Journal of Horticultural Research 2017, vol. 25(2): 67-74
DOI: 10.1515/johr-2017-0015

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EFFECTS OF *FUSARIUM VERTICILLIOIDES* AND *LACTOBACILLUS* STRAINS INOCULATION ON GROWTH AND ANTIOXIDANT ENZYMES ACTIVITY OF *ZEA MAYS* PLANTS

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Received: May 2017; Accepted: October 2017

Abstract

The current research based on greenhouse experiment evaluates the impact of the *Lactobacillus* strains (*Lactobacillus plantarum*, *Lactobacillus paralimentaris*, *Lactobacillus fermentum*, *Lactobacillus pentosus*, and *Lactobacillus buchneri*) previously isolated from maize silage on the *Fusarium verticillioides*-infected maize plants. The growth parameters as well as catalase, superoxide dismutase, ascorbate peroxidase, and peroxidase antioxidant enzymes activity were investigated in one-month old seedlings, after inoculations with *Fusarium* or co-inoculations with *Fusarium* and the *Lactobacillus* strains. Application of *Lactobacillus* strains in maize seedlings significantly enhanced the plant growth and biomass. The best effect was observed when the *L. buchneri* was applied. It was revealed that inoculation with *Fusarium* stimulated antioxidant enzyme activity and co-inoculation with *Lactobacillus* strains reduced the enzyme activity, compared to *Fusarium* treatment alone. This is the first report that revealed the bioprotective role of *Lactobacillus* strains against *F. verticillioides*.

Key words: biocontrol, *Fusarium* disease, lactic acid bacteria, maize, plant growth promotion

INTRODUCTION

Maize (*Zea mays* L.) is one of the most important and the third most traded cereal grain in the world (Pereira et al. 2011b). *Fusarium verticillioides* (Sacc.) Nirenberg (syn. *Fusarium moniliforme*) is known as one of the most frequent fungal pathogens in maize worldwide. In the suitable conditions, the pathogen induces root, stalk, ear, kernel, and seedling rot, which causes serious production losses. *F. verticillioides* secretes several toxins that are potentially toxic for humans and farm animals. The most important of these toxins produced by *F. verticillioides* are mycotoxins, the fumonisins (Oren et al. 2003), possessing carcinogenic effects (Pereira et al. 2011b). This species, in association with maize, can appear as both a pathogen or a symptomless intercellular endophyte, depending on diverse factors such as plant and fungal genotypes, environmental conditions, fungal inoculum size, and the presence of antagonists (Bacon et al. 2001; Pereira et al. 2011a). The contamination of maize and wheat fields with *Fusarium* strains, particularly *F. verticillioides* and *F. proliferatum*, is commonly reported (Mohammadi-Gholami et al. 2013). This contamination is a serious public health hazard because of the food spoilage and the presence of carcinogenic fumonisin B1 in high levels. Biological control of crops' disease and pets using microbial inoculants is being increasingly noticed as a feasible, ecofriendly alternative that limits the enormous use of the synthetic chemical pesticides (Gajbhiye & Kapadnis 2016; Oliveira et al. 2014; Pereira et al. 2011a).

Lactic acid bacteria (LAB) are a family of gram-positive, non-spore forming, cocci- or rod-shaped, catalase (CAT)-negative organisms (Patil et

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al. 2010). LAB have been widely and safely used in the food and feed industries as probiotics or starters during the past decades (Franz et al. 2010; Oliveira et al. 2014). Recently, some studies reported the antifungal activities of these bacteria against some plant pathogenic fungi (Gajbhiye & Kapadnis 2016; Gupta & Srivastava 2014; Kharazian et al. 2017; Kıvanc et al. 2014; Oliveira et al. 2014; Tropcheva et al. 2014; Varsha et al. 2014).

The resistance of plants to fungal colonization is often manifested by the hypersensitive reaction (HR) of challenged plant cells and the reactive oxygen species (ROS) production. It is the evidence of successful recognition of infection and activation of plant defenses. The excess of ROS causes damage to proteins, lipids, carbohydrates, DNA and finally results in cell death (Torres 2010). The role of the ROS family is that of a double-edged sword; while they act as secondary messengers in various key physiological phenomena, they also induce oxidative damages under several environmental stress conditions (Das & Roychoudhury 2014).

The induction of ROS-scavenging enzymes, such as superoxide dismutase (SOD), peroxidases (PODs), and CAT, are the most important and common mechanism for detoxifying ROS, synthesized during stress responses. These enzymes act by either the partial suppression of ROS production or the scavenging of the ROS already produced (Torres 2010).

Many references report the impact of *Fusarium* maize pathogens on antioxidative responses of the plants (García-Limones et al. 2009; Gherbawy et al. 2012; Sorahinobar et al. 2015), but there is no report on the effects of LABs as biocontrol agents on the antioxidant enzymes in the *Fusarium*-infected plants. So the objective of the present study was to evaluate the impact of the *Lactobacillus* strains previously isolated from maize silage (Kharazian et al. 2017) on the physiological responses and growth parameters of *Fusarium*-infected maize plants.

MATERIAL AND METHODS

Microbial strains

The *F. verticillioides* was kindly provided by the Maize & Forage Crops Research Department, Seed and Plant Improvement Institute (SPII), Karaj, Iran. This strain was previously isolated from diseased maize plants in the fields. For spore production, the fungus was grown in the Potato Dextrose Broth medium at 28 °C, and the spores were collected by filtration.

The *Lactobacillus* strains used in the present study were isolated from Iranian maize silages, and their high antifungal activities against some plant pathogenic fungi, including *F. verticillioides*, *Penicillium* sp., *Pythium aphanidermatum*, and *Verticillium dahliae* have been confirmed (Kharazian et al. 2017). The *Lactobacillus* strains used in the present work were *Lactobacillus plantarum* E2, *Lactobacillus pentosus* E4, *Lactobacillus paralimentaris* Q2, *Lactobacillus fermentum* Q4, and *Lactobacillus buchneri* (*sunkii*) Q6 with NCBI nucleotide sequence databases (https://www.ncbi.nlm.nih.gov) accession numbers KJ736725, KJ736733, KJ736727, KJ736732, and KJ736735, respectively (Kharazian et al. 2017).

The *Lactobacillus* strains were inoculated into De Man, Rogosa, and Sharpe (MRS**)** broth and cultivated overnight at 37 °C. Bacterial suspensions were centrifuged at 10,000 *g* for 20 min to remove the nutritional medium, and then they were washed twice with sterile water. The bacterial pellets were suspended in sterile water to the volume of about 10^8 (CFU/ml) and immediately used for inoculation of the seedlings.

In the treatment with the combination of five *Lactobacillus* strains, equal amount of each strain was given to the final concentration of about 10^8 (CFU/ml).

Plant material

The seeds of *F. verticillioides* – susceptible maize line K74/1 were kindly provided by the Maize & Forage Crops Research Department of SPII. The seeds were surface sterilized by bleach (5.25% sodium hypochlorite) for 10 min and then were rinsed several times in sterile water. The kernels beaker was placed in 60 °C water bath for 3 min, then the water was removed, and the kernels were transferred to a Petri dish and covered with water. For germination, the kernels were incubated in the dark for two days at 25 °C and then for two days at 4 °C (Bacon et al. 1994).

Table 1. Design of the greenhouse experiment

Treatment	Strains	
	Fungus (Pathogen)	Bacteria (Antagonists)
T1	-	-
T2	*F. verticillioides*	*L. plantarum* E2, *L. pentosus* E4, *L. paralimentaris* Q2, *L. fermentum* Q4, *L. buchneri* (*sunkii*) Q6
T3	-	*L. plantarum* (E2), *L. pentosus* E4, *L. paralimentaris* Q2, *L. fermentum* Q4, *L. buchneri* (*sunkii*) Q6
T4	-	*L. buchneri* (*sunkii*) Q6
T5	*F. verticillioides*	*L. buchneri* (*sunkii*) Q6
T6	*F. verticillioides*	Benomyl (Fungicide)
T7	-	Benomyl (Fungicide)
T8	*F. verticillioides*	-

Plant microbe interactions and growth parameters analysis

Eight different combinations of the *Lactobacillus* strains were used for inoculation of the maize seedlings under greenhouse conditions (Table 1). For the treatments 6 and 7, a solution of the fungicide benomyl (commercial powder Benlate) with a concentration of 100 $mg \cdot ml^{-1}$ was added to the soil substrate for controlling *Fusarium*.

Maize seedlings with aerial parts of 2.0–3.5 cm in length were placed in Petri dishes together with *Lactobacillus* strains suspensions and left for 4 h at 25 °C and then 4 h with *F. verticillioides* spore suspensions and then transferred to pots in the greenhouse. In the control samples, the seedlings were soaked in water instead of bacterial suspensions. The greenhouse experiment was conducted in pots containing a mixture of 40% peat, 30% loam, 20% vermiculite, and 10% compost. There were three seedlings per pot and three pots for each treatment. The greenhouse temperature was 25–27 °C with 12-h photoperiod. The plants were irrigated twice in a week. After one month, the maize plants were harvested. Then different growth parameters, including shoot and root length and fresh and dry weights of plants were measured.

Measurement of enzymes activity

To prepare crude enzyme extracts, fresh leaves (0.05 g) were ground with 2 ml of 0.1 M cool phosphate buffer (pH 6.8) as described by Kar and Mishra (1976). The obtained homogenate was then centrifuged at 15,000 *g* for 15 min at 4 °C. The clear supernatant was used for assaying the enzyme activities.

CAT activity was determined by monitoring the destruction of H_2O_2 at 240 nm. The reaction mixture in a final volume of 3 ml contained 50 mM phosphate buffer (pH 6.8), 100 µl enzyme extract, and 15 mM H_2O_2. The decrease in absorbance at 240 nm was recorded with a spectrophotometer (Shimadzu UV-160) (Aebi 1984). The POD reaction mixture in a final volume of 3 ml contained 20 mM guaiacol, 25 mM phosphate buffer (pH 6.8), 40 mM H_2O_2, and 10 µl of the crude enzyme extract. The increase in absorbance at 470 nm because of tetra-guaiacol formation was recorded spectrophotometrically (Chance & Maehly 1955). Superoxide dismutase (SOD) activity was measured by using the photochemical nitro blue tetrazolium (NBT) method (Beauchamp & Fridovich 1971). The SOD reaction mixture in a final volume of 1 ml contained 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 20 µl of the extract, 75 µM NBT, 13 mM methionine, and 4 µM riboflavin. One unit of SOD was defined as the quantity of enzyme required to inhibit the reduction of NBT by 50%. Total ascorbate peroxidase (APX) activity was measured spectrophotometrically by detecting the absorbance at 290 nm during oxidation of ascorbic acid, using the method described by Nakano and Asada (1981). One milliliter of the reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.45 mM l-ascorbic acid, 0.3 mM H_2O_2, and 30 µl of the extract. One unit of APX was defined as the quantity of enzyme required to consume 1 µM of substrate.

Statistical analysis

The experiment was carried out in three replications. Analysis of variance, average comparing, and treatment groups score were obtained by using SAS (version 9.1) and the Duncan's Multiple range tests ($P < 0.05$).

RESULTS AND DISCUSSION

In the current study, we have made the greenhouse experiment to evaluate the impact of inoculations of the *F. verticillioides* with or without *Lactobacillus* strains (as biocontrol agent) on maize seedlings growth and antioxidant enzymes activity. Our previous data from *in vitro* experiments showed that all selected *Lactobacillus* strains can inhibit growth of *F. verticillioides* (Kharazian et al. 2017).

Root lengths increased in the seedlings that were soaked in suspensions of *Lactobacillus* strains (Figs. 1 and 2A). The treatments 2 (*Fusarium* + all 5 *Lactobacillus* strains) and 5 (*Fusarium* + *L. buchneri*) caused the longest roots (24 cm) compared to the control (T1) (11 cm). The treatment 6 (fungicide + *Fusarium*) resulted in the shortest roots, which was similar to that of the control with *Fusarium.* The root weight increased in all *Lactobacillus*-containing treatments (with or without *Fusarium* inoculation) compared to the control (Fig. 2B). The maximum root weight, 1.854 and 1.729 mg, belonged to the treatments 3 (all 5 *Lactobacillus*) and 4, respectively. The lowest root weight was recorded in the *Fusarium* treatment (81 mg fresh weight). The treatments 3 and 5 caused significant increase in the shoot length and weight, compared to the control (Fig. 3A and 3B). However, other treatments did not show any significant differences in shoot lengths compared to the control. Fresh weight of the shoots in all *Lactobacillus* treatments (also in the co-inoculation of *Lactobacillus* with *Fusarium*) was significantly bigger than that of the control, while dry weight of the shoots was higher only in treatments 3 and 5.

The effect of *Lactobacillus* strains on plant growth was previously described by Hamed et al. (2011), Limanska et al. (2013), and Narasimha Murthy et al. (2012). The positive effect of *Lactobacillus* strains inoculation on shoot growth and lateral root number was reported by Hamed et al. (2011). According to Limanska et al. (2013), the physiological response of seedlings for inoculation with suspensions of *Lactobacillus* depends on the tested strain.

Fusarium caused a significant increase in the activity of all enzymes (Fig. 4). All the treatments with microorganisms have increased POD and SOD activities compared to the control (Fig. 4A and 4B). The activity of APX and CAT was higher after inoculations with the mixture of bacteria and with *L. buchneri* (Fig. 4C and 4D).

The above results are in agreement with Gherbawy et al. (2012), who demonstrated that *F. moniliforme* inoculation resulted in enhanced activity of antioxidant enzymes (SOD, CAT, and APX) in the wheat shoots. Meanwhile, Pereira et al. (2011a) demonstrated that inoculation of maize seeds with *F. verticillioides*, either alone or co-inoculated with the *Bacillus*, resulted in enhanced SOD activity. The chances of oxidative burst and programmed cell death are minimized because of the enhanced antioxidant enzymes activity. As a result, *F. verticillioides* can be protected from the oxidative damage during colonization (Kumar et al. 2009). Another interesting result of this study was that the antioxidant enzymes activity are decreased in plants that were co-inoculated with *F. verticillioides* and *Lactobacillus* strains as compared to plants inoculated with *F. verticillioides* only. Previously, two characteristics, including antagonistic effects against plant pathogenic fungi (Yan et al. 2017; Russo et al. 2017; Guo et al. 2012) and also high antioxidant activity, ROS scavenging and inhibition of the production of free radicals have been reported for different *Lactobacillus* species (Virtanen et al. 2007; Xing et al. 2015).

Our experiments confirmed the high antifungal activity of the selected *Lactobacillus* strains against *F. verticillioides*, which may be caused by the secretion of antifungal substances by *Lactobacillus* strains. Some of the known secreted substances by *Lactobacillus* strains are cyclic dipeptides, proteinaceous compounds, organic acids, fatty acids, nisin, and reuterin (Crowley et al. 2013; Gajbhiye & Kapadnis 2016; Limanska et al. 2013). Further experiments are needed to determine how *Lactobacillus* strains prevent *F. verticillioides* infection.

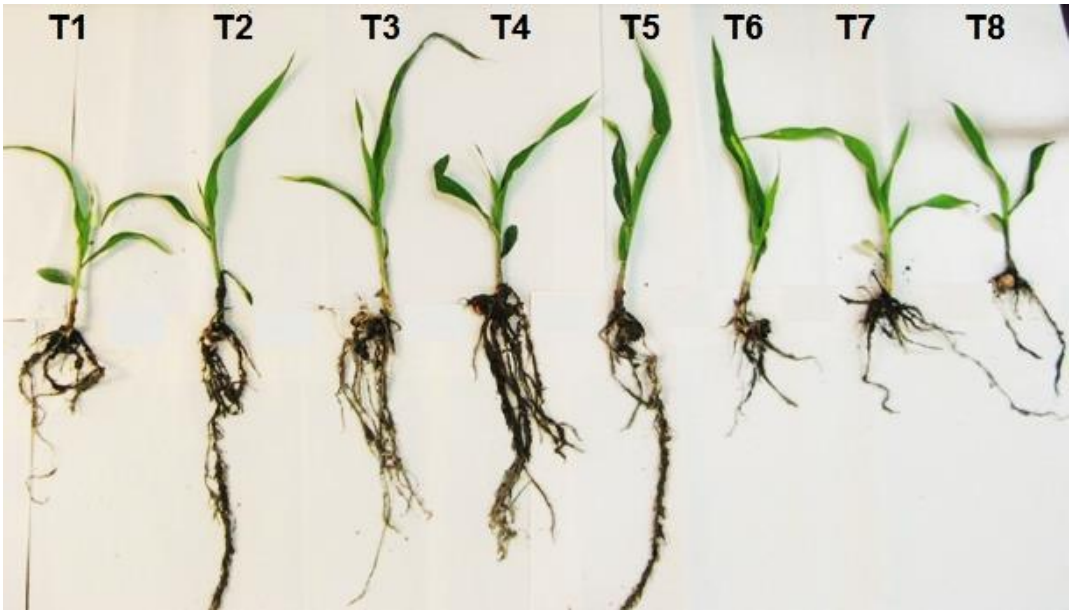

Fig. 1. The effect of *Lactobacillus* strains and *Fusarium verticillioide*s inoculation on maize seedlings growth in the greenhouse for 4 weeks. T1: control, T2: *F. verticillioides* + *L. plantarum* + *L. pentosus* + *L. paralimentaris* + *L. fermentum* + *L. buchneri*, T3: *L. plantarum* + *L. pentosus* + *L. paralimentaris* + *L. fermentum* + *L. buchneri*, T4: *L. buchneri*, T5: *F. verticillioides* + *L. buchneri*, T6: *F. verticillioides* + fungicide, T7: fungicide, T8: *F. verticillioides*

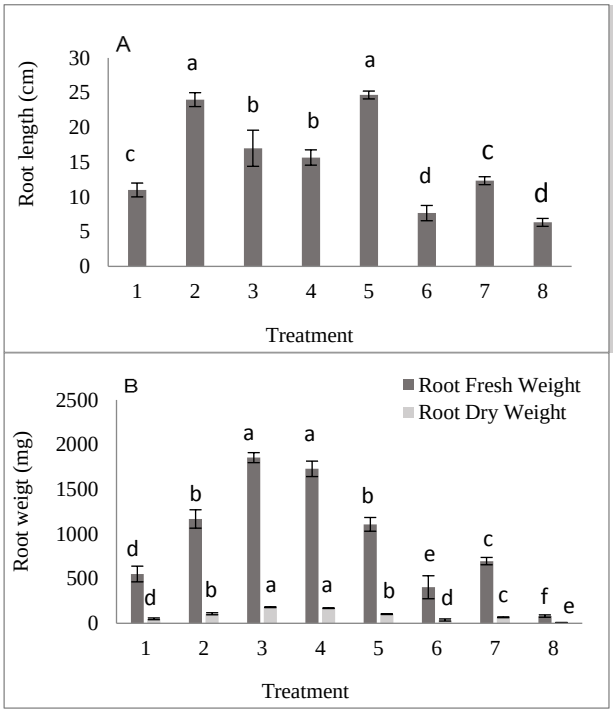

Fig. 2. A) Root length and B) root fresh and dry weight of maize seedlings inoculated with *Lactobacillus* strains and *F. verticillioides* after 4 weeks of growth in the greenhouse. The results are the means of three replicates of experiment ± SE. Different letters above the columns indicate significant differences between treatments (P ≤0.05) according to Duncan's multiple range tests. For treatments see Table 1 and Fig. 1.

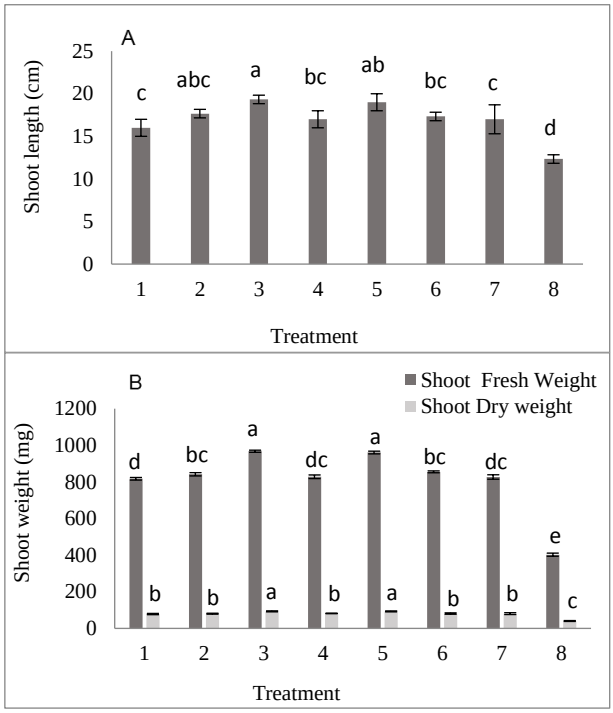

Fig. 3. A) Shoot length and B) shoot fresh and dry weight of maize seedlings inoculated with *Lactobacillus* strains and *F. verticillioides* after 4 weeks of growth in the greenhouse. The results are the means of three replicates of experiment ± SE. Different letters above the columns indicate significant differences between treatments (P ≤ 0.05) according to Duncan's multiple range tests. or treatments see Table 1 and Fig. 1.

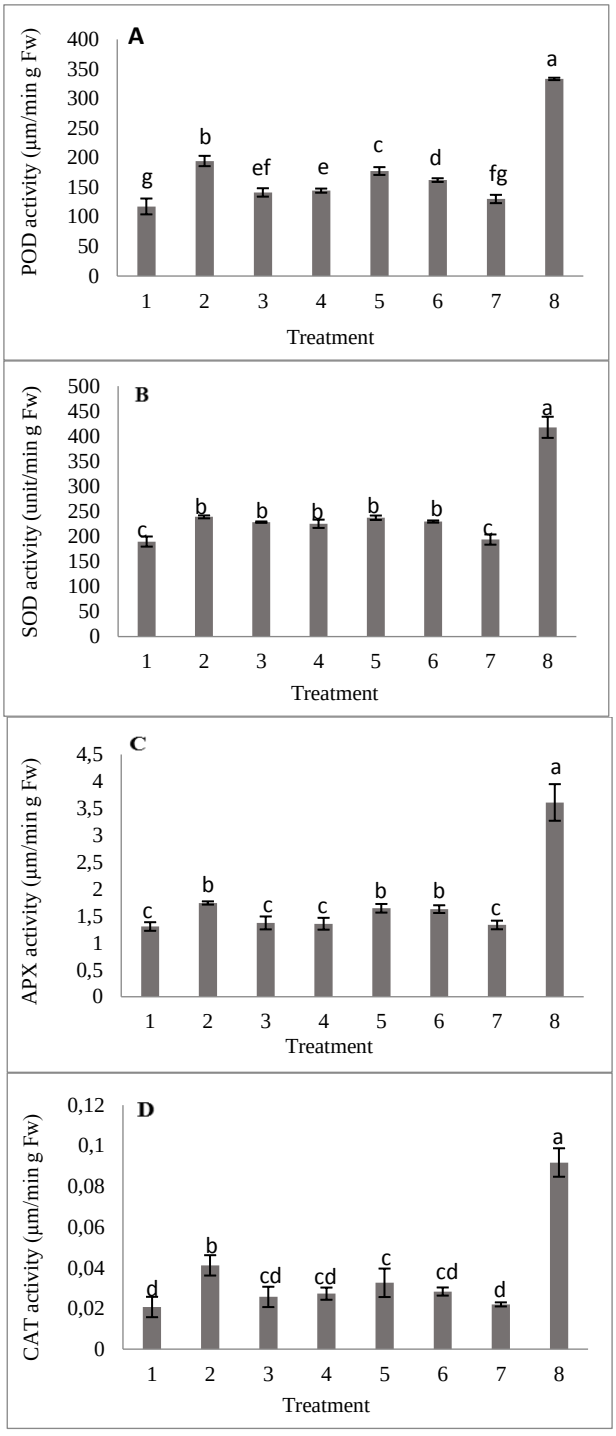

Fig. 4. A) Peroxidase (POD), B) superoxide dismutase (SOD), C) ascorbate peroxidase (APX) and D) catalase (CAT) activities in shoots of maize seedlings inoculated with *Lactobacillus* strains and *F. verticillioides* after 4 weeks of growth in the greenhouse. The results are the means of three replicates of experiment ± SE. Different letters above the columns indicate significant differences between treatments ($P \leq 0.05$) according to Duncan's multiple range tests. For treatments see Table 1 and Fig. 1.

CONCLUSION

The current results of the greenhouse experiment on maize seedlings suggest that studied *Lactobacillus* strains may have potential to be used as biocontrol agents. Field experiments are needed to propose using of these strains in crop farming.

Acknowledgment

We thank the Golestan University Deputy of Research and Office of Higher Education for the financial support to Zohreh Akhavan Kharazian in the form of grants for PhD research project.

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