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Testing Biological Agents and Methods to Control Fusarium Wilt of Alfalfa Plants (*Medicage sativa* L.)

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Abstract: Three strains of rhizobacteria, namely *Azospirillium brasilense, Azotobacter chroococcum* and *Klebsilense peneumoneae* and a commercial product of HALEX® (rhizobacteria mixture) were tested for health and growth promotion of alfalfa plants and for controlling *Fusarium oxysporum f.* sp. *Medicaginis* the causal agent of fusarium wilt disease in Alfalfa. *In vitro*, the rhizobacteria reduced the dry weight of fusarium mycelia by 31.3 to 63.7%, therefore, the highest suppression was achieved by *K. peneumoneae* and the three mixture isolates. *In vivo* a significant decrease in disease severity was observed when alfalfa seeds were coated with HALEX®. This treatment also, increased the plant fresh and dry weight. Dry weight increased from 3.31, 2.16, 1.2 to 3.8, 2.17, 1.76 g in noninfected soil with the pathogen and from 1.81, 1.07, 1.01, to 2.45, 1.33, 1.28 g in soil treated with pathogen on Najdy, Hassawy and Max cultivars, respectively. Also, significant increases were observed in the number of root nodules from 74.6, 200.7, 161.6 to 190.2, 301.2, 274.6 in noninfected soil with the pathogen and from 61.0, 185.6, 124.3 to 159.2, 288.4, 251.3 nodules in soil treated with the pathogen but only when seeds were treated with HALEX® and respectively on the tested alfalfla cutivars.

Key words: Alfalfa, fusarium wilt, *Azotobacter, Azospirillium, Klebsiella*

INTRODUCTION

Alfalafa (*Medicage sativa* L.) is one of the most important forage crops in the world and is the primary hay crop supporting dairy production[1]. Diseases and other stress factors can severally reduce the production and life cycle of alfalfa stand *Fusarium* spp. that cause crown and root rot as well as fusarium wilt, has been implicated as the cause of alfalfa plant death and decreased yield quantity and quality in large areas of United States[2]. Fusarium wilt of alfalfa is a systemic disease, caused by *Fusarium oxysporum* Schechted, Fr. *f.* sp. *Medicaginis* [J.L. Weimer W.C. Snyder and H.N. Hans]. This disease occurs throughout many of the world's alfalfa-growing regions and is most severe in the warmer areas[3]. Symptoms of fusarium wilt, which include chlorosis, wilting, stunting and death of plant, increase in severity and incidence during subsequent growing season[4]. Numerous investigations have been conducted to develop systems for effective utilization of microbial amendments for biocontrol of soilborne plant pathogens. Induced systemic resistance in plants by growth promotion or by antagonism to pathogen has been demonstrated in alfalfa and many crops studies. Hwang *et al.*[5] used mycorrhizal fungi (*Glomus* spp., *G. fasciculatus* and *G. mosseae*) to suppressed fusarium wilt of alfalfa. A mixture of Plant Growth Promoting Rhizobacteria (PGPR)[6] consisting of N_2–fixing *Azotobacter, Azospirillium* and *Klebsiella* has been developed by Hassouna and registered as HALEX®[7]. Hassouna and Aboul-Nasr[8] used HALEX® against soilborne plant pathogen in soybean plants. Aboul-Nasr *et al.*[9] used PGPR against fusarium root rot of cucumber. Hassouna *et al.*[10] used PGPR to increased yield of alfalfa in Egypt. All these researches were conducted in cold or moderate climate. Since alfalfa cultivated mainly in wormer areas, the objectives of this study were focused on testing indigenous N_2–fixers *Azotobacter, Azospirillium, Klebsiella* isolates and the commercial product HALEX® on wormer conditions for their potential use in protecting alfalfa against fusarium wilt caused by *F. oxysporum f.* sp. *medicaginis*.

MATERIALS AND METHODS

Source of rhizobacterium and HALEX®: Three growth promoting nitrogen fixing rhizobacteria, namely *Azospirillium barasilense, Azotobacter chroococcum* and *Klebsiella pneumoneae*, engineered for increasing the N_2 fixation, were chosen for this study, in addition to HALEX®, (a mixture of the above rhizobacteria and chemically inert talc powder, which served as a carrier). All the bacteria inocula were kindly provided by M.G. Hassouna, Plant Pathogen Department, Faculty of Agriculture, Alexandaria University, Egypt.

Isolation the pathogen from infected plants: Fungus was isolated from roots of an infected alfalfa plants in Al-Qassim region, Saudi Arabia. A single spore isolate was cultured, 7-days-old, were maintained on Potato Dextrose Agar (PDA) medium at 27°C to serve as a source of inoculum Isolates were identified according to Booth[11] as *Fusarium oxysporum f.* sp. *medicaginis*.

Pathogenicity tests: *F. oxysporum f.* sp. *medicaginis* isolate was grown on sterilized barley grain in 250 mL flasks for 10 days. Inoculum was transferred from the flasks to autoclaved aerated potted soil at the rate of 4.5 g/15 cm pot, then sown with alfalfa seeds at the rate of 25 seeds/pot. Three alfalfa cultivars were used, Najdy, Hassawy and Max. The seeds of these cultivars were obtained from Agronomy Department, College of Agriculture, Al-Qassim University, Saudi Arabia. Ten replicates (pots) were used for each cultivar. In check treatment the seeds were sown in autoclaved soil which no fungi were added. Number of wilted and diseased plants were recorded after 28 days from sown.

***In vitro* assays**

Inhibition zones: PDA medium was poured into 9 cm diameter sterilized petri dishes. Two straight lines, 5 cm long and 3 cm apart, were streaked with a loopfull of the investigated bacterial suspension according to the method of reddy and patrick[12]. The dishes were incubated at 30°C for 48 h before the fungus (*F. oxysporum f.*sp. *medicaginis*) was introduced. As a disc, 0.7 cm in diameter, in a central position between the two lines. The fungal growth was followed daily for 5 days. The suppressive affects were visually assessed by comparing 10 dishes with their controls (i.e., no bacterial inoculation). The reactions were scored as (-) no suppression (= no inhibition zone), (+) minimal, (++) moderate and (+++) high suppression (i.e., clear inhibition zone), for four (4).

Fungal dry weight: Fifty milliliters of PD medium were poured into each of five 100 mL conical flasks. The medium was sterilized subsequently, inoculated with cell suspensions of the rhizobacteria individually. Inoculum density was approximately $1.6x10^8$ cells/flask. A set of flasks was left uninoculated to serve as a control. All flasks were incubated at 30°C for 48 h and then were inoculated with 7-day-old fungal culture as 0.7 cm diameter disk to each flask. Each of the three rhizobacteria and HALEX® were tested against the pathogenic fungus. Flasks were incubated at 30°C for 7 days and the hyphal mat of each flask was obtained by filtration, dried at 50°C overnight and placed in a desiccator. The dry weight was then recorded.

Field experiment: The experiment was carried out in the experimental station, College of Agriculture and Vet. Medicine, Al-Qassim University, Saudi Arabia during 2000 and 2001 growing seasons. The field plot of 3x5 m was divided into twelve rows. Six plots were used, three plots were received the pathogen and each plot sown with one cultivar. The other plots were used as a control without the pathogen. Three alfalfa cultivars Najdy, Hassawy and Max were tested. The isolated fungus *F. oxysporum f. sp. medicaginis* was grown on autoclaved barley grains for 10 days and then applied to the soil at the rate of 4.5 g dried inocula/row. Seeds of the three cultivars were slightly moistured and completely with the HALEX® powder at the rate of 7 g kg^{-1} seeds. The treatments were 1) rows with non-seed treatment, 2) rows with seed coated with HALEX® and 3) rows with seeds coated with vitavax fungicide at the rate of 3 g kg^{-1} seeds. Four replication were used. Rows were sown with seeds at the rate of 200 seeds/meter. Fresh, dray weight and disease severity to each cultivar were recorded. The obtained data were statistically analyzed using a computer Statistical Analysis System package (SAS)[13].

RESULTS AND DISCUSSION

Growth promotion and greater yield can be achieved by plants escaping the attach of soilborne pathogens, which the cause when Reddy and Patrich[12] used the three plant growth promoting N_2-fixing rhizobacteria and the commercial product HALEX®. In the present study the PDA medium test showed that the 5 day old fungal growth completely covered the control plates, while the area of the fungal growth was restricted in the plates when the rhizobacteria isolates were used, showing zones of inhibition. Table 1 shows the suppressing effect of rhizobacteria on fungal growth on PDA medium. The effect of rhizobacteria on fungal mat (dry weight) of *F. oxysporum f.* sp. *medicaginis* was studied and the data in Table 2 showed that the reduction of fungal growth was reduced to 34.8% for *A. brasilense*, 31.3% for *A. chroococcum*, 60.8% for *K. pneumoneae* and 63.7% for mixture rhizobacteria, compared with the control. Khot *et al.*[14] suggested that the rhizobacteria produced chitinase β-1, 3-glucanase and sidero-phores which may be involved in suppression of plant diseases and inhibition growth of fungal and this chemical production can be maximazed in a suitable climate, similar to Saudi Arabia alfalfa fields and such the temperature that we used in our experiments (30°C).

Table 1: Suppression effect of rhizobacteria on *F. oxysporum f.* sp. *medicaginis* growth on PDA medium

Rhizobacteria	Level of suppression
Azospirillium brasilensa	++
Azobacter chroococcum	++
Klebsiella pneumoneae	+++
A. brasilena + A. chroococcum	++
A. brasilense + K. pneumoneae	+++
A. chroococcum + K. pneumoneae	+++
Mixture*	+++
Control**	-

Level of suppression : (-) no suppression, (+) minimal, (++) moderate, (+++) high. *Mixture = *A. brasilense + K. pneumoneae A.+ chroococcum.* **Control = no rhizobacteria.

Table 2: Dry weight of the fungal mycelial mat and reduction percent as affected by rhizobacteria

Rhizobacteria	Dry weight (g)	Reduction (%)
Control	0.33^{A}	0.0
Azospirillium brasilense	0.21^{B}	34.8
Azotobacter chroococcum	0.22^{B}	31.3
Klebsiella pneumoneae	0.13^{C}	60.8
Mixture*	0.12^{C}	63.7

Data are based on average of five replicates. Mean in column followed by a common letter are not significantly different according to Duncan's Multiple Range Test. *Mixture = *A. brasilense + K. pneumoneae + A. chroococcum.*

In pathogenicity tests and field experiments several symptoms were found affecting alfalfa plants, which were designated as wilt. Wilt symptoms include reducing the stand of alfalfa seedlings, chlorosis, wilting, stunting and deth of plants. Similar symptoms were reported by many investigators[1,3-5]. Fusarium wilt caused by *F. oxysporum f.* sp. *medicaginis* reduced alfalfa yield by increasing disease severity and decreasing the number of nodules which content rhizobacteria to N_2-fixation in root plants and decreasing fresh, dry weight. Table 3 showed that significant decrease in disease severity occurred when alfalfa seeds were coated with rhizobacteria (HALEX®). Disease severity decrease from 3.4 to 2.2 in Najdy Cv., from 3.1 to 1.9 in Hassawy Cv. and from 3.2 to 2.1 in Max Cv. as a result of fusarium soil infestation. The obvious, results indicate that rhizobacteria decreased the disease severity of *F. oxysporum f.* sp. *medicaginis*. Similar results were reported by Khot *et al.*[14] they found that rhizobacteria reduced the wilt of Chickpea plants caused by *F. oxysporum f.* sp. *ciceris* under field condition. Also, the results show that no significant different in disease severity between the treated seeds with HALEX® and fungicides to control *F. oxysporum f.* sp. *medicaginis* in the three alflalfa cultivars, which proved the positive effects of HALEX® as biocontrol of soilborne plant pathogens. Table 4 showed the significant increase fresh and dry weight of alfalfa plant in three cultivars when treated with rhizobateria (HALEX®) when soil non treated with *F. oxysporum f.* sp. *medicaginis*. But when soil treated with *F. oxysporum f.* sp. *medicaginis* significant decrease were occurred in fresh and dry weight of the three alfalfa plant cultivars. The lower decrease was found when seed treated with HALEX®. Similar results were reported by Hassouna and

Table 3: Effect of seed inoculation with HALEX® and *F. oxysporum f.* sp. *medicaginis* on disease severity and number of nodules/10 plants on alfalfa plants

	Disease severity*			No. root nodules/10 plants		
Treatments	Najdy	Hassawy	Max	Najdy	Hassawy	Max
Control (no treatment)	1.3^{BC}	1.1^{C}	1.2^{BC}	74.6^{D}	200.7^{D}	161.6^{D}
Seed with HALEX®	1.1^{C}	1.0^{C}	1.1^{C}	190.2^{A}	301.2^{A}	274.6^{A}
Seed with fungicide	1.1^{C}	1.0^{C}	1.1^{C}	116.1^{C}	253.0^{B}	211.6^{C}
Non treated seed In infested soil	3.4^{A}	3.1^{A}	3.2^{A}	61.0^{E}	185.6^{D}	124.3^{E}
Seed with HALEX® In infested soil	2.2^{B}	1.9^{B}	2.1^{B}	154.2^{B}	288.4^{A}	251.3^{B}
Seed with fungicide In infested soil	1.8^{B}	1.4^{BC}	1.7^{B}	104.7^{C}	229.3^{C}	209.0^{C}

*Disease severity was assessed on a 0.0 to 4.0 scale, according to the percentage of wilting of plant. Score 0.0=0.0%, 1=1 to 25%, 2=26 to 50%, 3=51 to 75% and 4= 76 to 100%. Mean in column followed by a common letter(s) are not significantly different according to Duncan's Multiple Range Test.

Table 4: Effect of seed inoculation with HALEX® and *F. oxysporum f.* sp. *medicaginis* on fresh and dry weight of alfalfa plants

	Fresh weight*(g/10 plants)			Dry weight**(g/10 plants)		
Treatments	Najdy	Hassawy	Max	Najdy	Hassawy	Max
Control (no treatment)	15.8^{B}	8.2^{C}	6.7^{C}	3.3^{B}	2.2^{A}	1.2^{D}
Seed with HALEX®	16.2^{A}	10.8^{A}	9.6^{A}	3.8^{A}	2.2^{A}	1.8^{B}
Seed with fungicide	15.7^{B}	9.2^{B}	8.7^{B}	3.3^{B}	1.8^{B}	1.9^{A}
Non treated seed in infested soil	8.3^{E}	4.2^{E}	4.9^{E}	1.8^{D}	1.1^{E}	1.0^{E}
Seed with HALEX® in infested soil	13.2^{C}	7.4^{D}	5.5^{D}	2.5^{C}	1.3^{D}	1.3^{C}
Seed with fungicide in infested soil	12.0^{D}	6.7^{E}	5.1^{E}	2.4^{C}	1.6^{C}	1.2^{D}

* ' ** Fresh and dry weight/ 10 plants after 2 month from sowing. Mean in column followed by a common letter are not significantly different according to Duncan's Multiple Range Test.

Hassanein[15], they found increased in number of tillers, spike length, grain yield of wheat and increased in plant height and dry weight of barley plants. Also, Hassouna *et al.*[16] found that the application of HALEX® give significantly higher growth values of sorghum fresh weight.

It can be concluded that under alfalfla field condition in Saudi Arabia, the promoting N_2-fixing rhizobacteria and HALEX® succeeded in controling the *F. oxysporum f.* sp. *medicaginis* by decrease disease severity and increase yield by increase root nodules which content rhizobacteria. These results are achieved while hopefully, avoiding the use of fertilizers to produce fresh food uncontaminated with pesticides to encourage propagating and augmenting biocontrol of soilborne fungi in crop rhizospheres.

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