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Suppressing pathogenic fungi associated with stored garlic bulbs causing cloves rot and decreasing disease development during storage by *Bacillus subtilis* and *Trichoderma harzianum*

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Abstract

This study investigated the pathogenic fungi associated with stored garlic bulbs causing cloves rot (CR) disease. First, 25 fungal isolates were obtained from naturally diseased samples of stored garlic bulbs showing cloves rot symptoms collected from different counties of Sohag governorate and identified as *Aspergillus niger* van Tieghem (4 isolates), *Botrytis allii*. (4 isolates), *F. oxysporum* Schlecht. (5 isolates), *F. proliferatum* (Matsush.) Nirenberg (4 isolates), *F. solani* (Mart.) Sacc. (4 isolates), and *Penicillium* sp. (4 isolates). Ihe pathogenicity test conducted on cloves and seedlings under ambient laboratory and greenhouse conditions. All isolates belonging to *Fusarium* spp. were superior to other tested fungal isolates, causing the highest infection of CR and recovered from infected tissues of garlic cloves. Also, all isolates of *F. oxysporum* caused seedlings' damping-off and were superior to other tested isolates of *F. solani.* Under the greenhouse conditions, a significant decline in cloves germination and increased CR values of the disease severity index (DSI) occurred on garlic plants inoculated with all isolates of *F. proliferatum* and *F. oxysporum*. On the other hand, all isolates of *F. proliferatum* and *F. oxysporum* exhibited high values of the DSI after 60 days of bulb storage at room conditions. *In vitro* tests, all tested bacterial and fungal isolates significantly inhibited the mycelial growth of *F. oxysporum* and *F. proliferatum*. However, isolates of *T. harzianum* were more effective in reducing the mycelial growth of both fungi than isolates of *B. subtilis*. In the greenhouse trial, both tested antagonists, *B. subtilis* (isolate No. 2) and *T. harzianum* (isolate No. 5), significantly increased cloves germination, reduced the DSI of cloves rot caused by both fungi, and decreased cloves rot disease development of garlic during storage. Under field conditions, both tested antagonists significantly increased cloves germination and reduced the DSI of cloves rot caused by both fungi, as well as decreased the development of garlic cloves rot disease during storage under room conditions.

Keywords: garlic cloves rot, *F. oxysporum, F. proliferatum. Trichoderma sp., B. subtilis.* Bological control.

INTRODUCTION

In Egypt, the garlic (*Allium sativum* L.) plant is considered a major vegetable cultivated crop, with 333,543 tonnes produced from 15,719 Ha of area harvested (FAOSTAT, 2021). Factors responsible for the low yield of grown garlic in Egypt and worldwide are pests and diseases. However, fungal diseases are the most damaging among other disorders (Schwartz and Mohan, 2008). Pathogenic fungi belonging to *Fusarium* spp. are the main causes of garlic clove/bulb rot disease in the field and storage, and the most important pathogens causing severe yield loss and bulb loss during storage worldwide (Yun-ying and Zhi-gang, 2001; Galal *et al.*, 2002; Koleva, 2004; Dugan *et al.*, 2007; Palmero *et al.*, 2010 and 2012; Xu-shuang *et al.*, 2012; Mishra *et al.*, 2013; Moharam *et al.*, 2013; Oh and Kim, 2016; Ignjatov *et al.*, 2018; Arifin *et al.*, 2021; Chrétien *et al.*, 2021; Mondani *et al.*, 2021; Gálvez and Palmero, 2021 and 2022). Various fungi associated with clove/bulb rot in the field and storage have been identified in Egypt. In this regard, *F. solani* (*Fs*) has been reported as the causal organism of dry rot disease of garlic cloves and *Penicillium allii* was reported to cause postharvest decay of garlic cloves (Vincent and Pitt, 1989). *Aspergillus niger* and *F. oxysporum* (*Fo*) have also been reported as the most pathogenic fungi causing bulb rots of garlic during storage conditions (Abdel-Al *et al.*, 1991). Later, *F. moniliforme* was stated to be the incitant of postharvest cloves decay of garlic bulbs (Galal *et al.*, 2002), and *F. proliferatum* (Matsush) Nirenberg has been recently reported as the primary causal pathogen of garlic clove rot (CR) in the field and storage (Moharam *et al.*, 2013; Elshahawy *et al.*, 2017). Moreover, *F. proliferatum* (*Fp*) is a worldwide causal pathogen of rotting on various important crops. This fungus has been known as a causal agent of garlic dry rot in Germany in the past few years (Seefelder *et al.*, 2002), and later, it was stated as the main causal agent of CR disease of stored garlic bulbs (SGB) in many countries around the world (Dugan *et al.*, 2003; Stankovic *et al.*, 2007; Palmero *et al.*, 2010; Stepien *et al.*, 2011; Sankar and Babu, 2012; Tonti *et al.*, 2012; Xu-shuang *et al.*, 2012; Fuentes *et al.*, 2013; Moharam *et al.*, 2013; Elshahawy *et al.*, 2017; Chrétien *et al.*, 2021; Anisimova, Olga *et al.*, 2021). This pathogenic fungus may contaminate garlic seed cloves, infects and colonizes plant roots in the field soil during growth, and later causes CR of SGB (Stankovic *et al.*, 2007; Gálvez and Palmero, 2022). The biological control approach offers an environment-friendly alternative to using fungicides to control plant diseases. A few studies have reported the biocontrol agents *in vitro* and their capability for controlling *Fusarium* spp., but their use in garlic crops has not been yet tested (Kavitha *et al.*, 2013; Evangelista-Martínez, 2014; Ghanbarzadeh *et al.*, 2014; Ju *et al.*, 2014; Samsudin and Magan, 2016). However, several antagonistic bacteria and fungi as biocontrol agents have played an important role in the biological control of other soil-borne pathogenic fungi affecting garlic and onion plants in the field and storage. In this concern, *Trichoderma* spp. and *Bacillus subtilis* have been the most promising active ingredients commercially available for biocontrol use. Therefore, the current study was intended to identify the pathogenic fungi associated with stored garlic bulbs causing cloves rot disease. Another objective was to study the antagonistic activity of some microorganisms against the growth of fungal pathogens *in vitro* and biocontrol of cloves rot disease under greenhouse and field conditions

MATERIALS AND METHODS

1. Isolation and identification of the causal pathogens of garlic cloves rot disease:

Diseased samples of stored garlic bulbs (SGB) showing CR symptoms were collected from the farmers living in different areas of Sohag governorate. Cloves were surface sterilized after immersing in 0.5% sodium hypochlorite solution for 3 min, rinsed four times in sterile distilled water (SDW), and left to air dry under sterile conditions. Then, cloves were cut into small pieces and immediately transferred into 9.0 cm Petri dishes containing potato dextrose agar (PDA) medium amended with antibiotic streptomycin sulfate (400 mg L-1 medium). The dishes were incubated at 28±1 °C for 4-7 days. Afterward, the growing fungal colonies were checked and purified by single spore and hyphal tip techniques and cultured on new PDA plates in the same growth conditions. Pure cultures of all isolates of all

obtained fungi were then identified based on their described morphological characteristics of colony, mycelia, and spores (Domsch *et al*., 1980; Nelson *et al*., 1983; Nirenberg and O'Donnell, 1998; Leslie and Summerell, 2006). Cultures of all obtained fungal isolates were preserved at 5 °C in the PDA slants until use.

2. Pathogenicity tests:

2.1. Testing the pathogenic ability of the isolated fungi on garlic cloves under ambient laboratory conditions:

The pathogenic abilities of all fungal isolates obtained from rotting garlic cloves were determined on the garlic Balady cultivar following the method described by Dugan (2007) with insignificant changes (Palmero, 2010; Moharam *et al.*, 2013). All isolated fungi were grown in PDA medium dishes at 28±1°C for seven days till the fungal spores were densely formed. Each tested fungal isolate was inoculated into 15 garlic cloves in 25 cm Petri plates, and three plates were used for each isolate. Before inoculation, cloves were surface sterilized in 0.5% SH solution for 45 seconds, rinsed in four alterations of SDW, and injured to a depth of 4.5 mm with a 1 mm diameter probe. Then the wounded cloves were inoculated with a PDA medium colonized by each fungal isolate. The inoculated cloves with a sterile PDA medium served as control. All cloves were then incubated at 28±1°C in a growth chamber for symptom development. The experiment was conducted with 3 replicates of each tested isolate in a completely randomized experimental design. After 21 days, the CR symptoms similar to the original appeared symptoms were visually examined on all treated cloves. The percentage of clove rot was then calculated, and the main pathogen was recovered from infected clove tissue.

2.2. Testing the pathogenic capability of the fungal isolates on garlic seedlings under ambient laboratory conditions:

The pathogenic ability of selected 13 fungal isolates belonging to *Fusarium* spp. that cause clove rot was also determined on the garlic Balady cultivar (*cv*) seedlings according to the technique described by Moharam *et al*. (2013). Garlic cloves were surface sterilized, washed in four alterations of SDW as mentioned before, and then placed on sterile plastic trays filled to two-thirds capacity with autoclaved vermiculite. To prepare fungal inocula, the conidia of each tested isolate were harvested vigorously from the PDA growing cultures (14-day-old) in SDW and filtered through two layers of muslin cloth. The conidial suspension of each tested isolate was adjusted to a concentration of 106 CFU/ml using a hemocytometer. Then it was immediately supplied with 50 mg of streptomycin sulfate (Lin *et al*., 1995). Cloves were soaked in the conidial suspension of each isolate for 24 h before planting in trays. Each tray was cultivated with 30 cloves, and 3 trays were used for each isolate. Then the planted cloves were covered with a 1 cm deep layer of vermiculite. Planted cloves treated with SDW served as control. Trays of inoculated and control cloves were preserved in the growth chamber at 25-28 °C under a 14-h- and 10-h dark photoperiod. Three weeks later, the growing seedlings in trays were rated for damping-off disease (Schumann and D'Arcy, 2006) after germination of garlic cloves according to the recommendations described by the International Seed Testing Association standards (ISTA, 2004).

2.3. Testing the pathogenic capability of the fungal isolates on garlic plants under greenhouse conditions:

The pathogenic ability of selected 13 fungal isolates belonging to *Fusarium* spp. was determined on the garlic Balady *CV* during the 2019/2020 growing season in the greenhouse at the Experimental Farm, Faculty of Agriculture, Sohag University, Sohag. As mentioned before, cloves of Balady cv. were disinfected and inoculated with conidial suspension (106 CFU/ml) of each tested fungal isolate before planting in 30 cm sterilized plastic pots containing 8 kg of autoclaved clay loam soil. Cloves treated with SDW served as control. The experiment used a completely randomized experimental design with six pots (replicates) of each tested isolate. Five disinfected cloves were sown in each pot, and the pots were irrigated when necessary. Three sowed pots were used to assess cloves germination and clove rot disease after 21 days of planting, and the rest were left to get mature bulbs. Clove rot symptoms were visually examined and graded on five scales according to Stankovic *et al*. (2007) as follows: 1 =

no rot symptoms; 2 = < 10% rotted cloves; 3 = 10–50% rotted cloves; 4 = > 50% rotted cloves; 5 = completely rotted cloves.

The disease severity index (DSI) of each tested fungal isolate in each pot (replicate) was then calculated by the formula:

$DSI = \Sigma (Si \times Ni) / (5 \times Nt) \times 100$

Si is the severity rating 0-5, Ni is the number of cloves in each rating, and Nt is the total number of rated cloves.

At the end of the experiment, garlic bulbs were harvested, left in the drying shed, and then stored under room conditions. After 60 days of storage, the bulbs were visually examined for CR symptoms, and the DSI of stored bulbs was calculated as described above.

3. Biocontrol of garlic cloves rot disease:

3.1. The antagonistic activity of some microorganisms against *Fo* and *Fp in vitro*:

In this study, eight antagonistic bacterial and fungal isolates belonging to *Bacillus subtilis* Cohn (4 isolates) and *Trichoderma harzianum* Rifai (4 isolates), kindly obtained from the cultures collection of the Plant Pathology Department, Faculty of Agriculture, Sohag University (Moharam and Negin, 2012), were used to investigate their antagonistic activity against *Fo* (isolate No. 11) and *Fp* (isolate No. 14). Sterilized Petri plates containing PDA medium were inoculated with 5-mm discs of both fungi obtained from the 7-day-old culture on one side of the plates. The opposite side was inoculated with a disc of the fungal isolates or a streak of the bacterial isolates. Control plates were only inoculated with *Fo* and *Fp*. Four plates were used as replicates for each treatment in a completely randomized design. Inoculated plates were then incubated at 28±1 °C till the control plates were wholly covered with mycelium. The inhibition zone (cm) of *Fo* and *Fp* was then measured.

3.2. Effect of some selected antagonists on the infection with *Fo* and *Fp* and development of garlic cloves rot disease during storage:

A- Greenhouse experiments:

The following experiments were conducted in the open greenhouse at the Experimental Farm, Faculty of Agriculture, Sohag University, during the 2020/2021 and 2021/2022 growing seasons. The sowing date of both seasons was the 10th of October. In this study, the disinfected garlic seed cloves of Balady *cv* were treated with the most antagonistic bacterial and fungal isolates belonging to *B. subtilis* (isolate No.2) and *T. harzianum* (isolate No.5) by immersing the cloves in each antagonist suspension after inoculation with cloves rot pathogens. The disinfected garlic seed cloves of Balady *cv* were inoculated with *Fo* and *Fp* for 24 h before treating with bacterial and fungal antagonists and sowing in 30 cm pots, as mentioned before. Inocula of *B. subtilis* were prepared by growing the bacteria on the nutrient broth medium at 25 °C for two days. Then the bacterial suspension was prepared using SDW and adjusted to 5 × 106 CFU ml-1. Also, the inocula of *T. harzianum* were prepared by growing on the PDA broth medium and shaking after placing it on a rotary checker at 3.000 rpm and 25 °C for ten days. The fungal growth was washed several times with SDW and blended using a sterilized blender. Then the fungal suspension was adjusted to 5 × 104 CFU ml-1 using SDW. Cloves treated with inocula of the pathogens served as controls. The trials were carried out in a completely randomized block experimental design with eight treatment pots. Five cloves were sown in each pot, and the pots were irrigated every other day. After 21 days of planting, cloves germination and DSI of cloves rot were assessed, as mentioned before. At the end of the trial, garlic bulbs of each cultivar were harvested, left in the drying shed, and then stored under room conditions. After 30, 60, and 90 days of storage, the bulbs were visually examined for CR symptoms, and the DSI was calculated as described before. Then means over the two growing seasons were calculated and used in the static analysis.

B- Field experiments:

Under field conditions and artificial infestation, the following experiments were conducted in the Experimental Farm, Faculty of Agriculture, Sohag University, during the 2020/2021 and 2021/2022 growing seasons. The sowing date of both seasons was the 10th of October. As mentioned before, the garlic seed cloves of Balady cv were inoculated with *Fo* and *Fp* before being treated with bioagents. The applied experiments were conducted in a

completely randomized block experimental design. Three plots, 1.5 × 2.4 m each, were used as replicates for each treatment. Each experimental plot had two rows with 60 cm apart space between rows, 20 cm apart distance between hills, and ten hills in each row. Two cloves per two opposite hills in each row were planted. Inocula of *B. subtilis* (isolate No. 2) and *T. harzianum* (isolate No. 5) were prepared, as mentioned before. Then the bacterial suspension was prepared using SDW and adjusted to 5 × 106 CFU ml-1. Also, the fungal suspension was adjusted to 5 × 104 CFU ml-1 using SDW. Inoculated cloves with pathogens were treated with each bioagent suspension by adding 20 ml to 60 cloves in a glass bottle and shaking carefully. Cloves treated with inocula of the pathogens served as controls. All common cultural practices recommended for garlic production were carefully followed. After 21 days of planting, cloves germination and DSI of cloves rot were assessed, as mentioned before. At the end of the trial, garlic bulbs of each cultivar were harvested, left in the drying shed, and then stored under room conditions. After 30, 60, and 90 days of storage, the bulbs were visually examined for CR symptoms, and the DSI was calculated as described before. Then means over the two growing seasons were calculated and used in the static analysis.

Statistical analysis:

Data obtained in this study were statistically analyzed by the MSTAT-C program version 2.10. Duncan's multiple range tests for means comparing and the least significant difference (L.S.D.) at the p= 0.05 probability level was used as described by Gomez and Gomez (1984). Values shown in the Figures are the means, and the bars show the standard error.

RESULTS

1. Isolation and identification of the causal pathogens of garlic cloves rot disease:

Table 1 shows that 25 fungal isolates were obtained from naturally diseased samples of stored garlic bulbs showing cloves rot symptoms in Akhmem, Dar Elsalam, Baliana, El Maragha, Tahta, and Tema of the Sohag governorate. All isolates of obtained fungi were identified based on their morphological characteristics of the colony, mycelia, and spores. The fungi were identified as *Aspergllus niger* van Tieghem (4 isolates), *Botrytis allii* (4 isolates), *Fusarium oxysprum* Schlecht. (5 isolates), *F proliferatum* (Matsush.) Nirenberg (4 isolates), *F solani* (Mart.) Sacc. (4 isolates), and *Penicillium* sp. (4 isolates).

Table 1: Identifying fungi isolated from diseased samples of stored garlic bulbs showing cloves rot symptoms collected from different localities in the Sohag governorate.

Identification of isolated fungi	Isolates						
	Locality						Total
	Akhmem	Dar Elsalam	Baliana	Maragha	Tahta	Tema	
Aspergillus niger van Tieghem	1*	-	2	3	-	4	4
Botrytis allii Munn.	5	-	6	7	8	-	4
Fusarium oxysporum Schlecht.	9	10	-	11	12	13	5
Fusarium proliferatum (Matsush.) Nirenberg	-	14	15	-	16	17	4
Fusarium solani (Mart.) Sacc.	18	19	-	-	20	21	4
Penicillium sp.	22	-	23	24	25	-	4
Total							25

*1 - 25 = Isolates number; - = Absent.

2. Pathogenicity tests:

2.1. Testing the pathogenic ability of the fungal isolates on garlic cloves under ambient laboratory conditions: The pathogenic abilities of all fungal isolates obtained from rotten garlic cloves were tested on the garlic Balady *cv in vitro*. Inoculated cloves were incubated at 28±1° C in a growth chamber for symptom development. After 21 days, cloves rot symptoms similar to the original observed symptoms were visually examined on all inoculated cloves. Data in Table 2 indicate that all isolated fungi from SGB significantly varied in their ability to cause CR under ambient laboratory conditions.

All isolates of *Fusarium* spp. (Figure 1) were superior to other tested fungal isolates of *A. niger*, *Botrytis allii*, and *Penicillium* sp., and induced the highest CR infection and recovered from tissues of infected garlic cloves. Isolate No. 14 of *Fp* caused the highest CR infection (94.45%), followed by the isolate No. 11 of *Fo* (85.55%), whereas isolate No. 20 of *F. solani* caused the lowest CR infection (5.45%).

Table 2: Pathogenic capability of the fungal isolates on garlic cloves under ambient laboratory conditions.

Number of fungal isolates	Clove rot (%)
A. niger	
1	2.22
2	0.00
3	0.00
4	2.22
Botrytis sp.	
5	4.45
6	2.22
7	2.22
8	2.22
F. oxysporum	
9	28.89
10	31.11
11	85.55
12	28.89
13	26.67
F. proliferatum	
14	94.45
15	33.33
16	31.11
17	22.22
F. solani	
18	38.11
19	8.89
20	5.45
21	6.67
Penicillium sp.	
22	2.22
23	2.22
24	0.00
25	0.00
General control*	0.00
L.S.D. at 5%	5.24

* Uninoculated cloves.

2.2. Testing the pathogenic capability of the fungal isolates on garlic seedlings under ambient laboratory conditions:

The pathogenic capability of selected 13 fungal isolates belonging to *Fusarium* spp. was also determined on garlic seedlings of Balady cv. under ambient laboratory conditions. Three weeks after cloves inoculation with the tested fungal isolates and incubation in a growth chamber at 28±1 °C, the growing seedlings were visually examined and rated for damping-off disease. Results in Table 3 show that all tested fungal isolates significantly varied in their potential to reduce clove germination. Isolates of *Fp* were superior to other tested fungal isolates of *Fo* and *Fs*, affecting cloves' germination. Isolate No. 14 of *Fp* caused the lowest clove germination (44.44%), followed by isolate No.11 of *Fo* (51.11%). Whereas isolate No. 20 of *Fs* caused cloves germination reached 77.78% compared with complete germination (100%) of control. Results also indicate that all tested isolates of *Fo* caused seedlings' damping-off of garlic and were superior to other tested fungal isolates of *Fs*. Isolate No.11 of *Fo* caused the highest seedlings damping-off (18.89%), followed by isolate No.10 (15.56%), whereas isolate No. 20 of *Fs* exhibited seedlings damping-off reached 6.67%. Furthermore, all isolates of *Fp* did not exhibit damping-off symptoms on garlic seedlings.

2.3. Testing the pathogenic capability of the fungal isolates on garlic plants under greenhouse conditions:

The pathogenic ability of selected 13 fungal isolates belonging to *Fusarium* spp. was determined on potted garlic plants of Balady *cv.* under greenhouse conditions in the 2019/2020 growing season. Data in Table 4 show that a decline in cloves germination and increased DSI values have occurred in cloves of garlic bulbs originating from inoculated cloves with all isolates of *Fp* and *Fo* compared to the control. The highest reduction in cloves germination and DSI were recorded after inoculations with isolate No. 14 of *Fp* (43.33 and 65.67%, respectively), followed by isolate No. 11 of *Fo* (50.00 and 25.33%, respectively). Moreover, isolate No. 20 of *Fs* caused a reduction in cloves germination and DSI of 76.67 and 14.67%, respectively.

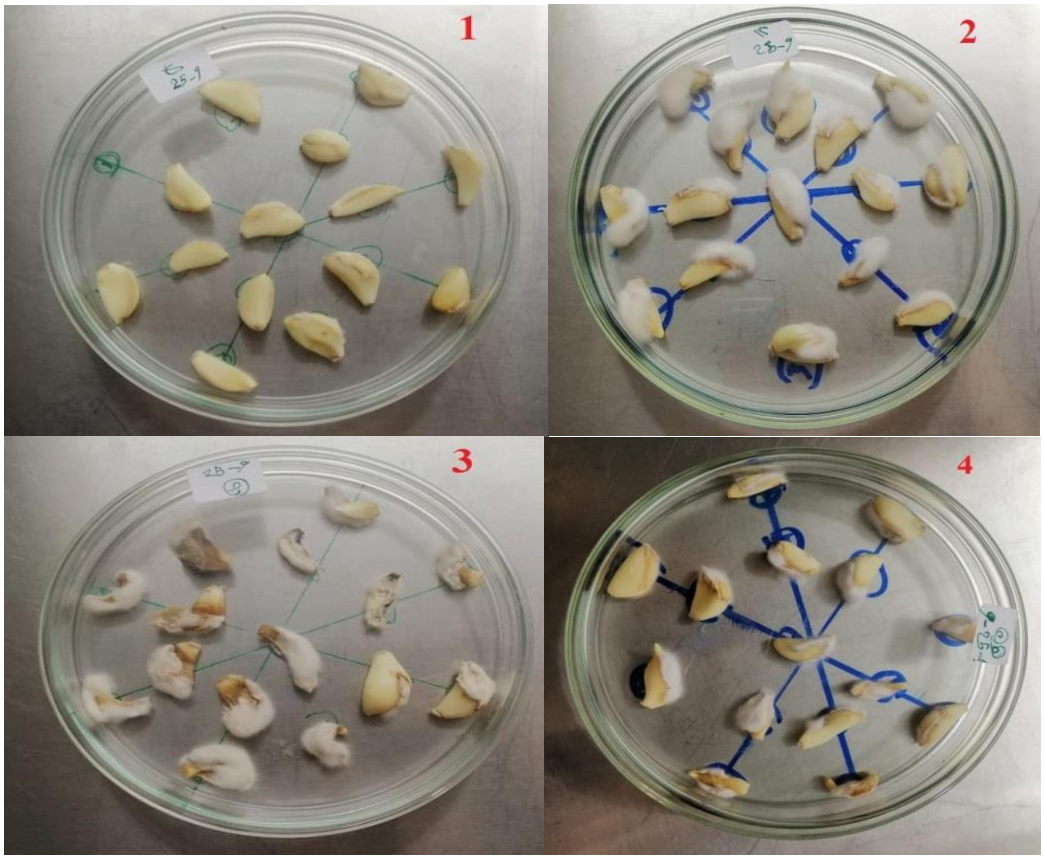


Fig. 1: The pathogenicity test of isolated *Fusarium* spp. on garlic Balady cv. cloves in Petri plates (25 cm diam.) performed under ambient laboratory conditions. The wound-inoculated garlic cloves with sterile PDA medium as control (1), and the wound-inoculated cloves covered with mycelium of *F. proliferatum* (2), *F. oxysporum* (3), and *F. solani* (4) after 21 days of inoculation.

Table 3: Pathogenic capability of *Fusarium* spp. isolates on garlic seedlings of Balady *cv.* under ambient laboratory conditions.

***Fusarium* spp. (Isolate No.)**	**Cloves germination (%)**	**Seedlings damping-off** (%)**
F. oxysporum		
9	74.44	11.11
10	65.56	15.56
11	51.11	18.89
12	73.33	10.00
13	76.67	13.33
F. proliferatum		
14	44.44	0.00
15	62.22	0.00
16	55.56	0.00
17	62.22	0.00
F. solani		
18	90.00	3.33
19	95.56	1.11
20	77.78	6.67
21	97.78	0.00
General control*	100.00	0.00
L.S.D. at 5%	3.10	2.10

* Uninoculated cloves; ** Seedlings' damping-off was estimated after 21 days of inoculation.

On the other hand, all tested isolates of *Fp* and *Fo* exhibited high DSI values of cloves rot of stored garlic bulbs after 60 days of storage under room conditions. Isolate No.14 of *Fp* exhibited the highest DSI (68.33%) of cloves rot after 60 days of storage under room conditions, followed by isolate No. 11 of *Fo* (48.67%). Moreover, isolate No. 20 of *Fs* exhibited a DSI value (12.33%) of cloves rot after 60 days of storage.

Table 4: Pathogenic capability of *Fusarium* spp. isolates on garlic Balady *cv.* under greenhouse conditions in the 2019/2020 growing season.

***Fusarium* spp. isolates**	**Cloves germination (%)**	**DSI****	**DSI*** of stored bulbs**
F. oxysporum			
9	73.33	16.33	18.67
10	66.67	19.67	21.33
11	50.00	25.33	48.67
12	73.33	19.67	20.33
13	80.00	15.33	17.67
F. proliferatum			
14	43.33	65.67	68.33
15	63.33	29.67	37.67
16	63.33	28.33	32.33
17	70.00	26.67	30.67
F. solani			
18	86.67	1.33	0.33
19	93.33	0.33	0.00
20	76.67	14.67	12.33
21	93,33	0.33	0.00
General control*	100.00	0.00	0.00
L.S.D. at 5%	2.83	2.53	1.79

* Uninoculated cloves.
** DSI of rotted cloves after 21 days of planting.
*** DSI of rotted cloves of stored garlic bulbs after 60 days of storage under room conditions.

3. Biocontrol of garlic cloves rot disease:

3.1. The antagonistic activity of some microorganisms against *Fo* and *Fp in vitro*:

Eight bacterial and fungal isolates belonging to *B. subtilis* (4 isolates) and *T. harzianum* (4 isolates) were tested for their antagonistic activity against *Fo* and *Fp in vitro*. Results in Table 5 and Fig. 2 showed that the tested bacterial and fungal isolates significantly inhibited the mycelial growth of *Fo* and *Fp in vitro*. However, isolates of *T. harzianum* were more effective in decreasing the mycelial growth of both fungi than isolates of *B. subtilis*. Isolate No. 5 of *T. harzianum* caused the highest inhibition zone of 7.8 and 7.4 cm of both fungi, respectively, followed by isolate No. 8 of *T. harzianum* and isolate No. 2 of *B. subtilis*, where they caused inhibition zone of (6.1 and 6.2 cm) and (5.7 and 5.5 cm) of both fungi, respectively. In contrast, isolate No. 4 of *B. subtilis* caused the lowest inhibition zone of 3.3 and 3.1 cm of both fungi, respectively, followed by isolate No. 1 of *B. subtilis*, where it caused an inhibition zone of 4.3 and 3.9 cm of both fungi, respectively.

Table 5: Antagonistic effect of some bacterial and fungal isolates against *F. oxysporum* and *F. proliferatum in vitro*.

Isolate No.	Tested bacteria/fungi	Inhibition zone (cm)	
		F. oxysporum	***F. proliferatum***
1	*B. subtilis* Cohn	4.3	3.9
2		5.7	5.5
3		5.4	5.2
4		3.3	3.1
5	*T. harzianum* Rifai	7.8	7.4
6		5.1	5.2
7		4.8	4.6
8		6.1	6.3
Control		0.0	0.0
L.S.D. at 5%		0.14	0.27

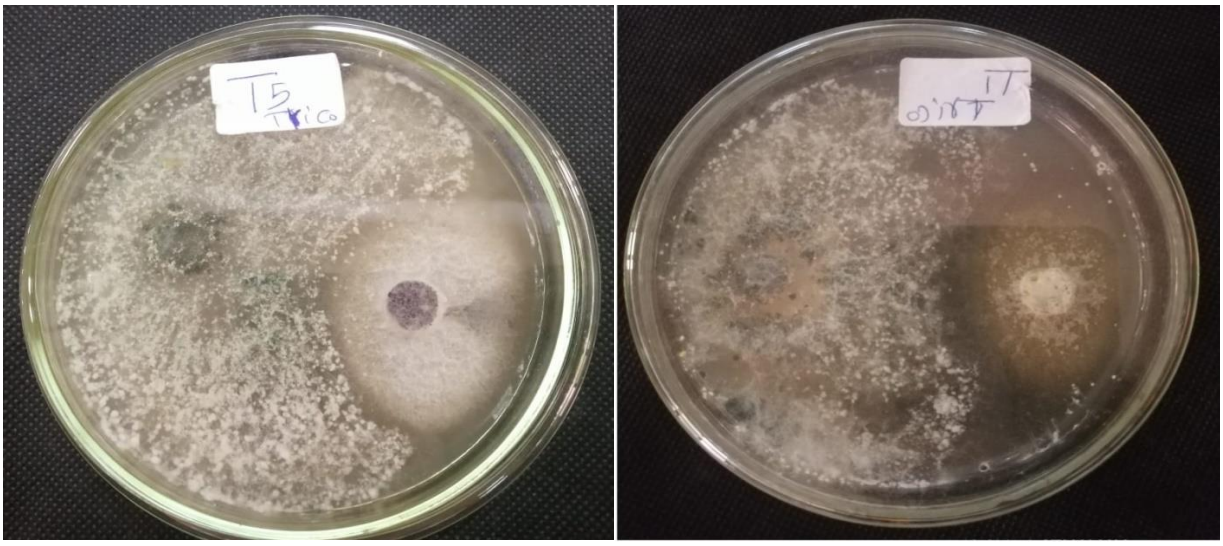


Fig. 2: Antagonistic effect of *T. harzianum* (isolate No. 5) against *F. oxysporum* and *F. proliferatum in vitro*: The left plate is *T. harzianum* (left) against *F. oxysporum* (right). The right plate is *T. harzianum* (left) against *F. proliferatum* (right).

3.2. Effect of some selected antagonists on the infection with *Fo* and *Fp* and development of garlic cloves rot disease during storage:

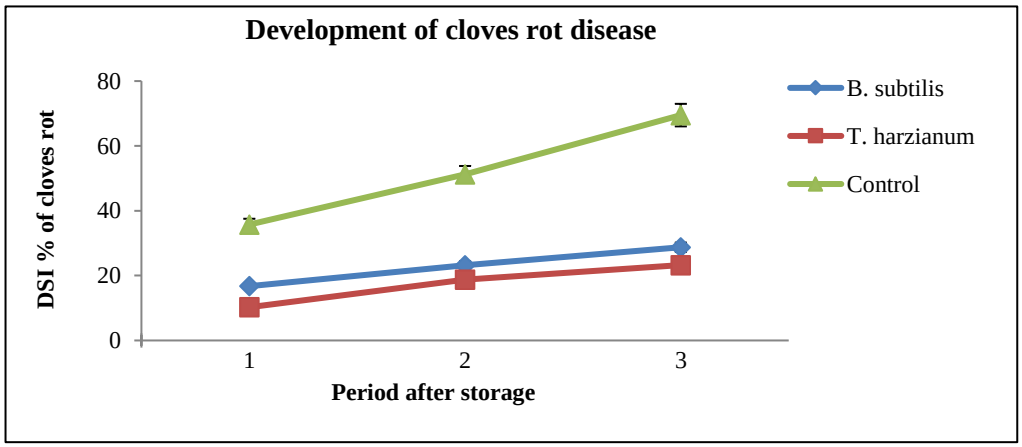
A. Greenhouse experiments:

The antagonists *B. subtilis* (isolate No. 2) and *T. harzianum* (isolate No.5) were tested in the open greenhouse during the 2020/2021 and 2021/2022 growing seasons for their effects on infection with *Fo* and *Fp* of garlic and the development of cloves rot disease during storage. Results presented in Table 6 and Fig. 3 indicate that both tested antagonists significantly varied in controlling cloves rot disease of garlic. Both tested antagonists significantly increased cloves germination, decreased the DSI of cloves rot caused by fungi, and decreased clove rot disease development of garlic during storage. However, *T. harzianum* was more effective than *B. subtilis*. Treating garlic cloves with *T. harzianum* increased the cloves' germination to 77.50 and 66.25% in cloves inoculated with both fungi and reduced the DSI caused by both fungi to (16.75 and 18.25, respectively) compared with the control. Furthermore, *T. harzianum* highly decreased the progress of garlic cloves rot disease caused by fungi to (21.25, and 23.25%, respectively) after 90 days of storage under room conditions compared with control (51.50, and 69.50%, respectively).

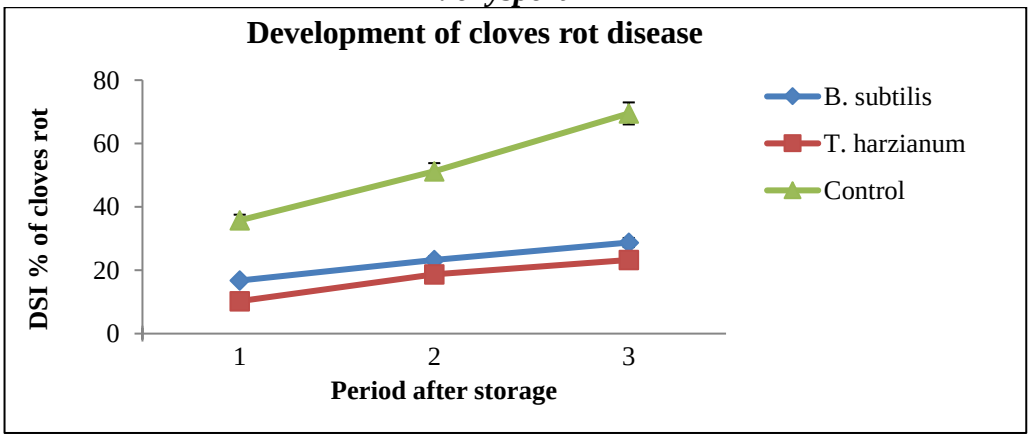
Table 6: Effect of some selected antagonists on the infection with *F. oxysporum* and *F. proliferatum* under greenhouse conditions during the 2020/2021 and 2021/2022 growing seasons and development of cloves rot disease of stored garlic bulbs after 30, 60, and 90 days of storage under room conditions.

Antagonists	*F. oxysporum*					*F. proliferatum*				
	Cloves germination (%)	DSI*	DSI** of stored bulbs after			Cloves germination (%)	DSI*	DSI** of stored bulbs after		
			30 days	60 days	90 days			30 days	60 days	90 days
B. subtilis No. 2	71.25***	27.75	11.25	25.25	34.75	52.50	21.25	16.75	23.25	28.75
T. harzianum No. 5	77.50	16.75	8.25	16.75	21.25	66.25	18.25	10.25	18.75	23.25
Control	52.50	39.50	23.75	38.75	51.50	46.25	67.50	35.75	51.25	69.50
L.S.D. at 5%	4.11	4.72	2.01	1.91	3.97	3.58	1.87	1.14	1.65	1.95

* DSI of rotted cloves after 21 days of planting.
** DSI of rotted cloves of stored garlic bulbs after 30, 60, and 90 days of storage under room conditions.
*** The values are the means over the two growing seasons.



F. oxysporum



F. proliferatum

Fig. 3: Effect of *B. subtilis* (isolate No. 2) and *T. harzianum* (isolate No. 5) on the development of cloves rot disease of stored bulbs of garlic Balady *cv*. caused by *F. oxysporum* and *F. proliferatum* after (**1**)= 30, (**2**)= 60, and (**3**)= 90 days of storage under room conditions. (After greenhouse experiments).

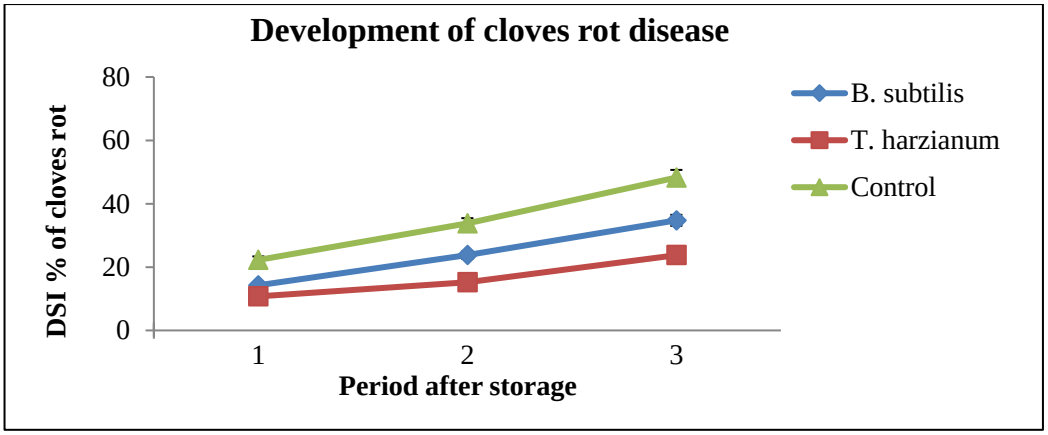
B. Field experiments:

Under field conditions during the 2020/2021 and 2021/2022 growing seasons, the antagonists *B. subtilis* (isolate No. 2) and *T. harzianum* (isolate No.5) were tested for their effects on infection with *Fo* and *Fp* of garlic and the development of cloves rot disease during storage. Results presented in Table 7 and Fig. 4 indicate that both tested antagonists significantly varied in controlling cloves rot disease of garlic. Both tested antagonists significantly increased cloves germination, reduced the DSI of cloves rot caused by both fungi and decreased the development of garlic clove rot disease during storage under room conditions. However, *T. harzianum* was more effective than *B. subtilis*. Treating garlic cloves with *T. harzianum* increased the cloves' germination to 75.42 and 57.08%, respectively, in cloves inoculated with both fungi and reduced the DSI caused by both fungi to (19.25 and 22.75, respectively) compared with the control. On the other hand, *T. harzianum* highly decreased the progress of garlic cloves rot disease caused by both fungi to (23.75, and 25.25%, respectively) after 90 days of storage under room conditions compared with control (48.25, and 65.75%, respectively).

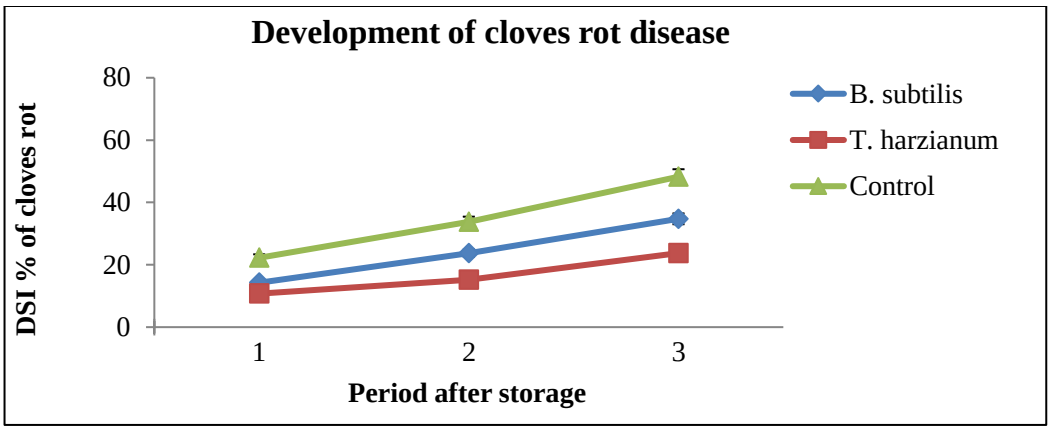
Table 7: Effect of some selected antagonists on the infection with *F. oxysporum* and *F. proliferatum* of garlic under field conditions during the 2020/2021 and 2021/2022 growing seasons and development of cloves rot disease of stored garlic bulbs after 30, 60, and 90 days of storage under room conditions.

Antagonists	*F. oxysporum*					*F. proliferatum*				
	Cloves germination (%)	DSI*	DSI** of stored bulbs after			Cloves germination (%)	DSI*	DSI** of stored bulbs after		
			30 days	60 days	90 days			30 days	60 days	90 days
B. subtilis No. 2	66.25***	24.50	14.25	23.75	34.75	46.25	27.25	19.25	25.75	29.75
T. harzianum No. 5	75.42	19.25	10.75	15.25	23.75	57.08	22.75	13.75	20.25	25.25
Control	50.42	38.25	22.25	33.75	48.25	41.25	62.25	37.25	43.75	65.75
L.S.D. at 5%	4.83	3.23	2.41	2.11	4.01	3.96	2.69	1.92	2.37	2.65

* DSI of rotted cloves after 21 days of planting.
** DSI of rotted cloves of stored garlic bulbs after 30, 60, and 90 days of storage under room conditions.
*** The values are the means over the two growing seasons.



F. oxysporum



F. proliferatum

Fig. 4: Effect of *B. subtilis* (isolate No. 2) and *T. harzianum* (isolate No. 5) on the development of cloves rot disease of stored bulbs of garlic Balady *cv*. caused by *F. oxysporum* and *F. proliferatum* after (1)= 30, (2)= 60, and (3)= 90 days of storage under room conditions. (After field experiments).

DISCUSSION

In this study, 25 fungal isolates were obtained from naturally diseased samples of stored garlic bulbs showing cloves rot symptoms collected from different counties of Sohag governorate. Isolated fungi were identified as *A. niger* (4 isolates), *Botrytis* sp. (4 isolates), *Fo* (5 isolates), *Fp* (4 isolates), *Fs* (4 isolates), and *Penicillium* sp. (4 isolates) Various pathogenic fungi associated with clove/bulb rot in the field and storage have been previously isolated in Egypt and worldwide (Moharam *et al.*, 2013; Oh and Kim, 2016; Elshahawy *et al.*, 2017; Ignjatov *et al.*, 2018; Arifin *et al.*, 2021; Chrétien *et al.*, 2021; Mondani *et al.*, 2021and Ahmed, Naglaa *et al.*, 2022).

Under ambient laboratory conditions, the pathogenicity test of isolated fungi was performed on cloves of garlic Balady *cv* and showed that all isolated fungi significantly varied in their ability to induce clove rot symptoms. However, all isolates belonging to *Fusarium* spp. were superior to other tested fungal isolates of *A. niger*, *Botrytis allii*, and *Penicillium* sp., causing the highest infection of cloves rot and recovered from infected tissues of garlic cloves. Isolate No. 14 of *Fp* caused the highest cloves rot infection, followed by isolate No. 11 of *Fo*, whereas isolate No. 20 of *Fs* caused the lowest clove rot infection. In contrast, no fungi were recovered from control cloves. These findings could be interpreted in light of the similar conclusions previously stated by Elshahawy *et al.*(2017); Ignjatov *et al.*(2018); Horákova, Miriam *et al.*(2020); Mondani *et al.(*2020); Chrétien *et al.*(2021) and Ahmed, Naglaa *et al.*(2022). Moreover, isolates of *Fp*-induced cloves rot symptoms severely developed on all inoculated garlic cloves. In Egypt, symptoms of rotted garlic cloves induced by *Fp* were similar to those described earlier by Moharam *et al.* (2013), Elshahawy *et al.* (2017), and Ahmed, Naglaa *et al.* (2022), who also established different levels of virulence between the tested isolates, which may be due to the genetic structure of each pathogenic fungal isolate.

Under ambient laboratory conditions, the pathogenicity test of 13 isolates of *Fo*, *Fp*, and *Fs* was done on seedlings of garlic Balady *cv* showed that all tested fungal isolates significantly varied in their potential to reduce cloves germination and caused seedlings damping-off. In this concern, isolates of *Fp* were superior to other tested fungal isolates of *Fo* and *Fs*, affecting cloves' germination. Isolate No. 14 of *Fp* caused the lowest cloves germination,

In contrast, all tested isolates of *Fp* did not induce damping-off symptoms in garlic seedlings. These findings could also be interpreted in light of the similar conclusions stated by Dugan *et al.* (2007), Stankovic *et al.* (2007), Palmero *et al.* (2012), and Moharam *et al.* (2013). In the present study, *Fo* highly reduced cloves germination produced extensive seedlings damping-off and induced a high disease severity index of rotted

cloves similar to those reported by Moharam *et al.* (2013). In contrast, *Fp* did not prove to be aggressive and cause damping-off symptoms of garlic, contrary to other findings reported by Stankovic *et al.* (2007), who noted that *Fp* affects garlic plants during growth in the field. Elshahawy *et al.* (2017) also recognized that *Fp* affected the percentage of plant emergence and caused wilt symptoms, which were established progressively in survival garlic plants.

Under the open greenhouse conditions, results of the pathogenicity test of 13 isolates of *Fo*, *Fp*, and *Fs* on garlic plants of Balady *cv* showed a significant decline in cloves germination and an increase in values of the DSI of cloves rot that has occurred on the cloves of garlic plants originating from inoculated cloves with all isolates of *Fp* and *Fo* compared to the control of uninoculated plants. These findings also could be interpreted in light of similar other results stated by Tonti *et al.* (2012), Xu-shuang *et al.* (2012), Moharam *et al.* (2013) and Elshahawy *et al.* (2017). The fungus *Fp* is a worldwide causal pathogen of various diseased crops. This fungus has also been recognized as a dry rot agent of garlic in Germany in the past few years (Seefelder *et al.*, 2002), and later, it has been reported in many countries as the main causal agent of clove rot disease of stored garlic bulbs around the world (Moharam *et al.*, 2013; Elshahawy *et al.*, 2017; Chrétien *et al.*, 2021; Anisimova, Olga *et al.*, 2021and Ahmed, Naglaa *et al.*, 2022). This fungal pathogen may contaminate garlic seed cloves, infects and colonizes plant roots in the field during growth, and later causes clove rot of stored garlic bulbs (Stankovic *et al.*, 2007; Moharam *et al.*, 2013; Elshahawy *et al.*, 2017and Gálvez and Palmero, 2022).

In vitro tests, all tested isolates of *B. subtilis* and *T. harzianum* affected the mycelial growth of *Fo* and *Fp*. Inhibition zones formed between *Fo* and *Fp* and *B. subtilis* or *T. harzianum*. However, isolates of *T. harzianum* were more effective in decreasing the mycelial growth of *Fo* and *Fp* than *B. subtilis* isolates. The inhibitory effect of *B. subtilis* or *T. harzianum* against *Fo* and *Fp* could be attributed to the antibiotics and/or toxic substances secreted by these bioagents, limiting and inhibiting the fungal growth. Such results and others concerning the mode of action of these antagonistic bioagents were also reported by Samsudin and Magan (2016), Elshahawy *et al.* (2017), Bjelić *et al.* (2018), Mondani *et al.* (2021), and Poromarto *et al.* (2021).

Under greenhouse and field conditions during the 2020/2021 and 2021/2022 growing seasons, applying the tested biocontrol agents *B. subtilis* and *T. harzianum* on garlic plants immediately after inoculation with *Fo* and *Fp* gave positive results in reducing cloves rot incidence and reducing the progression of disease severity index of cloves rot during storage under room conditions. However, *T. harzianum* isolates were more effective than isolates of *B. subtilis*. Such positive effects could be to the antagonistic activity of these bioagents against *Fo* and *Fp*, which were also similar to those stated by Ahir and Maharshi (2008), El-Babley, Hala (2012), Bjelić *et al.* (2018), and Poromarto *et al.* (2021), who applied the same bioagents on onion and/or garlic against *Fusarium* spp. and other fungal pathogens causing basil rot, black mold, and neck rot diseases in the field and storage. A recent study has reported these bioagents and their ability to control dry rot disease of garlic caused by *Fo* and *Fp* (Mondani *et al.*, 2021) and confirmed the results obtained in this study.

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الملخص العربي

تثبيط ا لفطريات الممرضة المصاحبة ألبصال الثوم المخزونة المسببة لمرض عفن الفصوص وتقليل تتطور المرض أثناء التخذين بواسطه البكتيريا باسلس ساتلس و الفطر تريكودرما هارزيانم

تم فحص الفطريات الممرضه المصاحبة لأبصال الثوم المخزونة المسببة لعفن الفصوص، والعوامل التي تؤثر على الإصابة بالفطرين *Fusarium oxysporum* و *F. proliferatum* وتطور مرض عفن الفصوص أثناء التخزين. أولاً، تم الحصول على 25 عزلة فطرية من عينات مصابة طبيعياً من أبصال الثوم المخزونة التي تظهر أعراض عفن الفصوص والتي تم جمعها من مختلف مراكز محافظة سوهاج وتم التعرف عليها على أنها الفطر *Aspergillus niger* (4 عزلات)، الفطر *Botrytis allii* (4 عزلات)، الفطر *Fusarium oxysporum* (5 عزلات)، الفطر *Fusarium proliferatum* (4 عزلات) والفطر *Fusarium solani* (4 عزلات).تم أختبار القدره المرضيه على الفصوص والشتلات في ظل ظروف المختبر المحيطة ونباتات الثوم في الأصص تحت ظروف الصوبه. أظهرت النتائج أن جميع العزلات تنتمي إلى أنواع الفطر *Fusarium* كانت ممرضه ومتفوقة على العزلات الفطرية الأخرى المختبرة، مما تسببت في أعلى إصابة بمرض عفن الفصوص وتم عزلها مره أخرى من الأنسجة المصابة من فصوص الثوم. كما تسببت جميع عزلات الفطر *F. oxysporum* في موت البادرات وتفوقت على العزلات الأخرى المختبرة من الفطر *F. solani*. تحت ظروف الصوبه، حدث إنخفاض معنوي في إنبات الفصوص وزيادة في قيم معامل شدة المرض لعفن الفصوص في نباتات الثوم الملقحة بجميع عزلات الفطرين *F. proliferatum* و *F. oxysporum*. من ناحية أخرى، أظهرت جميع عزلات الفطرين *F. proliferatum* و *F. oxysporum* قيمًا عالية من معامل شده المرض لعفن الفصوص بعد 60 يومًا من تخزين الأبصال في ظروف الغرفة. . أظهرت النتائج أن كل العزلات البكتيريه والفطريه المختبرة ثبطت معنويا نمو الميسيلوم للفطرين. وبالرغم من ذلك، كانت عزلات الفطر *T. harzianum* أكثر فاعلية في إختزال نمو الميسيلوم للفطرين عن عزلات البكتيريا *B. subtilis* المختبرة. . وفى تجارب الصوبه والحقل أدت معاملة تقاوى فصوص الثوم بالكائنات الحية الدقيقة المضادة إلي زياده إنبات الفصوص وإختزال معامل شدة المرض لعفن الفصوص المتسبب عن الإصابة بالفطرين *F. oxysporum* و *F. proliferatum* وايضا إلي إختزال تطور مرض عفن الفصوص لأبصال الثوم اثناء التخزين تحت ظروف الغرفة. وبالرغم من ذلك، كان الفطر *T. harzianum* أكثر فاعلية من البكتريا *B. subtilis*.