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Evaluation of certain *Penicillium frequentans* isolates against Cercospora leaf spot disease of sugar beet

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Abstract

The impact of six local isolates of *Penicillium frequentans* recovered from healthy sugar beet (*Beta vulgaris* L.) leaves was evaluated against *Cercospora beticola*, the causal pathogen of Cercospora sugar beet leaf spot under laboratory and field conditions. In in vitro studies, all the six isolates were able to inhibit the mycelial growth of *C. beticola* with variation in their antagonistic capability. *P. frequentans* isolates produce pectinase and cellulase at different degrees. There was a correlation between enzyme activity and the antagonistic ability for each isolate. The high antagonistic ability isolates had the most enzyme activity. In field studies, some adhesives such as agar, starch flour, white glue, gum, and commercial adhesive (Triton Mok) were added to conidia spore suspensions of *P. frequentans* at 1% to improve conidial adhesion to sugar beet plant surface. Data also showed that all adhesives increased ($P = 0.05$) the efficiency of the spore suspension of *P. frequentans* to control the disease. The starch flour at 1% gave a significant reduction in disease severity from 43.23 to 10.42% pre-infection and from 43.23 to 10.52% post-infection. The application of *P. frequentans* led to improved root yield and the sugar percent of sugar beet crop in two tested seasons.

Keywords: Biological control, *Penicillium frequentans*, Isolates, Cercospora leaf spot disease, Sugar beet, Antagonist, Sugar content, Root yield

Background

Cercospora leaf spot caused by *Cercospora beticola* is the most destructive leaf disease of sugar beet in all production areas (Piszczek et al. 2017). It causes reduction in root weight and extractable sucrose yields increases impurity concentrations resulting in higher processing losses and can lead to reductions in gross sugar yield of up to 42% (Shane and Teng 1992).

The genus of *Penicillium* has major importance in the natural environment as well as food and drug production (Ropars et al. 2014). Carlton et al. (1976) reported that *Penicillium* species have been also suppressing the bacterial growth in a number of studies. A much diversified array of active secondary metabolites, including potent mycotoxins (Frisvad and Samson 2004) and antibacterial (Rancic et al. 2006; Lucas et al. 2007) and antifungal substances (Nicoletti et al. 2007). These secondary metabolites of *Penicillium* species have been identified as well as proved their biological activities (Silva et al. 2004). Antibiotic-based products are one of the biochemical mechanisms regulating antagonism between soil fungi that may also influence fungi stasis and the suppressive properties of certain soils toward plant pathogens (Frisvad and Samson 2004).

Penicillium frequentans Westling is reported as a biological control agent (De Cal et al. 1990). It has shown a good potential for development as a commercial biocontrol product against the brown rot of peach fruit (Guijarro et al. 2006, 2007). It produced high levels of extracellular pectinases after 24 h of incubation in submerged culture (Kawano et al. 1999). Antibiotics produced by *P. frequentans* are active against *Monilinia laxa* spore germination and germ-tube growth and may be related to the control of the pathogen (De Cal et al. 1988). Some preparations of *P. frequentans* gave a significant reduction in severity of the disease (from 38 to 80%) comparable to that given by the fungicide Captan (De Cal et al. 1990).

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Severe environmental conditions may drastically limit the establishment and survival of a biocontrol agent on a host target site (Schisler et al. 2004; Bonaterra et al. 2007). Therefore, formulation of biocontrol agents could be effective to protect them when applied on aerial plant parts. Adhesives improve spore adhesion to the target leaf and prevent wash-off by rain (Schisler et al. 2004).

The present work was planned to study the efficiency of *P. frequentans* isolates in controlling Cercospora leaf spot disease of sugar beet and evaluate the effect of different adhesives to improve conidial adhesion of *P. frequentans* to sugar beet plant surface.

Materials and methods

Isolation and identification of fungal isolates

Six isolates of *P. frequentans* recovered from healthy sugar beet leaves. The fungal isolates were identified according to their morphological and microscopic characteristics of conidia spores (Pitt 1979) and confirmed by Assiut University Mycological Center (AUMC), Assiut, Egypt. The pathogenicity of *P. frequentans* isolates was tested on sugar beet (*Beta vulgaris subsp. vulgaris* L.), table beet (*Beta vulgaris* L.), turnip (*Brassica rapa* L.), spinach (*Spinacia oleracea* L.), Swiss chard (*Beta vulgaris var. cicla* L.), and radish (*Raphanus sativas* L.) plants under greenhouse conditions to confirm that it is not pathogenic to plants. The spore suspension of *P. frequentans* isolates was prepared by growing it on PDA medium. The growth isolates were collected and blended using a warring blender. The concentration of spore suspension was adjusted to 1×10^5 per milliliter of water using a hemacytometer. The tested plants were sprayed with spore suspensions until runoff and covered with clear plastic bags to maintain high humidity. Covering bags were loosened after 24 h and removed after 48 h, and then inoculated plants were kept under natural humidity as mentioned by El-Fawy and Abo-Elyousr (2016). Control plants were sprayed with distilled water and three pots for each treatment as replicates. Regular observations were made to record appearance of symptoms or not.

A pathogenic isolate of *C. beticola* previously recovered from sugar beet plants, showing typical symptoms of Cercospora leaf spot disease, was used in this study (El-Fawy 2016).

In vitro, the antagonistic effect of *P. frequentans* on *C. beticola*

The antagonistic effect of six *P. frequentans* isolates against *C. beticola* was investigated in Petri dishes containing potato agar dextrose (PDA) medium. Each plate was inoculated on both sides with 6-mm discs from 7-day-old cultures of individual antagonistic fungal isolates. Also, one disc from 7-day-old cultures of *C. beticola* was placed in the center of the same plate. Four plates were used for each treatment as replicates. Inoculated plates with the pathogen only were used as a control. The inoculated plates were incubated at 25 °C. The antagonistic abilities of *P. frequentans* isolates were recorded when the growth of *C. beticola* isolates completely covered surface of control plates. The percentage of growth inhibition was calculated according to the equation of Abo-Elyousr et al (2014):

$$R = (C–B/C) \times 100$$

where R = % of growth inhibition, C = growth in the control, and B = growth in the treatment.

Effect of culture filtrate of *P. frequentans* on mycelial growth of *C. beticola*

The isolates of *P. frequentans* showed high antagonistic abilities (Nos. 2 and 4) were grown in a 250-ml flask containing 100 ml of malt extract (ME) broth and incubated for 15 days at 25 °C. At the end of the incubation period, *P. frequentans* liquid cultures were filtered first through filter paper to remove the mycelia. Then, the filtrates were centrifuged for 60 min at 3000 rpm to separate the fungal growth (Mohamed et al. 2008). Culture filtrate was sterilized using Seitz filter. The sterilized filtrates were added to PDA medium at concentrations of 5, 10, 20, 30, and 40% *v:v* (filtrate: medium) and mixed thoroughly before solidification. Petri dishes were inoculated in the center with 6-mm discs of *C. beticola* and incubated at 25 °C. Petri dishes without culture filtrate were used as a control. Four plates were used for each treatment as replicates. Data were recorded as a diameter of linear growth when the control plates were completely covered by the fungal mycelium. Different concentrations from culture filtrates were tried until complete inhibition of *C. beticola* growth was achieved. The percentage of growth inhibition was calculated as previously mentioned.

Pectinase and cellulase activity

The ability of the six isolates of *P. frequentans* to produce pectinase and cellulase enzymes was determined using enzyme activity-plate tests. Czapek Dox Agar (CZA) medium as described by Panda et al. (2012) containing 1% pectin and carboxymethyl cellulose was used for evaluating pectinolytic and cellulolytic activities, respectively. CZA plates were inoculated with 6-mm discs of *P. frequentans* isolates. Four plates for each isolate were used as replicates. Inoculated plates were incubated at 25 °C for one week. Pectinolytic and cellulolytic activities were determined by averaging diameters of clear zones around fungal colonies (Poloni et al. 2009).

Enhancing the adhesion of *P. frequentans* conidia to sugar beet plants and its relationship with the biocontrol of *C. beticola*

To study the efficiency of *P. frequentans* against *C. beticola*, field experiments were carried out at the Nubaria Research Station, El-Behera, Governorate, Egypt, during the sugar beet growing seasons 2014/15 and 2015/16. Seeds of Pleno sugar beet cultivar (multigerm seeds) obtained from Nubaria Sugar Refining Company (NSRC) were sown directly in plots of 3×3.5 m^2 arranged in a completely randomized design, with three plots for each treatment as replicates. Sixty days old plants were sprayed by *C. beticola* spore suspension at the concentration of 1×10^5 spores/ml as mentioned by El-Fawy (2016).

For obtaining conidial suspension, *P. frequentans* (isolate No. 4) was grown on PDA plates for 7 days at 25 °C in the dark (Guijarro et al. 2017). The concentration of spore suspension was adjusted to 1×10^5 per milliliter of water, using a hemacytometer. Agar, starch flour, white glue, gum, and a commercial adhesive (Triton Mok: 5% K_2O *w*/*v* obtained from Moka Group Company, Egypt) were added at 1% to conidia spore suspensions of *P. frequentans* before inoculation. The sugar beet plants were treated with a spore suspension of *P. frequentans* pre- and post-infection with *C. beticola*. Each treatment received two sprays (10 days between each one). The fungicide Score 25% (Difenoconazole 25%) obtained from Syngenta Company, Switzerland, was applied at concentration of 0.50 cm^3/l water as a control El-Fawy and Abo-Elyousr (2016).

Disease severity was calculated a week after the second spray, using the diseased scales by Jones and Windels (1991) as follows: 0 = no leaf lesions, 1 = 25% or less-infected leaf area, 2 = 26 to 50%, 3 = 51 to 75% and 4 = 76 to 100% infected leaf area. At the end of the experiment, root yield and sugar percent were determined. Sugar percent was measured at the sugar factory laboratory at Nobaryia Sugar Refining Company, using standard polarimetric method estimated by Schneider et al. (2002).

Statistical analysis

The obtained data were subjected to statistical analysis using the MSTAT-C (1991) program version 2.10. Least significant difference (LSD) was employed to test for significant difference between treatments at $P = 0.05$ (Gomez and Gomez 1984).

Results and discussion

Isolation and identification of fungal isolates

The fungal isolates were identified as *P. frequentans* according to their morphological and microscopic characteristics of conidia spores (Pitt 1979) and confirmed by Assiut University Mycological Center (AUMC). The pathogenicity of *P. frequentans* isolates was tested on different species of plants, i.e., sugar beet, table beet, turnip, spinach, Swiss chard, and radish under greenhouse conditions to determine the pathogenic capability of the fungal isolates. All *P. frequentans* isolates were non-pathogenic to the tested plant species (unpublished data).

Antagonistic effect of *P. frequentans* isolates on *C. beticola* in vitro

The tested isolates of *P. frequentans* were able to inhibit the mycelial growth of *C. beticola* but the isolates varied in their ability to antagonistic effect (Figs. 1 and 2). In general, *P. frequentans* isolate No. 4 exhibited the highest antagonistic effect toward the tested isolate of the pathogen, followed by isolate No. 2. The high ability of

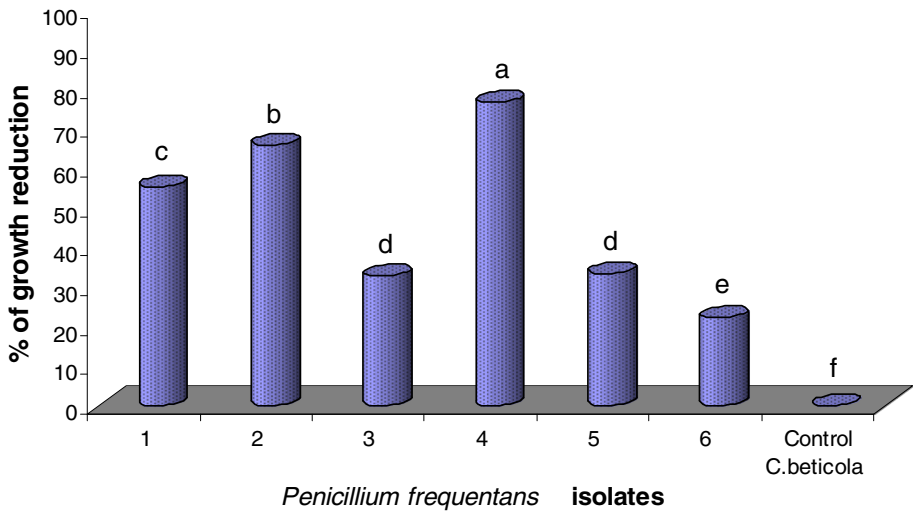

Fig. 1 Antagonistic effect of *Penicillium frequentans* isolates on the growth of *Cercospora beticola* in vitro. Different letters indicate significant differences among isolates according to Duncan's multiple range test ($P < 0.05$)

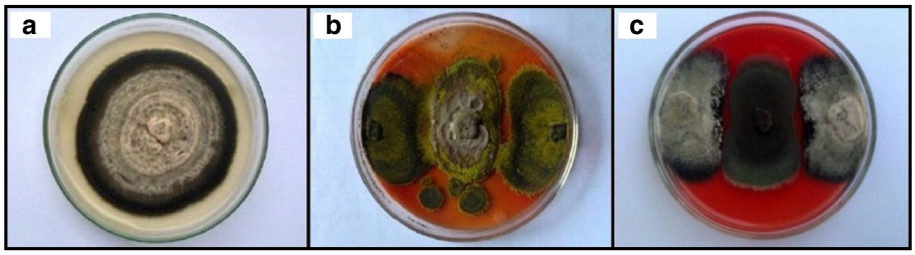

Fig. 2 Inhibition of mycelial growth of *Cercospora beticola* by *Penicillium frequentans*. **a** Control with *C. beticola*. **b** *P. frequentans* has grown rapidly over the mycelium of *C. beticola*. **c** Inhibition zone between *P. frequentans* and *C. beticola*

these isolates to inhibit the pathogen is due to the production of some toxic substances and hydrolytic enzymes such as pectinase and cellulase, which could degrade the cell wall of the pathogen. Obtained results agree with those reported by several authors, (Rancic et al. 2006), who found that *Penicillium* species produce a much diversified range of active secondary metabolites, including antibacterial (Lucas et al. 2007) and antifungal substances (Nicoletti et al. 2007) and also potent mycotoxins (Frisvad and Samson 2004).

Effect of culture filtrates of *P. frequentans* on mycelial growth of *C. beticola* in vitro

The results of this experiment in Table 1 indicate that addition of culture filtrate of *P. frequentans* to the medium significantly reduced the mycelial growth of the pathogen. Culture filtrate of *P. frequentans* reduced ($P = 0.05$) the mycelial growth of the target fungal pathogen at the tested concentrations of culture filtrate. The highest growth inhibition was reported at 40% culture filtrate concentration (*v*/*v*). These results are in agreement with those obtained by M-Sagasta (1986) who found that the isolate of *P. frequentans*, from olive fruits, was shown to produce frequent and highly antagonistic to *Geotrichum candidum*.

Table 1 Effect of different concentrations of culture filtrate of *Penicillium frequentans* isolates on mycelial growth of *Cercospora beticola* in vitro

Culture filtrate concentration %	Mycelial growth inhibition %		
	Penicillium frequentans		Mean
	Iso. No. 2	Iso. No. 4	
0	0.00 g	0.00 g	0.00 f
5	23.70 f	22.22 f	22.96 e
10	31.48 e	30.74 e	31.11 d
20	37.04 c	34.81 d	35.93 c
30	57.41 b	55.93 b	56.67 b
40	67.41 a	68.52 a	67.97 a

Values in the column followed by different letters indicate significant differences among treatments according to LSD at 0.05

In the present study, culture filtrate of isolate No. 2 was more effective on the pathogen than the culture filtrate of isolate No. 4. Data also indicated that mycelial growth of *C. beticola* significantly decreased as the concentration of culture filtrate increased and reached its maximum reduction at concentration of 40% (Fig. 3). There is a highly significant difference between the all tested concentrations ($P = 0.05$). Ali et al. (2011) found that culture filtrates of *P. citrinum*, *P. digitatum*, *P. expansum*, *P. verrucosum*, and *P. viridicatum* were the most effective to control *Salmonella gallinarum*. Three of Penicillium species isolated from *Picea glehnii* seeds produced antifungal compounds: patulin, citrinin, palitantin, and frequentin (Yamaji et al. 2001). *Pythium vexans* was more sensitive to these antifungal compounds. Also, Abdel-Rahim and Abo-Elyousr (2018) found that endophytic *Talaromyces pinophilus* had high activities of cell wall-degrading enzymes and it may be a promising biocontrol agent of phytopathogenic fungi.

Pectinase and cellulase activity

All *P. frequentans* isolates were able to produce pectinase and cellulase at different degrees. Data in Fig. 4 indicate that isolate No. 4 has the best producing

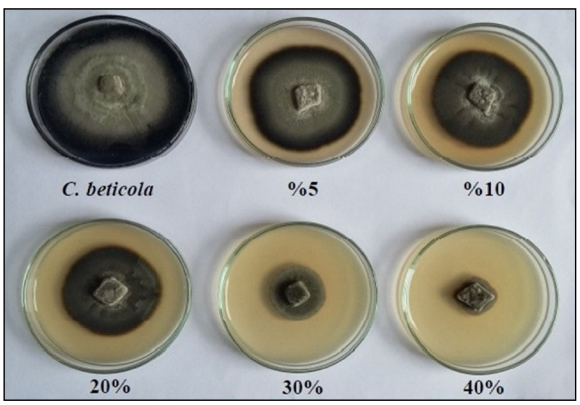

Fig. 3 Effect of culture filtrate of *Penicillium frequentans* isolate No. 4 on mycelial growth of *Cercospora beticola* in vitro

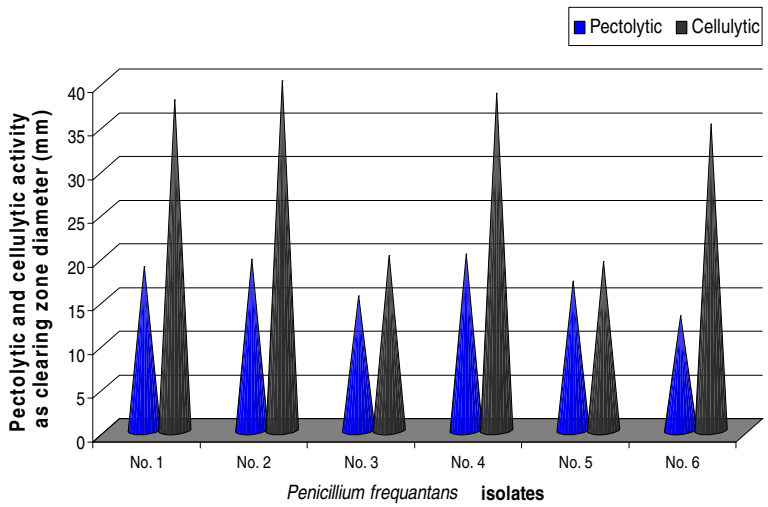

Fig. 4 Screening test of pectinase and cellulase activity of *Penicillium frequentans* isolates

pectinase (20.20 mm), followed by No. 2 (19.60 mm). However, isolate No. 6 had the lowest producing pectinase (13.10 mm). Data also indicated that cellulase activity of all isolates ranged from 19.30 to 40.00 mm. Isolate No. 2 had the best producing cellulase (40 mm), while isolate No. 6 had the lowest (19.30 mm). These results are in agreement with those obtained by Kawano et al. (1999) who noted that *P. frequentans* produced high levels of extracellular pectinases after 24 h of incubation in submerged culture. Moreover, many researches have shown the ability of different *Penicillium* species to produce some enzymes, such as *P. italicum* (Alana et al. 1990), pectolytic molds (Fawole and Odunfa 1992), *P. viridicatum* RFC3 (Silva et al. 2002), *P. roqueforti* (Pericin et al. 2007), and *P. expansum* (Cardoso et al. 2007). From these results, it is clear that there was a correlation between enzyme activity and the antagonistic ability for each isolate. The isolates with high antagonistic ability were the most enzyme activity. A cellulase enzyme had the ability to degrade the fungal cell wall which is an important mechanism of fungal inhibition (Reetha et al. 2014).

Effect of foliar spraying with spore suspension of *P. frequentans* on reducing Cercospora leaf spot disease of sugar beet under field conditions

The application of the tested adhesive (agar, starch flour, white glue, gum, and Triton Mok) to the spore suspension of *P. frequentans* was very effective in reducing the disease severity of Cercospora leaf spot of sugar beet. Data in Table 2 indicate that amendment of the spore suspension with adhesives was very beneficial for increasing effectiveness of the suspension of *P. frequentans* for controlling the disease than the spore suspension alone. Moreover, treatment with adhesives pre-infection was more effective than that post-infection. Adding starch flour at 1% pre-infection caused the highest reduction of

Table 2 Effect of foliar treatment with spore suspension of *Penicillium frequentans* on Cercospora leaf spot of sugar beet under field conditions during 2014/15 and 2015/16 growing seasons

Adhesives	Disease severity (%)					
	Season 2014/15			Season 2015/16		
	Before infection	After infection	Mean	Before infection	After infection	Mean
Agar	11.25 ef	12.50 fce	11.88 cd	12.08 e	17.19 c	15.43 d
Starch flour	10.42 ef	10.52 ef	10.47 de	10.31 f	10.33 f	10.57 f
White glue	10.94 ef	11.98 e	11.46 d	11.15 ef	15.63 c	13.39 e
Gum	13.02 ce	14.17 c	13.60 c	15.10 c	16.25 d	15.68 d
Triton Mok	15.21 c	15.52 c	15.37 c	15.73 c	18.75 bc	16.46 bc
Without adhesives	16.67 b	18.75 b	17.71 b	17.50 c	19.19 b	18.34 b
Score 25%	7.81 g	8.44 g	8.13 f	8.33 f	9.17 f	8.75 g
Control (untreated)	43.23 a	43.23 a	43.23 a	42.19 a	42.19 a	42.19 a

Values in the column followed by different letters indicate significant differences among treatments according to LSD at 0.05

disease severity (10.42 and 10.31%) at both seasons, followed by white glue (10.94 and 11.15%). On the other hand, the fungicide Score 25% gave the highest reduction of disease severity at concentration 0.50 cm^3/l water (7.81 and 8.33%) at both seasons 2014/15 and 2015/16, respectively, when used pre-infection. Triton Mok as a commercial adhesive gave the lowest reduction of disease severity (15.21 and 15.73%). The results showed that there were significant differences among the adhesives ($P = 0.05$) in increasing the effectiveness of the spore suspension in controlling the disease. Such results are in line with those reported by Larena et al. (2010) who found that incorporation of 2.5% methylcellulose into an *Epicoccum nigrum* conidial formulation; the adhesion of *E. nigrum* conidia to peach surfaces improves and results in effective biocontrol of brown rot. McGuire and Shasha (1995) found that the use of the pregelatinized flour formulation promoted *B. thuringiensis* adhesion even after multiple rain events. *P. frequentans* produced antifungal frequentin and palitantin and inhibited spore germination and germ-tube growth of *M. laxa* (De Cal et al. 1988). *P. frequentans* increased the average percentage of surviving seedlings when inoculated together with *Pythium vexans*, but the increase was not significant (Yamaji et al. 2004). The peach trees inoculated with *P. frequentans* inhibited the infection by a pathogenic fungus, *M. laxa* (Melgarejo et al. 1986). *P. frequentans* produced antifungal frequentin and palitantin and inhibited spore germination and germ-tube growth of *M. laxa* (De Cal et al. 1988).

Effect of foliar spraying with spore suspension of *P. frequentans* on root yield and sugar percent of sugar beet

Data presented in Table 3 show that treatment with *P. frequentans* spore suspension significantly ($P = 0.05$) improved agronomic characters of the sugar beet crop, i.e., root yield and sugar content at both seasons (2014/15 and 2015/16). Data also indicated that spraying the spore suspension showed a significant positive effect on the root yield and sugar percent. From the previous results, it is a clear that root yield and sugar percent were reduced by increasing disease severity. Data also showed that the highest root yield was recorded in plots treated with a spore suspension amended with white glue at concentration 1% being 36.90 and 34.04 ton/feddan at seasons 2014/15 and 2015/16, respectively, compared with the other adhesives ($P = 0.05$). The increase in root yield was due to the increased effectiveness of the spore suspension in the control of the disease. The sugar percent was highest with Score 25% treatment (21.84 and 21.97%), followed by white glue (21.82 and 21.74%) at the two seasons, respectively, ($P = 0.05$) than the control treatments (17.41 and 16.03%). These results are in agreement with those reported by Ziedan and Farrag (2011) who found that application of yeasts as biocontrol agents for controlling foliar diseases on sugar beet was effective in increasing root yield and sugar percent. However, adding Triton Mok to the spore suspension at 1% gave the lowest values of root yield and sugar percent at both seasons.

Conclusions

From the results, it could be concluded that the application of the adhesives to spore suspension before application led to improve target coverage, adhesion of conidia to sugar beet plants, and increased effectiveness in controlling the disease. The use of *P. frequentans* in the biological control of foliar diseases should be given more attention.

Table 3 Effect of foliar treatment with spore suspension of *Penicillium frequentans* on root yield and sugar percent of sugar beet under field conditions during 2014/15 and 2015/16 growing seasons

Adhesives	Root yield (tons/feddan)				Sugar %			
	Before infection Seasons		After infection Seasons		Before infection Seasons		After infection Seasons	
	2014/15	2015/16	2014/15	2015/ 16	2014/15	2015/ 16	2014/ 15	2015/ 16
Agar	33.75 bc	33.09 bc	29.20 ef	26.40 ef	19.06 bc	21.46 a	19.60 bc	19.54 bc
Starch flour	34.60 b	33.02 bc	32.72 cd	29.81 ef	21.82 a	21.11 b	21.74 a	20.20 b
White glue	36.90 ab	34.04 b	35.41 ab	33.60 bc	21.02 b	21.51 a	20.14 b	21.63 a
Gum	34.87 b	34.13 b	29.12 de	26.71 ef	19.78 bc	19.80 bc	19.56 bc	18.08 de
Triton Mok	33.67 bc	29.78 de	26.30 ef	25.03 ef	17.56 f	18.74 d	18.79 d	18.34 de
Without adhesives	24.90 fg	30.34 d	30.64 d	28.11 de	17.41 f	18.11 de	18.45 de	16.25 g
Score 25%	38.85 a	37.90 da	37.39 a	36.44 a	21.82 a	21.97 a	21.74 a	20.34 b
Control (untreated)	20.45 b	22.02 h	20.45 h	22.02 h	17.41 f	16.03 g	17.41 f	16.03 g

Values in the column followed by different letters indicate significant differences among treatments according to LSD at 0.05

Abbreviation
ME: Malt extract

Authors' contributions
All authors contributed equally in the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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