed inoculation technique was used under greenhouse conditions*.

Treatment	% Rotted seed[1]	% Infected seedling[2]	% Healthy seedling
Control	1.60b	0.0b	98.4 a
C. trifolii	26.45a	21.1a	52.45c
F. equiseti	17.45ab	16.45ab	66.1b
F. incarnatum	15.7ab	9.8b	74.5b
R. solani	31.6a	26.1a	42.3c

*Affected plants with different fungi in the pathogenicity test were determined during seedling growth stage as:
[1]Pre-emergence damping- off (rotted seeds)
[2]Post- emergence damping- off (infected seedlings).
Values within a column followed by the same letter(s) are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

Table 2. Incidence of the pathogenic fungi in different parts of alfalfa.

Fungus	Fungal incidence (%)					
	Root	Crown	Basal stem	Middle stem	First internode from shoot tip	Stem apex
Control	0.0d	0.0d	0.0c	0.0b	0.0b	0.0a
C. trifolii	83.3a	50.0b	33.4a	11.7a	3.3a	0.0a
F. equiseti	34.1c	17.0c	0.0c	0.0b	0.0b	0.0a
F.incarnatum	35.0c	67.0a	0.0c	0.0b	0.0b	0.0a
R. solani	66.2b	67.0a	11.4b	0.0b	0.0b	0.0a

Values within a column followed by the same letter(s) are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

equiseti (16.45%). The lowest pathogenic one was *F. incarnatum* (9.8%). Disease symptoms were expressed as necrotic root tissue varied from light brown to explicit black. Necrotic tissue of the root was rotten and decayed (Table 1).

Transmission of the pathogenic fungi

Data presented in Table 2 show the incidence of the tested pathogens on different parts of the alfalfa plants. The highest incidence percentage on the root part was that of *C. trifolii* (83.3%) while, *F. incarnatum* and *R. solani* were found on the crown part (67%). On the other hand, *C. trifolii* solely recovered from middle and upper stem parts (11.7 and 3.3%, respectively). Isolation trials from different plant parts showed that *F. equiseti* and *F. incarnatum* were restricted to root and crown parts. However, results indicated that the recovery percentages of the tested pathogens gradually decreased from root up to the upper stem, and did not reach to the stem apex.

Evaluation of the antifungal activity of the tested substances *in vitro*

Antifungal activity of the tested substances against *F. equiseti*, *F. incarnatum*, *C. trifolii* and *R. solani* at different concentrations (4, 6, 8, 10, 12 and 14 mM) is presented in Table 3. Data show that there is an inverse relationship between the concentration of the tested substances and the linear growth of the tested fungi, so as when the concentration increases the linear growth decreases. Comparing with the control, SM was the best effective substance to decrease the growth of all tested fungi. Treatment with SM at a concentration of 10 mM completely inhibited the growth of all tested fungi. Treatment with HQ came at the second rank, while SS showed to be the lowest substance as antifungal activity even at high concentrations.

Monitoring disease severity

In greenhouse, the best substances (HQ at 10 and 12mM and SM at 8 and 10 mM) were chosen according to *in vitro* experiments. The results presented here indicate that HQ and SM at different concentrations significantly reduced the disease incidence in alfalfa plants infected with the tested pathogens (Table 4). Regarding to *R. solani* and *F. equiseti*, treatments of HQ at 10 and 12 mM and SM at 8 mM recorded the highest significant increase in the percentage of survived plants and consequently, the highest significant decrease in disease incidence percentage. SM at 8 and 10 mM were the most effective

Table 3. *In vitro* evaluation of the antifungal activity of tested substances.

Fungus		Colony diameter (cm)							
		F. equiseti		*F. incarnatum*		*C. trifolii*		*R. solani*	
Treatment	Conc. (mM)	3 day	5 day	3 day	5 day	3 day	5 day	3 day	5 day
Control	0	5.6 a	8.9 a	4.4 a	8.1 a	3.4 a	5.6 a	7.9 a	9.0 a
Hydroquinone	4	3.0 ef	5.8 e	2.9 f	4.7 f	1.5 e	3.6 d	6.0 e	8.4 c
	6	2.9 f	5.7 e	2.4 g	4.4 f	1.3 f	3.4 d	5.0 g	7.2 d
	8	2.6 g	5.2 f	1.8 h	3.6 g	1.1 g	2.9 e	4.0 h	6.7 e
	10	1.9 h	5.0 f	1.3 i	3.4 g	1 .0 h	2.5 f	3.8 i	5.4 f
	12	1.7 i	4.4 g	1.1 j	2.4 h	0.7 i	1.3 g	3.4 j	5.3 f
	14	1.3 j	3.5 h	0.8 k	1.4 i	0.0 k	0.0 h	3.1 k	4.6 g
Sodium metabisulfite	4	3.7 d	7.7 c	1.4 i	4.4 f	0.5 j	1.2 g	3.5 j	6.5 e
	6	0.7 k	1.3 i	0.0 l	0.0 j	0.0 k	0.0 h	1.3 l	3.4 h
	8	0.0 l	0.0 j	0.0 l	0.0 j	0.0 k	0.0 h	0.9 m	1.7 i
	10	0.0 l	0.0 j	0.0 l	0.0 j	0.0 k	0.0 h	0.0 n	0.0 j
	12	0.0 l	0.0 j	0.0 l	0.0 j	0.0 k	0.0 h	0.0 n	0.0 j
	14	0.0 l	0.0 j	0.0 l	0.0 j	0.0 k	0.0 h	0.0 n	0.0 j
Sodium salicylate	4	5.4 a	8.4 b	4.4 a	8.1 a	3.4 a	5.6 a	7.7 ab	9.0 a
	6	5.1 b	8.3 b	4.1 b	7.4 b	3.4 a	5.6 a	7.6 b	9.0 a
	8	4.1 c	7.9 c	3.9 c	6.8 c	3.3 b	5.5 ab	7.2 c	9.0 a
	10	3.7 d	7.0 d	3.6 d	6.7 c	3.3 b	5.4 b	6.3 d	8.6 b
	12	3.2 e	5.8 e	3.4 e	6.4 d	3.2 c	5.3 b	6.0 e	7.3 d
	14	1.7 i	4.6 g	3.0 f	6.0 e	3.1 d	4.8 c	5.3 f	6.6 e

Values within a column followed by the same letter(s) are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

treatments in case of *C. rolfesii*, while, treatment of HQ at 12 mM was the most effective one in case of *F. incarnatum*.

Effect on growth parameters

Data presented in Table 5 reveal that no significant differences were recorded on growth parameters of alfalfa plants (shoot length, branches and leaves number and shoot dry weight) when treated by HQ or SM at any concentration. Except treatments of HQ at 12 mM and SM at 8 mM, no significant difference was recorded in the leaf area of alfalfa plants treated with the other treatments.

Comparing to the untreated infected plants, treatments of HQ or SM at any concentration increased all investigated growth parameters (except the number of branches) of alfalfa plants infected with *C. trifolii* or *R. solani.* On the other hand, no significant differences were recorded in all investigated growth parameters (except leaf area) of alfalfa plants infected with *F. incarnatum* and treated with HQ or SM at any concentration. In contrast, treatment with HQ or SM at any concentration of alfalfa plants infected with *F. equiseti* significantly decreased both of number of branches and shoot dry weight, while,

no significant difference was recorded in the shoot length when compared with the untreated-infected plants.

Photosynthetic pigments

The photosynthetic pigments of alfalfa plants showed different responses to the tested substances (Table 6). No significant differences were founded in the photosynthetic pigments of the non infected alfalfa leaves when treated with HQ at 10 and 12mM or SM at 10 mM, while, treatment with SM at 8 mM led to a significant decrease in the photosynthetic pigments.

Results obtained indicated that, treatments with HQ or SM at any concentration led to a significant increase in the photosynthetic pigments of alfalfa plants infected with *C. trifolii, R. solani* or *F. incarnatum*. Except the treatment with SM 8 mM, all treatments led to a significant increase in the photosynthetic pigments of alfalfa plants infected with *F. equiseti*.

Total phenol content

No significant differences were recorded in the total phenol content of the non-infected alfalfa plants treated

Table 4. Effect of HQ and SM on disease incidence of alfalfa under greenhouse conditions.

Fungus	Symptom	Treatment					
		P*	F+P	P+ HQ10	P+ HQ12	P+ SM 8	P+ SM10
C. trifolii	Pre-emergence	37.7	21.9	22.3	20.0	18.3	18.0
	Post-emergence	22.3	18.0	14.4	15.0	7.7	12.0
	Survival	40.0	60.1	63.0	65.0	73.3	70.0
R. solani	Pre-emergence	33.3	22.0	14.0	18.3	16.2	18.3
	Post-emergence	21.1	15.9	11.7	11.7	15.1	17.1
	Survival	45.6	62.1	70.0	70.0	68.8	64.6
F. equiseti	Pre-emergence	16.7	15.7	10.5	16.6	16.1	17.8
	Post-emergence	14.4	9.2	9.5	11.1	7.2	13.3
	Survival	68.9	75.0	80.0	72.3	76.7	68.9
F. incarnatum	Pre-emergence	26.6	14.1	13.9	11.1	18.1	18.8
	Post-emergence	15.1	10.4	9.4	7.8	11.9	12.2
	Survival	58.3	75.4	76.7	81.1	70.0	68.9
Control	Pre-emergence	2.7					
	Post-emergence	0.0					
	Survival	97.3					

Each value represents the mean of 10 replicates (10 seeds each), where, P= pathogen, F= chemical fungicide, HQ10 - HQ12= hydroquinone at 10 and 12 mM, and SM8 - SM10= sodium metabisulfite at 8 and 10mM, respectively.
L.S.D. at 0.05= 6.016 for pre-emergence damping-off, 5.499 for post-emergence damping-off and 7.442 for Survival seedlings.

with HQ or SM at any concentration (Table 7). On the other hand, treatments with HQ or SM at any concentration led to a significant increase in total phenol contents in shoots of alfalfa plants infected with *C. trifolii* and *F. equiseti*. Except the treatment with SM 10 mM, all treatments led to a significant increase in the total phenol content of alfalfa plants infected with *F. incarnatum* when compared to the untreated–infected treatment. Regarding to alfalfa plants infected with *R. solani*, treatment with HQ at 10 or 12 mM showed significantly increases in the total phenol content, while, no significant difference was recorded when seeds were treated with SM at 8 or 10 mM.

DISCUSSION

Pathogenicity test indicated that *R. solani* and *C. trifolii* were the most devastating fungi causing 26.45, 26.1%, seed infection and 31.6, 21.1% seedling mortality, respectively. This was followed by *F. equiseti* and *F. incarnatum*, in seed inoculation experiments. Results of the transmission study showed that the recovery percentages of the tested pathogens gradually decreased from root apex up to the first internodes below the shoot tip, but did not reach to the shoot apex. These results are in agreement with that of Frate and Davis (2007) who reported that *C. trifolii* spores may spread by seed during the threshing process to infect the growing stem and

petioles of alfalfa plant. Al-Kherb et al. (1997) confirmed these results by positive serial isolations made up of *R. solani* from roots, crowns and stems of infected alfalfa plants. There was a direct correlation between seed infection and loss in germination. Moreover, Krnjaja et al. (2005) studied the virulence of 91 isolates of different species of *Fusarium* on alfalfa seedlings. Isolates of *F. equiseti* demonstrated weak virulence (necrosis on the tip of the root), while isolates of species *F. incarnatum* demonstrated medium virulence (root and low part of the stem while necrosis did not spread on leaves or on the upper section of stem). These results were previously explained by Neergaard (1979), since he reported that the pathogens either extra-embryonal or embryonal, since infection was able to cause seed rot, seedling mortality and finally death of seedlings. In this case the pathogen may spread from primary infection (seeds) to leaves, petioles and stems, where they germinate, the hypha penetrating the host and producing local infection (example *A. alternata*) or lives for period of time saprophytically, persists in a resting stage in the soil or in plant residues and infects the host at a later time example, *Phoma* sp., *R. solani* and *Fusarium* species. Since most fungi affect alfalfa is seed-borne, the movement of seeds for commerce exchange greatly increases opportunities for long distance dispersal and permanent establishment of pathogens in new areas (Vasić et al., 2011).

Table 5. Growth parameters of infected alfalfa plants treated with different treatments.

Fungus	Growth parameter	Treatment					
		P	F+P	P+HQ10	P+HQ12	P+SM8	P+SM10
C. trifolii	Shoot length (cm)	20.6	32.3	25.7	24.9	23.2	24.8
	No. of branches	1.3	2.0	2.2	2.3	1.8	2.3
	No. of leaves	12.8	16.5	18.2	19.5	17.8	20.8
	Leaf area (cm2)	25.3	65.4	69.1	48.6	43.8	59.3
	Shoot dry weight (g)	0.31	0.33	0.39	0.48	0.47	0.38
R. solani	Shoot length (cm)	25.3	23.2	26.5	25.0	23.9	25.1
	No. of branches	2.1	2.3	2.0	2.1	2.8	2.3
	No. of leaves	17.8	18.0	18.3	17.0	21.1	20.6
	Leaf area (cm2)	39.1	56.7	68.4	45.8	72.4	70.8
	Shoot dry weight (g)	0.20	0.30	0.31	0.27	0.26	0.30
F. equiseti	Shoot length (cm)	26.3	25.2	35.2	28.8	27.7	27.0
	No. of branches	2.7	1.2	1.8	1.3	2.1	2.0
	No. of leaves	21.0	18.6	18.5	24.5	21.1	17.1
	Leaf area (cm2)	79.4	72.4	80.9	43.9	69.4	84.6
	Shoot dry weight (g)	0.49	0.25	0.39	0.37	0.30	0.37
F. incarnatum	Shoot length (cm)	20.6	21.3	33.0	31.5	24.	25.7
	No. of branches	1.5	1.8	1.8	1.9	2.0	2.7
	No. of leaves	14.3	16.7	21.7	21.7	17.0	21.7
	Leaf area (cm2)	29.3	36.1	54.1	41.4	37.4	48.1
	Shoot dry weight (g)	0.22	0.28	0.36	0.37	0.37	0.39
		C	F	HQ10	HQ12	SM8	SM10
Controls	Shoot length (cm)	26.3	29.4	26.3	29.6	26.1	29.3
	No. of branches	2.7	2.5	2.7	2.2	19.0	2.0
	No. of leaves	21.0	21.5	22.7	20.2	94.2	19.0
	Leaf area (cm2)	79.4	76.5	83.9	94.4	0.40	65.8
	Shoot dry weight (g)	0.49	0.48	0.53	0.41	0.40	0.35

Each value represents the mean of 10 replicates (10 seeds each), where, C=control, F= chemical fungicide, P= pathogen, HQ10 - HQ12 = hydroquinone at 10 and 12 mM, and SM8 - SM10= sodium metabisulfite at 8 and 10mM, respectively.
 L.S.D. at 0.05= 3.632 for shoot length, 0.873 for no. of branches, 4.282 for no. of leaves, 11.311 for leaf area and 0.118 for dry weight.

The antifungal activity of the three tested substances (HQ, SS and SM) investigated in this study showed that SM was the most effective one in inhibiting the target fungi. This result is in agreement with that of Mills et al. (2004) who received the same results on *Alternaria alternata*, *Botrytis cinerea*, *F. solani*, *Phytophthora erythroseptica*, *P. infestans*, *Verticillium albo-atrum*, and *V. dahlia*. Also, Latifa et al. (2011) and Talibi et al. (2011) reported that SM has completely inhibited growth and spore germination of *Penicillium italicum* and *Geotrichum candidum,* respectively. The mechanisms thought to be responsible for the sodium metabisulfite inhibition of fungal growth were explained on the basis of cell disruption and compromised membrane permeability (Avis et

al., 2009). An investigation using transmission electron microscope showed some ultrastructural alterations including membrane retraction, undulation, and invagination, loss of membrane integrity, cytoplasmic leakage, and cell rupture in the affected cells (Avis et al., 2009). In addition, recent results with *F. sambucinum, P. infestans,* and *R. solani* exposed to sodium metabisulfite indicated that lipid peroxidation damage of fatty acids or fatty acyl chains would be involved in the antifungal mode of action (Avis et al., 2007).

Hydroquinone comes at the second rank of SM in inhibiting the growth of alfalfa seed-borne pathogens. In this connection, at 14mM, they completely inhibited the linear growth of *C. trifolii*. The obtained results are in

Table 6. Photosynthetic pigments (mg g^{-1} fresh weight) in infected alfalfa leaves treated with different treatments.

Fungus	Growth parameter	Treatment					
		P	F+P	P+HQ10	P+HQ12	P+SM8	P+SM10
C. trifolii	Chl. a	1.09	1.36	1.58	1.36	1.46	1.29
	Chl. b	0.78	0.96	1.28	1.27	1.15	1.07
	Total Chl.	1.87	2.32	2.86	2.62	2.61	2.36
	Carotenoids	0.57	0.69	0.84	0.79	0.76	0.7
R. solani	Chl. a	1.23	1.34	1.51	1.37	1.59	1.54
	Chl. b	0.87	0.88	1.22	1.09	1.34	1.24
	Total Chl.	2.1	2.22	2.73	2.46	2.93	2.78
	Carotenoids	0.65	0.66	0.82	0.72	0.86	0.82
F. equiseti	Chl. a	1.27	1.58	1.69	1.59	1.39	1.58
	Chl. b	1.01	1.18	1.38	1.36	0.76	1.36
	Total Chl.	2.28	2.76	3.07	2.95	2.16	2.94
	Carotenoids	0.68	0.81	0.89	0.9	0.56	0.87
F. incarnatum	Chl. a	1.18	1.26	1.43	1.47	1.4	1.67
	Chl. b	0.71	0.92	0.88	1.14	1.28	1.25
	Total Chl.	1.89	2.18	2.31	2.61	2.68	2.92
	Carotenoids	0.57	0.67	0.69	0.79	0.81	0.85
Controls		**C**	**F**	**HQ10**	**HQ12**	**SM8**	**SM10**
	Chl. a	1.74	1.25	1.62	1.57	1.23	1.62
	Chl. b	1.08	0.95	1.29	1.26	0.76	1.26
	Total Chl.	2.81	2.19	2.9	2.86	2	2.88
	Carotenoids	0.84	0.67	0.83	0.84	0.59	0.85

Each value represents the mean of 10 replicates (10 seeds each), where, C= control (uninfested sterilized soil), F= chemical fungicide, P= pathogen, HQ10 - HQ12 = hydroquinone at 10 and 12 mM, and SM8 - SM10= sodium metabisulfite at 8 and 10mM, respectively.
L.S.D. at 0.05= 0.2060 for chl. a, 0.2341 for chl. b, 0.3778 for total chl. and 0.1048 for carotenoids.

Table 7. Total phenol content (mg 100g^{-1} fresh weight) in infected alfalfa leaves treated with different treatments.

Fungus	Treatment					
	P	F+P	P+HQ10	P+HQ12	P+SM8	P+SM10
C. trifolii	68.4e	91.2a-c	97.9ab	79.7c-e	99.8ab	105.8a
R. solani	66.7c	71.5c	96.6a	75.5bc	71.2c	67.4c
F. equiseti	58.9f	96.5ab	92.0a-c	83.7b-e	105.4a	92.3a-c
F. incarnatum	77.3b-d	99.9a	85.5a-d	92.3ab	85.6a-d	78.8b-d
Controls	**C**	**F**	**HQ10**	**HQ12**	**SM8**	**SM10**
	72.1de	87.4b-d	80.9c-e	87.7b-d	77.4c-e	70.2e

Each value represents the mean of 10 replicates (10 seeds each), where, C= control (uninfested sterilized soil), F= chemical fungicide, P= pathogen, HQ10 - HQ12 = hydroquinone at 10 and 12 mM, and SM8 - SM10= sodium metabisulfite at 8 and 10mM, respectively.
 Values within a raw followed by the same letter(s) are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

agreement with the finding of El-Wakil and El-Metwally (2000) against seed-borne pathogenic fungi (*Cephalosporium* sp, *F. moniliforme, F. oxysporum, F. solani, R. solani, Sclerotium bataticola* and *Verticillum* sp.) of peanut. Also, Ali et al. (2009) reported that HQ reduced significantly the mycelial growth of root rot pathogenic fungi

attacking lupine plants. Cowan (1999) explained the mechanisms thought to be responsible for the phenolics toxicity to microorganisms on the basis of enzyme inhibition by the oxidized compounds, possibly through reaction with sulfohydryl groups or through more nonspecific interactions with the proteins. The sites and number of hydroxyl groups on the phenol compounds like hydroqui-none are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation associated with increased toxicity. In addition, Scalbert (1991) has found that the more highly oxidized phenols are the more inhibitory effect to the pathogen.

Under greenhouse conditions, treatment with HQ at 12 mM was the best. Where, the treated-infected alfalfa plants showed the lowest level of mortality percentage and the highest plant survival percentage. Moreover, treatment with HQ significantly improved the tested growth parameters (shoot length, number of leaves and shoot dry weight) when compared with the control. The obtained results are in agreement with the findings of El-Wakil (2003) who showed that hydroquinone not only inhibits the peanut seed-borne fungi but also has an effect in improving the growth of the plants and raise the yield by up to 50% more comparing to the untreated treatments. This phenomenon has also been shown by Chinese authors (Boeckx et al., 2000) who indicated that hydroquinone function as urease inhibitor and improve the urea N recovery by spring wheat with 34% as com-pared to the treatment where only urea was applied. It also improved the quality (protein N content) of the grain.

Alfalfa plants that were treated with HQ showed significant increase in the total photosynthetic pigments and the total phenol contents. Increasing photosynthetic pigments will expected to increase carbohydrate content in plant tissues. This important action greatly affects pectin that consider a barrier against plant pathogens invasion and phenolic compounds, which play a major and important role in plant defense (Hahlbrock and Scheel, 1989). In addition, the enhancement in chlorophell content increases disease resistance as well as decreases photophosphorylation rate, which occurred after infection (Amaresh and Bhatt, 1988). Plants can tolerate infection by increasing the chlorophyll concentration in leaves. Most plants produce a broad range of secondary metabolites (ex. phenolic and polyphenolic compounds) that are toxic to pathogens, either as part of their normal program of growth and development or in response to biotic stress (Lattanzio et al., 2006). In this connection, Ali et al. (2009) reported that, total phenols content in lupine leaves significantly increased by HQ application. Phenolics are well-known as antifungal, antibacterial and antiviral compounds occur naturally in plants (Lattanzio et al., 2006). According to Vermerris and Nicholson (2006), the first step of the defense mechanism in plants involves a rapid accumulation of phenols at the infection site, which restricts or slows the growth of the pathogen. Yet total phenols have long been considered as important

defense-related compounds whose levels are naturally high in resistant varieties of many crops (Vermerris and Nicholson, 2006). Based on these results we recommend applying these treatments in order to control the seed-borne diseases of alfalfa as alternative to chemical fungicides due to its safety, effectiveness and inexpensiveness.

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