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Research Article

Biological control of *Fusarium* basal rot of onion using *Trichoderma harzianum* and *Glomus mosseae*

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Abstract: *Fusarium proliferatum*, as a toxigenic fungus, is one of the important agents of onion basal rot (FBR). Among the control methods of the disease, biological control is considered as one of the best options. In this study, *Trichoderma harzianum* strain T100 at the rate of 1×10^6 cfu/g was mixed with pot soil artificially infested with *F. proliferatum*. Also *Glomus mosseae* was applied to each pot at the rate of three grams of soil containing 80 chlamydospores/ml. Combination of T100 and *G. mosseae* was used as well. Onion seeds were disinfected & planted in pots, arranged in completely randomized design in 4 replicates under greenhouse condition and finally, the individual or combined effects of these bioagents were assessed on FBR control and also root colonization by *Glomus* 23, 30 and 36 days after sowing. Inoculation of Arbuscular mycorrhizae improved onion growth effectively, but its biocontrol effect was not considerable. *Trichoderma* amended soil decreased disease incidence by 25% but its usefulness as biocontrol agent was reduced in the course of time. AM root colonization was decreased in plants in presence of *Trichoderma*. Nevertheless, the disease control in combination of *Trichoderma* and *Glomus* treatment was better than the treatments by each one of the agents singly. The disease control achieved by fungicide seed treatment was inferior to that of *Trichoderma* and *Glomus* in combination.

Keywords: biocontrol, *Fusarium proliferatum*, red onion, *Trichoderma harzianum*, *Glomus mosseae*

Introduction

Onion *Allium cepa* L., one of the oldest vegetables, has been used as spice and medicine for thousands of years (Keusgen, 2002). *Fusarium* basal rot of onion is an economically important disease to which onion bulbs and shallots are sensitive during all their growth stages (Cramer, 2000). Some *Fusarium* species causing the disease are: *F. oxysporum*, *F. solani*, *F. acuminatum*, *F. culmorum*, *F. equiseti*, *F. proliferatum*, *F. subglutinans*, *F. redolens* and *F. tricinctum* (Bayraktar and Dolar, 2011).

To manage the disease, chemical control is very effective, but it is not economical and also pollutes the environment. Use of resistant cultivars is another acceptable strategy of control however onion cultivars with acceptable level of resistance are limited. Researchers have recently considered biological control as a complementary approach for controlling this disease (Coşkuntuna and Özer,

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2008; Cramer, 2000). *Trichoderma* species, with high rate of growth, are common in all soils and root ecosystems, and have been recognized to control soil-borne fungal diseases caused by genera of *Fusarium*, *Pythium*, *Sclerotinia*, *Rhizoctonia, Gaeumannomyces* and others (Howell, 2003). The beneficial effects of these microorganisms last longer than that of chemicals and can therefore protect the plant throughout all growth stages (Rojo *et al*., 2007). Mycorrhizal fungi have long been known as plant-growth-promoters, also their ability to induce plant defense system, change the rhizosphere exudates and to produce antibiotics, phenolic and other suppressive compounds is reported by many researchers (Mar Vázquez *et al*., 2000; Pozo *et al*., 2002).

To ensure the stability of biocontrol agents in the rhizosphere and for the purpose of protecting the plant against various pathogens, it is recommended to use mixture of antagonists (Mazen *et al*., 2008). However, before developing such combination systems it should be confirmed that these biocontrol compounds have no adverse or antagonistic effect on each other (Martinez-Medina *et al*., 2009). Therefore, in this study it has been attempted to apply a combination of known antagonistic (*Trichoderma*) and beneficial (Arbuscular Mycorrhiza) fungi, with the aim to enhance the biocontrol of red onion basal rot.

Materials and Methods

Plant material, pathogen and mycorrhizae strains

Onion (*Allium cepa* L.) cv. Red Excel, sensitive to *Fusarium* basal rot, was used in the experiment. *F. proliferatum* (isolate 13C), was isolated from naturally infected red onion bulbs in an earlier study. This isolate was chosen for *in vivo* experiments, because its high virulence on onion seeds cv. Red Excel (disease severity of 63%) was confirmed and, it was also determined as the most destructive isolate on onion bulbs (Ghanbarzadeh *et al*., 2013).

Glomus mosseae was prepared in trap culture as described by Rezaee Danesh *et al*. (2006). To determine the density of the inoculum, the chlamydospores in 100 gram of soil were extracted and counted according to Gerdemann and Nicolson (1963) method.

Antagonistic experiments *in vitro*

Trichoderma isolates selected for this study were obtained from the Department of Plant Pathology, Tarbiat Modares University, Tehran-Iran. Four species (*T. viride*, *T. harzianum* strain T100, *T. haematum* and *T. harzianum*) were investigated in dual culture tests against *F. proliferatum*, using the method of Zlata *et al*. (2008). All fungi were cultured on PDA medium amended with chloramphenicol. Then 3-day-old cultures of *Trichoderma* and 5-day-old cultures of *Fusarium* were used in the experiment. The antagonistic effect of *Trichoderma* species was examined daily by measuring the mycelial growth inhibition rate of *Fusarium*. The experimental design used was completely randomized with three replications for each *Trichoderma* species. Data were analyzed by SAS software using Duncan's multiple range test at 5% probability.

In vivo experiments

Inoculum preparation

Fusarium and *Trichoderma* isolates, to be applied to soil, were grown on wheat kernels as described by Wacker *et al*. (1990) with some modifications. In this study, five (5-mm-diameter) plugs of *Fusarium* or *Trichoderma* cultures were added into separate flasks each containing 100 g of autoclaved wheat kernels. After two weeks, the inoculated kernels were dried, ground and finally stored in aluminum foils at 4 ºC, until application. To determine the spore density of inoculums, a 1: 10 dilution of each inoculum was prepared and shaken at 80 rpm for about 1h. Finally, the spore concentration was estimated by using haematocytometer.

Pot experiment

In pot experiment, 300g of a steam-sterilized soil mixture of field soil and sand (1: 1 v/v) was added to the bed of the pots and the upper section was filled with only sand. The pathogen and antagonistic fungus at the rates of 2×10^4 and 1×10^6 cfu/g (Rojo *et al*., 2007) were mixed with the upper layer of sand (approximately 100 g) one and

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two weeks before sowing, respectively, and kept at the moderate temperature of 26 ºC in greenhouse. Red onion seeds were washed under running tap water for 20 min, surface sterilized by 70% ethanol and rinsed thoroughly with sterile distilled water. Simultaneous with sowing the onion seeds (ten seeds per pot), three grams of mycorrhizal inoculums (containing 1300 propagules/g) was applied to the seed bed. Finally, eight treatments (NC, G, T100, G + T100, P, P + T100, P + G, P + T100 + G; see Table 1), with four replications, were performed in a completely randomized design. Pots were kept in greenhouse at 26 ± 1 ºC Irrigation was done regularly, and the percent of germination and pre/post-emergence damping-off were calculated 23, 30 and 36 days after sowing. MSTATC software was used for statistical analysis and grouping of treatments was done using Duncan's tests.

Symbiotic interaction of *G. mosseae* with onion roots

Root colonization of *G. mosseae* was determined using the method of Phillips and Hayman (1970). Root pieces were mounted in lactophenol and the chlamydospores and mycelia were observed under stereomicroscope with the magnitude of 10-40X. The proliferation of mycorrhiza in the soil was determined by counting the chlamydospores extracted from the soil following Gerdemann and Nicolson (1963) 36 days after inoculation.

Results

Seed germination and the fungal inoculums density

Onion seed germination used in this study was approximately 60%. Extraction and counting the chlamydospores of mycorrhiza determined that the inoculum density, added to the soil, was approximately 1300 propagules/g, the density of *Fusarium* and *Trichoderma* inoculums, after two weeks incubation at 25 ºC, was about 2×10^6 and 1×10^7 spores/g, respectively.

Antagonistic activity of Trichoderma species in vitro

Comparing the antagonistic activity of four *Trichoderma* species (based on the mycelial growth inhibition rate) against *F. proliferatum* indicated different degrees of antagonism (Fig. 1). The antagonistic activity of *T. harzianum* strain T100 was moderate (24.7%), but in comparison with other *Trichoderma* spp. it was able to overgrow *F. proliferatum* mycelia, produce conidia on their surface, and lyse its mycelia at the confronting colony edge (Fig. 2). It could therefore be speculated that *T. harzianum* strain T100 would be able to parasitize *F. proliferatum*, even in cases where the pathogen had colonized the rhizosphere in advance.

Table 1 Effect of *Trichoderma harzianum* strain T100 and *Glomus mosseae*, as single and combined treatments, on onion growth, seed germination and *Fusarium* basal rot control (expressed as surviving plants).

Treatments	23 DAS[1]	30 DAS[1]	36 DAS[1]	Seed germination (%)
	Onion Growth (%)[2]			
N.C.	100.0^{ab}	100.0^{ab}	100.00^{ab}	100.0^{ab}
G	125.0^{a}	133.0^{a}	133.00^{a}	102.5^{ab}
T100	62.5^{ab}	70.5^{bc}	64.25^{bc}	92.5^{ab}
G + T100	75.0^{ab}	83.0^{abc}	83.00^{bc}	87.5^{b}
	Surviving plants (%)[2]			
P	50.75^{b}	38.00^{c}	33.00^{c}	110.0^{ab}
P + T100	75.00^{ab}	41.25^{c}	41.25^{c}	102.5^{ab}
P + G	45.75^{b}	45.50^{bc}	39.25^{c}	112.5^{ab}
P + T100 + G	92.75^{ab}	87.25^{abc}	66.25^{bc}	120.0^{a}

[1] Days after sowing.
[2] Means followed by the same letters in each column are not significantly different (Duncan's test, $P < 0.05$).
Abbreviations: N. C.: Negative Control, G: *G. mosseae*, T100: *T. harzianum* strain T100, P: Pathogen (*F. proliferatum*).

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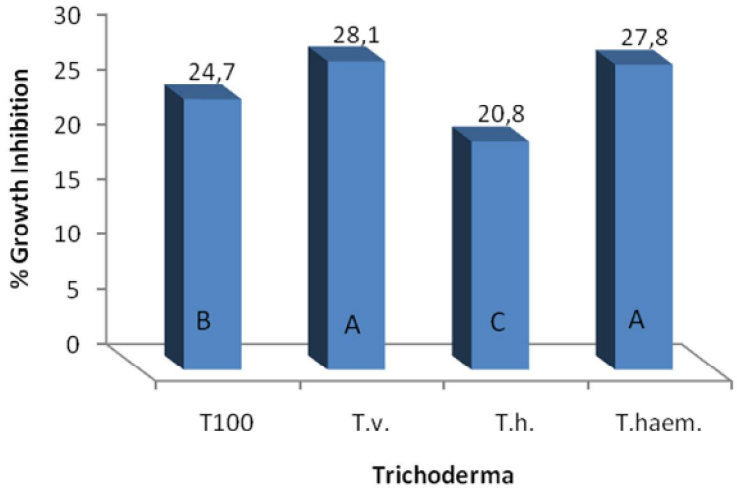

Figure 1 Inhibitory effect of *Trichoderma* species on colony diameter of *Fusarium proliferatum*, expressed as a percentage of the control. T100: *T. harzianum* strain T100, T. v.: *T. viride*, T. h.: *T. harzianum*, T.haem.: *T. haematum*.

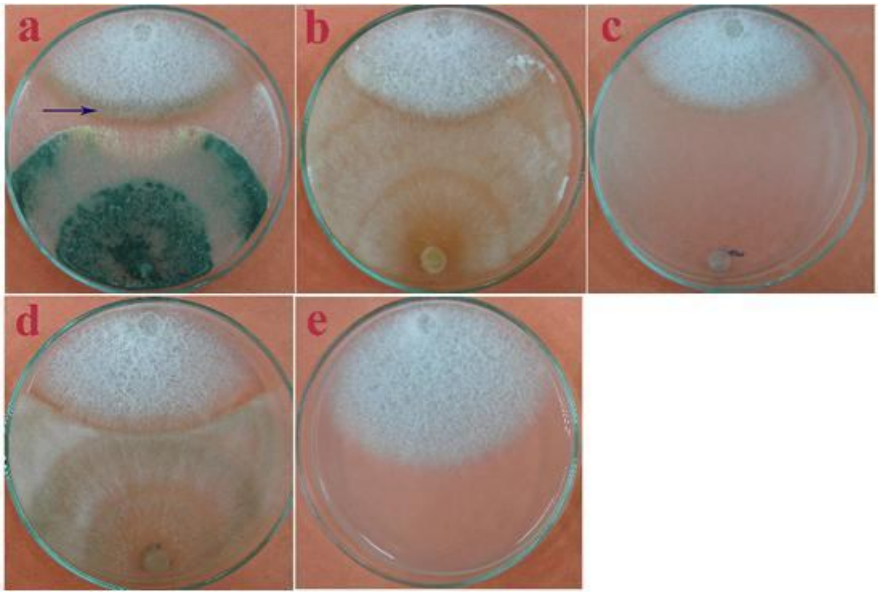

Figure 2 Effect of *Trichoderma* species on mycelial growth inhibition of *Fusarium proliferatum in vitro*, after four days incubation at 25 ± 1 ºC. *T. harzianum* strain T100 lysis of the *F. proliferatum* mycelia at the confronting colony edge, recognizable as brown color (a). The ability of *T. viride* just to overgrow *F. proliferatum* mycelia (b). *T. haematum* (c) and *T. harzianum* (d) unable to overcome *F. proliferatum* compared with control (e).

Pot experiment

Data in Table 1 indicate that all treatments amended with *G. mosseae* had no significant effect on seed germination, but improved the growth and increased plant survival. *Trichoderma* as single or combined with *G. mosseae*, at first stages of seedling growth, were ineffective on plant growth or health, but later on, the negative

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effect of *Trichoderma* on onion growth appeared and also its biocontrol effect was decreased. We also observed that the rate of onion growth in *Trichoderma* and mycorrhizae treatments was less than the average effect of either *Trichoderma* or mycorrhizae treatments. Also, the lowest seed germination occurred in the treatment of *Trichoderma* and mycorrhizae combined. Totally, the growth rate of *Trichoderma* treatments, with or without mycorrhizae, was less than mycorrhizae as single treatment (Fig. 3).

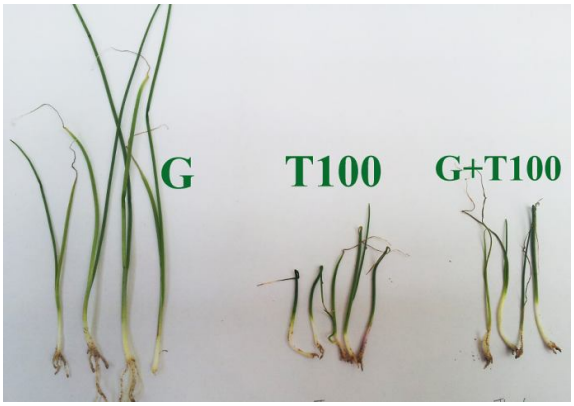

Figure 3. *Glomus* (G) and *Trichoderma* (T) effect on onion growth.

Inoculation of *F. proliferatum* caused 49.25%, 62% and 67% post-emergence damping-off and mortality compared to the uninoculated control plants on days 23, 30 and 36 after sowing, respectively. Inoculation of the soil with *Trichoderma*, significantly, reduced the disease severity at the early stages, but later its biocontrol ability declined from 25% to 3% and it was classified in the same group as the infected control plants. Surprisingly, an adverse result was gotten in infected treatments amended with mycorrhizae. Sice mycorrhizae controlled the disease at later stages. However, the best control of the disease was achieved in *Trichoderma* and mycorrhizae in combination that continued up to the end of the experiment.

The disease control achieved by Fungicide (Ortivatop), as seed treatment, ranged between 49.4% and 65.5% after 23 and 30 days of sowing, respectively. However, the disease control in *G. mosseae* and *Trichoderma* as combined treatment was more effective than fungicide during the experiment (data are not shown).

Mycorrhizal structures

All onions grown in AM infested soil were colonized by *G. mosseae*. In stained roots, mycorrhizal mycelia and chlamydospores were clearly observed in the cortex (Fig. 4). The aggregation of chlamydospores in various sections of the roots was different. Counting the chlamydospores, 40 days after sowing, in inoculated soil indicated that *G. mosseae* was able to proliferate in the soil successfully, as its density was increased from 80 propagules/ml, at the start of the experiment, to about 140 propagules/ml, in the pot soil at the end of the experiment.

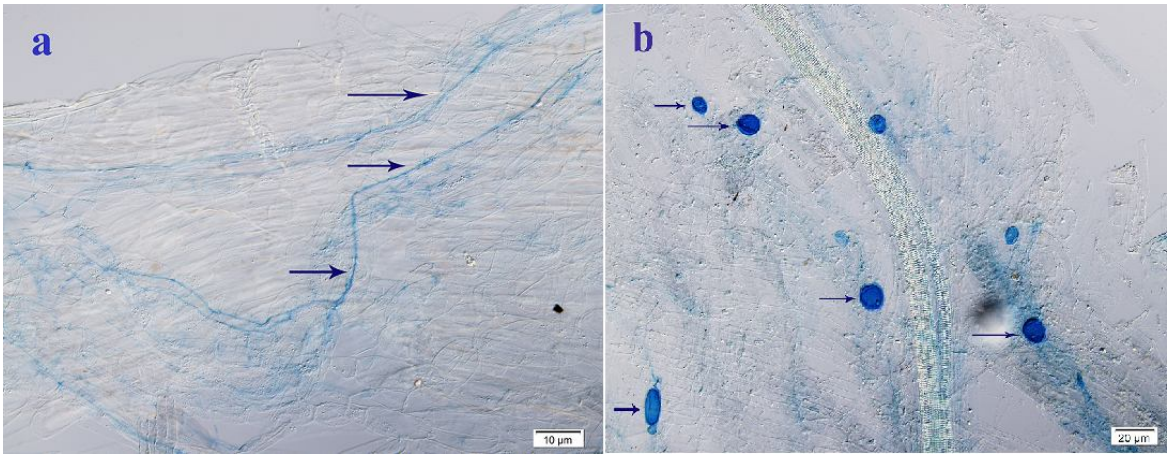

Figure 4 *Glomus* mycelia (a) and chlamydospores (b) in onion tissues.

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Discussion

Obligate biotrophic endosymbionts, Arbuscular Mycorrhizae, are known as plant growth promoting fungi through increasing uptake of mineral nutrients, especially phosphorus (Gill *et al*., 2002). So, as expected, *G. mosseae* improved onion seed germination and seedling stand in pot experiments. It is also reported that inoculation of mycorrhizae enhances the growth parameters of other vegetables such as lettuce (Kohler *et al*., 2006), tomato (Lingua *et al*., 2002) and onion cv. Yellow Granex (Brown *et al*., 2008). On the other hand, Mycorrhizal symbiosis can also increase the resistance of plants to root pathogens (Pozo *et al*., 2002; Vigo *et al*., 2001; Wacker *et al*., 1990). Our results also demonstrated the biocontrol effect of *G. mosseae* on *F. proliferatum*. However, its obvious effect was observed in later stages as, the functional establishment of the symbiosis between plant roots and mycorrhizal fungus took place as time went on (Koide and Elliott, 1989). Surprisingly, 36 days after sowing, the biocontrol ability of *G. mosseae* was decreased, which indicates that it has no impact (induction of defense mechanisms) on control of the disease when used alone (Srivastava *et al*., 2010).

Our results indicated the negative effect of *Trichoderma* on onion growth. Altintas and Bal (2008) reported that onion bulb and yield characteristics were not enhanced by *T. harzianum*, even at high dosages. The competition between plant root systems and microorganisms for nutrient (N) uptake and the possible role of volatile/non-volatile metabolites of antagonists can explain the negative effect of *Trichoderma* on plant growth in short (Hodge *et al*., 2000) and long-terms (McAllister *et al*., 1994), respectively. The reduction of nitrogen uptake in melon plants inoculated with *Trichoderma*, with or without mycorrhizae inoculation, is reported by Martinez-Medina *et al*. (2009). However, *Trichoderma* species are mostly known as biocontrol microorganisms as, in this research, it suppressed 25% of disease incidence in comparison with infected control, which corresponds to those of previous studies (Biswas and Das, 1999; John *et al*., 2010; Sivan and Chet, 1986; Šrobárová and Eged, 2005). Also, Ros *et al*. (2005) mentioned the ability of *Trichoderma* species to decrease *Fusarium* basal rot. However, the negative effect of *Trichoderma* on onion growth was the most likely reason for reduction of its biocontrol ability in later growth stages.

Finally, the percent of seed germination in combination treatments of *Glomus* + *Trichoderma*, was less than single treatments of *Trichoderma*. On the other hand, as mentioned before, onion growth in *Trichoderma* + *Glomus* treatments was less than the average growth in either *Trichoderma* or mycorrhizae treatments, that seems to be a direct consequence of the action of the antagonistic fungus on decreasing mycorrhizal root colonization. These results are in agreement with findings of Arriola *et al*. (2000), Mar Vázquez *et al*. (2000) and McAllister *et al*. (1994). However, it should be mentioned that the interaction between mycorrhizae and *Trichoderma* may be influenced by the species or even strains of either of the two fungi. As, root colonization level of melon plants with *G. intraradices*, *G. constrictum* and *G. claroideum* was increased by *T. harzianum* but not in *G. mossae*-inoculated plants (Martinez-Medina *et al*., 2009). Soluble exudates and volatile compounds produced by saprophytic fungi and also competition for space can be the causes of these findings (Calvet *et al*., 1992; Fracchia *et al*., 2004). The effect of the combined use of *Trichoderma* and *Glomus* on reducing the seedling mortality was confirmed as this combined treatment resulted in a general synergistic effect on disease control in pots. Obtained data agree with those reported earlier (Arriola *et al*., 2000; Datnoff *et al*., 1995; McAllister *et al*., 1994). In another study the combination of *Pseudomonas fluorescens*, *T. harzianum* and *G. intraradices* provided significantly better control of *Fusarium* wilt of

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tomato than with each one of the agents applied alone (Srivastava *et al*., 2010). However, combination use of biocontrol agents can sometimes provide no better or even worse biocontrol than single treatments (Singh *et al*., 2010).

In this study, we discovered that *T. harzianum* strain T100 and *G. mosseae* used in combination can control the onion basal rot better than when each one of them is used singly. However, owing to the negative effect of this *Trichoderma* strain on onion growth, application of other *Trichoderma* spp. is suggested for better results.

Acknowledgments

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کنترل بیولوژیک پوسیدگی فوزاریومی طبق پیاز با استفاده از *Trichoderma harzianum* و *Glomus mosseae*

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چکیده: قارچ *Fusarium proliferatum*، به‌عنوان یک قارچ توکسین‌زا، یکی از مهم‌ترین عوامل پوسیدگی قاعده پیاز (FBR) می‌باشد. در بین روش‌های کنترل بیماری، کنترل بیولوژیکی به‌عنوان یکی از مناسب‌ترین روش‌ها درنظر گرفته شده است. در این مطالعه، *Trichoderma harzianum* strain T100 به غلظت ۱×۱۰^{۶} cfu/g با خاک مایه‌زنی شده با *F. proliferatum*، به‌صورت مصنوعی، مخلوط شد. سه گرم از *Glomus mosseae* (حاوی ۸۰ پروپاگول/میلی‌لیتر) در بستر کشت به‌کار برده شد و در نهایت، تأثیر این عوامل بیوکنترل، به‌صورت تیمارهای ترکیبی و انفرادی، در کنترل پوسیدگی قاعده‌ای فوزاریومی و هم‌چنین کلنیزاسیون ریشه توسط *Glomus* مورد ارزیابی قرار گرفت. مایه‌زنی آربوسکولار مایکوریز رشد پیاز را به‌طور مؤثری بهبود بخشید، با این وجود کنترل بیماری در مراحل رشدی بعدی قابل مشاهده بود. شدت بیماری در خاک مایه‌زنی شده با *Trichoderma* تا ۲۵ درصد کاهش یافت اما تاثیر منفی تریکودرما در رشد پیاز توانایی بیوکنترلی آن را با گذشت زمان کاهش داد. میزان کلنیزاسیون ریشه با مایکوریز آربوسکولار در گیاهان مایه‌زنی شده با تریکودرما کاهش یافت. با این وجود، کنترل بیماری در تیمارهای ترکیبی *Glomus* و *Trichoderma* بهتر از سایر تیمارها بود، به‌طوری‌که کم‌ترین درصد بوته میری و بیش‌ترین درصد جوانه‌زنی بذر را به‌دنبال داشت. حتی میزان کنترل بیماری به‌صورت تیمار بذر با قارچ‌کش کم‌تر از ترکیب تریکودرما و *Glomus* بود.

واژگان کلیدی: بیوکنترل، *Fusarium proliferatum*، پیاز قرمز، *Trichoderma harzianum*، *Glomus mosseae*

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