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Effects of some Iranian *Trichoderma* isolates on maize seed germination and seedling vigor

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Trichoderma species are commonly used as biological control agents against phytopathogenic fungi and some of their isolates are able to improve plant growth. In the current study, we evaluated some Iranian *Trichoderma* isolate cultural filtrates as well as their direct effect on seed germination and seedling vigor of maize (*Zea mays* L. cultivar B73); they are *Trichoderma harzianum* T 969, *T. harzianum* T 447, *Trichoderma hamatum* T 614, *Trichoderma roseum* T678, *Gliocladium virens* G525 and the unknown *Trichoderma* species isolate (*Trichoderma* sp. T) obtained from the soil of Moghan Area, Ardabil Province of Iran. The culture filtrates reduced the speed of seed germination, but no influence ($p \geq 0.05$) was recorded at the final rate of the seed germination. When maize seeds were exposed to the *Trichoderma* spore suspension, all the *Trichoderma* isolates colonized the seed surface and inhibited the seed germination. The maize seed potted in inoculated soil did not emerge from the soil among all tested *Trichoderma* isolates, except for non-inoculated soil 30 days after potting. When *Trichoderma* conidia were added on the surface of soil near the emerged seed, significant ($p \leq 0.01$) decrease of seedling treated separately with *T. hamatum* T614 isolate was observed on the leaves area, fresh root and shoot weight, compared to the non-inoculated seedling. Whereas *T. hamatum* T447 and *T. harzianum* T969 reduced markedly ($p \leq 0.01$), seedling fresh root weight and fresh shoot weight were respectively increased as compared to the control. *Trichoderma* isolates had no influence on chlorophyll content in leaves and root length as well as stomata conductivity, except for *Trichoderma* spp. isolate T that increased stomata conductivity of seedling significantly ($p \leq 0.01$).

Key words: *Trichoderma* spp., maize, seed germination, seedling vigor.

INTRODUCTION

Trichoderma spp., which are common saprophytic filamentous fungi in almost any soil and rhizosphere microflora, are well recognized as biocontrol agents against various plant pathogenic fungi that caused a lot of soil-born, air-born and post-harvest diseases in several crops (Hajieghrari et al., 2008; Howell, 2003) for more than 75 years (Weindling, 1932). In addition to biocontrol ability, some *Trichoderma* species are able to promote plant growth (Hoyos-Carvajal et al., 2009; Shanmugaiah et al., 2009; Harman et al., 2004; Ousley et al., 1994; Baker, 1989; Baker, 1988) directly by colonization of rhizosphere change in microflora competition on roots and invade the superficial layers of root cortex (Yedidia et al., 1999) as opportunistic rhizosphere competence (Ahmad and Baker, 1987) and plant symbiont (Harman, 2006). Also, there is exudation of plant growth stimulating factors and

phytohormones such as indol acetic acid (IAA) and their analogs (Vinale et al., 2008a; Cutler et al., 1989; Windham et al., 1986), and vitamins (Inbar et al., 1994; Kleifeld and Chet, 1992). Production of some organic acids such as gluconic citric and/or fumaric acids (Vinale et al., 2008b) reduces the soil pH resulting in solubilization of phosphates. Micronutrients and minerals such as Fe, Mn and Mg have important role in plant growth and secretions of diffusible metabolites such as exogenous enzymes and siderophores (Jalal et al., 1987) leads to solubilization and increase in some nutrients resulting in stranger nutrient uptake of plant (Chet, 2001), as well as indirectly controlling the minor root infested pathogens (Harman et al., 2004). Moreover, *Trichoderma* spp. induces localized and systematic resistance to a variety of plant pathogens (Hoitink et al., 2006; Honson

and Howell, 2004; Yedida et al., 1999). The effects of *Trichoderma* isolates on plant growth and development have important economical implications such as shortening the plant growth period and time in nursery, as well as improving plant vigor to overcome biotic and/or abiotic stresses, resulting in increase plant productivity and yields. Therefore, the use of *Trichoderma* isolates in plant growth improvement is crucially important in sustainable agriculture system, because chemical fertilizer is not economical in the long run due to their cost and environmental pollution. Accordingly, reduction or elimination of synthetic fertilizer applications in agriculture is highly desirable. There are many abiotic and biotic factors such as plant species, the strain of *Trichoderma* used, the form of applied inoculum and its concentration and the soil environment as well as the rhizosphere microflora that may have an influence on *Trichoderma* activity. Therefore, it is very important to collect information about these factors. There have been many studies aimed at the characterization of biological and antagonistic aspects of *Trichoderma* spp. against plant pathogens and inhibition of the pathogen development by parasitism, competition and antibiosis (Verma et al., 2007; Howell, 2003). Recently, several attempts have been undertaken to survey *Trichoderma* spp. promotion of seedling establishment, enhancement of plant growth and elicit plant defense reaction in some crops such as cotton (Shanmugaiah et al., 2009), vegetables (Celar and Valic, 2005; Rabeerdran et al., 2000; Inbar et al., 1994; Lynch et al., 1991), bean (Hoyos-Carvajal et al., 2009) and corn (Windham et al., 1989).

The aim of this study is to evaluate some Iranian *Trichoderma* isolates on maize seed germination and seedling vigor through the analysis of some indicating factors such as germination rate, root elongation, plant height and fresh weight of the seedlings as well as chlorophyll content, stomata conductivity and leaves area.

MATERIALS AND METHODS

The experiment was carried out at the Biology Laboratory of Plant Production Department, Moghan Junior College of Agriculture, University of Mohaghegh Ardabili, in Ardabil, Iran during 2009.

The *Trichoderma* isolates selected for this study were obtained from collection of *Trichoderma* spp. in the Plant Pest and Disease Institute, Tehran, Iran; they are *Trichoderma harzianum* T 969, *T. harzianum* T 447, *Trichoderma hamatum* T 614, *Trichoderma roseum* T678, *Gliocladium virens* G525 and the unknown *Trichoderma* species isolate (*Trichoderma* spp. T) obtained from the soil of Wheat field in Moghan Area, Ardabil Province, Iran. The isolates were grown on potato dextrose agar (PDA, BDH Ltd, UK 39 g/l) medium, maintained on PDA medium and stored at 4°C for further use. Also, *Zea mays* L. cultivar B73 seed was used in the experiments.

To evaluate the influence of the *Trichoderma* metabolites on maize seed germination, five discs of mycelia agar plugs (5 mm diameter) were removed from the margin of 7 days old of each isolates colony by No. 3 cork borer. *Trichoderma* isolates were inoculated into 100 ml nutrient media (MgSO₄·7H₂O, 0.2 g/l, NH₄NO₃ 1 g/l, K₂HPO₄ 0.9 g/l, KCl 0.15 g/l, FeCl₂ 0.002 g/l, ZnSO₄

0.002 g/l and glucose 5 g/l in double distilled water (Celar and Valic, 2005)) sterilized (autoclaved at 121°C for 18 min) in 250 ml conical flasks and incubated at 25±1°C on a rotary shaker set at 100 rpm for 14 days. The control conical flasks were inoculated with five discs of 5 mm diameter sterile PDA medium. After incubation, the culture was filtered through millipore filter in order to remove mycelia mats and then sterilized by passing through 0.2 µm pore biological membrane filter (FP30/0.2 CA-S, Schleicher and Schuell Micro Science GmbH). One hundred maize surface seeds were disinfected by immersion in 0.5% hypochlorite sodium (NaClO) for 5 min before being rinsed and washed thoroughly in sterile distilled water thrice in a laminar air flow cabinet, placed on a sterile blotting paper in sterile dishes and watered with 10 ml of the filtrate. Two control sets were conducted. In one of the control treatments, 10 ml sterile distilled water and 10 ml non-inoculated medium in the other control were used. The Petri dishes were then sealed with parafilm and incubated in a growth chamber at 25±1°C and 80% relative humidity. Percentage of seed germination was counted 48, 72 and 96 h after incubation as well as the rootlet and coleoptiles length was measured after 96 h passed. The experimental design used was completely randomized (CRD) with four replicates.

In order to evaluate the effect of *Trichoderma* directly on seed germination, the surface disinfected seeds were soaked in the *Trichoderma* conidia suspension. For preparation of spore suspension, the 5 mm diameter mycelia disc of 7 days-old culture obtained from the margin of each *Trichoderma* isolate was centrally placed on the surface of 100 ml PDA in a 250 ml conical flask and incubated at 25±1°C for 7 days. In the control treatment, a 5 mm-diameter sterile PDA medium disc was placed on the surface of the medium. After the incubation period, 30 ml of double distilled water (ddH₂O) was added to each conical flask and shaken on a rotary shaker set at 80 rpm for 30 min. The concentration of *Trichoderma* spores in ddH₂O was counted using haemocytometer and adjusted to 10⁶ - 10⁷ spores per ml. Inoculated seeds were placed on a sterile blotting paper in sterile Petri dishes and incubated at 25±1°C in growth chamber after sealing with parafilm. Percentage of seed germination was counted 48, 72 and 96 h after incubation as well as the rootlet and coleoptile length was measured after 96 h. The means were analyzed by analysis of variance (ANOVA) and Fischer's protected LSD at 1 and 5% significant level and was used for separation of means with SAS software (SAS (1985) Institute Inc., Cary, NC, USA).

In order to reveal the effect of *Trichoderma* isolates on maize seedling growth directly, two inoculate applications were evaluated. In one set experiment, inoculums were presented after planting via spore presentation and in another set, the seeds were potted in inoculated soil. In the first experiment, three maize surface disinfected seeds (soaked in 0.5% hypochlorite sodium (NaClO) for 5 min then rinsed and washed thoroughly in sterile distilled water 3 times) were sown in each pot. Two weeks after potting, when the seedling was in two leaves stage, 10 ml spore suspension (prepared as mentioned above) was added on the surface of the soil near the emerged seedling. In the control pots, seed was watered with non-inoculated medium distilled water. One month after inoculating, plant response parameter such as total chlorophyll content and stomata conductivity were measured in each plant via chlorophyll meter (Model: SPAD 502 Konika Minolta Sensing Inc, Japan) and stomata conductivity (Model: SC-1 Eijkelpamp, Netherland) instruments respectively, as well as plant height, root height, aerial weight, root weight and total leaf area (Model: Li 3100, Area meter Licor Lincon Nebraska, USA), after uprooting the plants and washing them under running tap water to remove residual soil from the roots. In the second experimental set, *Trichoderma* inoculums were prepared by inoculation of sterilized wheat grains in 1 L conical flask (autoclaved at 121°C for 20 min twice with 24 h distance) by 5 mm *Trichoderma* isolate mycelia plug, before incubating at 25±1°C in a growth chamber for 14 days and then were mixed with soil in 1:10 w/w rate before seed potting. In the

Table 1. Effect of *Trichoderma* cultural filtrate on maize seed germination.

Treatment	%Seed germination after 48 h	%Seed germination after 72 h	%Seed germination after 96 h	Length of rootlet (mm after 96 h)	Length of coleoptile (mm after 96 h)	Length of rootlet / Length of coleoptile
Non-treated (Control 1)	31.1 ± 3.23 ^{ab**}	85.17 ± 2.05 ^{ab*}	90.98 ± 0.90 ^{a*}	18.4 ± 0.46 ^{ab**}	4.7 ± 0.23 ^{a*}	3.91 ± 0.09 ^{a**}
Non-inoculated medium (Control 2)	42.29 ± 2.48 ^a	92.43 ± 3.05 ^a	94.37 ± 2.69 ^a	22.03 ± 3.27 ^a	5.3 ± 0.36 ^a	4.15 ± 0.57 ^a
<i>Trichoderma</i> sp T	13.42 ± 2.49 ^c	86.70 ± 5.02 ^{ab}	88.47 ± 5.06 ^a	12.28 ± 0.3 ^{bc}	5.26 ± 0.25 ^a	2.35 ± 0.17 ^{bc}
<i>T. harzianum</i> T969	31.187 ± 4.03 ^{ab}	88.81 ± 2.91 ^{ab}	94.59 ± 0.93 ^a	16.97 ± 1.6 ^{ab}	5.13 ± 0.33 ^a	3.34 ± 0.42 ^{bc}
<i>T. hamatum</i> T614	20.78 ± 4.27 ^{bc}	80.28 ± 2.86 ^b	88.97 ± 3.32 ^a	8.2 ± 1.36 ^c	6.16 ± 0.74 ^a	1.44 ± 0.44 ^c
<i>T. harzianum</i> T447	30.22 ± 2.10 ^{ab}	80.37 ± 1.72 ^b	88.30 ± 2.72 ^a	11.93 ± 1.32 ^{bc}	7.39 ± 1.36 ^a	1.73 0.40 ^c
<i>T. roseum</i> T678	26.20 ± 0.178 ^{bc}	80.06 ± 3.21 ^b	90.81 ± 1.70 ^a	12.96 ± 1.30 ^{bc}	5.63 ± 0.35 ^a	2.32 ± 0.34 ^{bc}
<i>Gliocladium virens</i> G525	27.24 ± 4.78 ^b	85.78 ± 3.5 ^{ab}	90.88 ± 2.26 ^a	7.4 ± 1.16 ^c	7.22 ± 2.18 ^a	1.11 ± 0.15 ^c
LSD	13.42	9.52	8.27	6.54	2.92	4.03

Values with the same letter within the column are not significantly different (** P≤0.01, * P≤ 0.05) according to Fischer's protected LSD test; results are means of four replicates for each treatment; the value in parentheses is the standard error of the mean.

control treatments, the non-inoculated autoclaved wheat grains were used. One month after planting, plant response was evaluated through the above mentioned parameters. In both experiments, four replicates were conducted through completed randomized design (CRD). The means of the parameter from four replicates were statistically analyzed by ANOVA, followed by Fischer's protected LSD at 1 and 5% significant level with SAS software (SAS Institute Inc., Cary, NC, USA) to separate the means.

RESULTS AND DISCUSSION

Trichoderma spp. has long been known for their ability in the control of numerous plant pathogens (Verma et al., 2007; Howell, 2003). Recently, considerable efforts have been undertaken to study plant growth promotional activity of *Trichoderma* spp. (Hoyos-Carvajal et al., 2009; Harman et al., 2004; Lo and Lin 2002; Babeendean et al., 2000; Baker, 1988). *Trichoderma* species have evolved multiple mechanisms that are resulting in the improvement of seed germination and seedling vigor (Zheng and Shetty, 2000). *Trichoderma*

metabolites can influence the seed germination and seedling emergence (Clear and Valic, 2005). In this study, *Trichoderma* culture filtrates reduced maize seed germination rate significantly ($p \leq 0.01$) in comparison with non-inoculated medium treatment as a real control (rather than distilled double water treatment) at 48 and 72 h after potting in sterile blotting paper, except for *T. harzianum* T447 and *T. harzianum* T 696 (Table 1). *Trichoderma* sp. isolate T cultural filtrate reduced ($p \leq 0.01$) maize seed germination rate more than the other isolates at the first counting date. However, in the second counting date (72 h after potting), *T. hamatum* T614, *T. harzianum* T447 and *T. roseum* T678 cultural filtrates reduced markedly ($p \leq 0.05$) seed germination rate in relation to the controls (Table 1). In all the treated maize seeds with the *Trichoderma* culture filtrate, the metabolites affected the first and second count and no influence ($p \geq 0.05$) was recorded at the final rate of the seed germination by treating with *Trichoderma* isolates. The earlier germination of seed can be affected by the seedling height

and weight due to the short time for growing well. Negative efficacy of *Trichoderma* secondary metabolites was in agreement with Menzies (1993) reports that show *T. viride* cultural filtrate on cucumber, pepper and tomato seedling germination. Also, Celar and Valic (2005) showed significant inhibition of onion seed germination by *T. viride* culture filtrate, as well as in onion, chicory and lettuce seeds germination by cultural filtrate of *T. koningii*, which potted seeds on sterile blotting paper. On the other hand, cultural filtrates of some *Trichoderma* isolates were reported as stimulant seed germination in some seeds (Gupta and Sharma, 1995). Meanwhile, tested *Trichoderma* culture filtrate significantly decreased the length of the seedling roots 96 h ($p \leq 0.01$) after potting in sterile blotting paper except for *T. harzianum* T969; but no significant effect was observed on shoot length by treatment with any of the culture filtrates. *T. hamatum* T614 and *G. virens* G525 decreased the length of the seedling root more than other *Trichoderma* isolates. Also, the *Trichoderma* isolates decreased markedly ($p \leq 0.01$) in

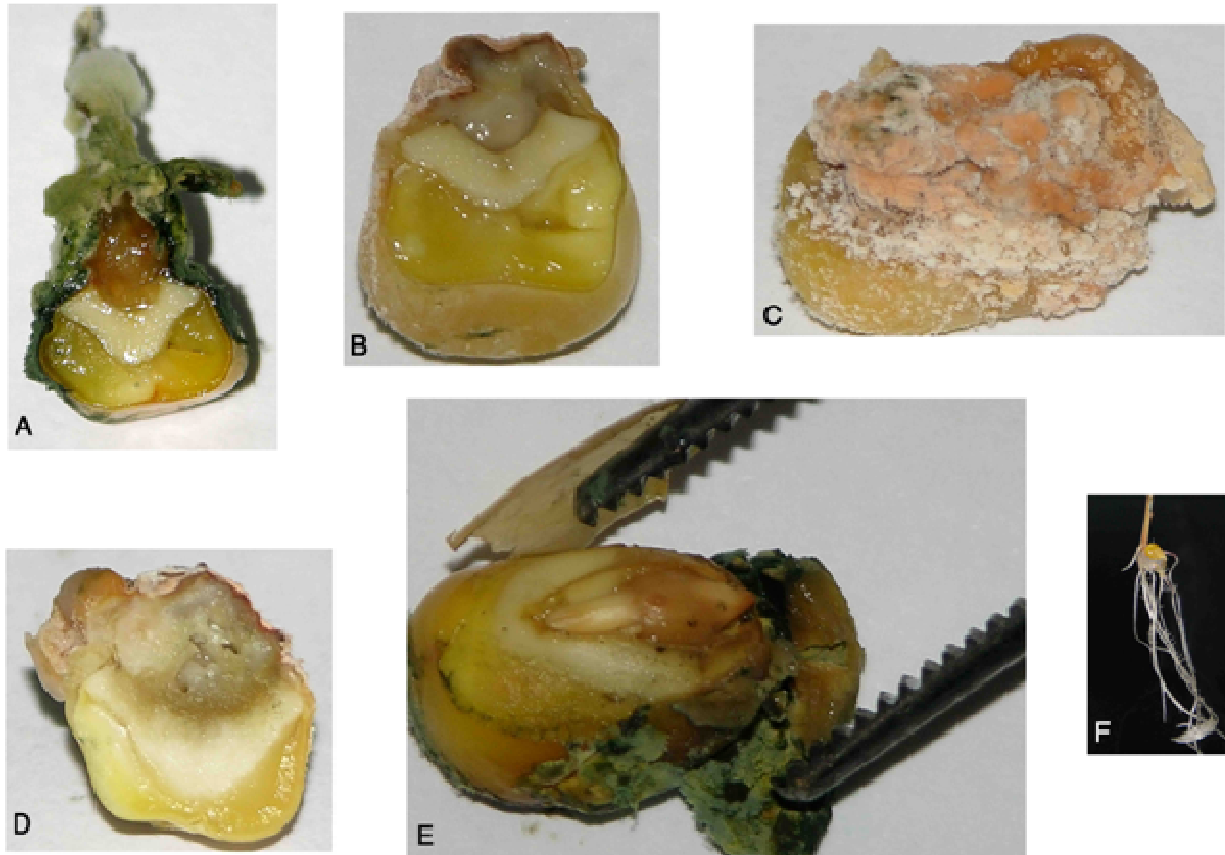


Figure 1. Injured seed, emerged shoot and rootlet by *Trichoderma* isolates when maize seeds were exposed with spore suspension on the sterile blotting paper for 14 days. **A**, *Trichoderma harzianum* T969; **B-D**, *T. roseum* T 678; **E**, *T. harzianum* T447 in comparison to the control (**F**).

the root- shoot ratio (length base) when compared with controls (Table1).

Available literature demonstrated that *Trichoderma* isolates produce *Trichoderma* species/strain-specific auxin-like and/or auxin inducer compounds that have inhibitory effect at the higher concentration than optimal doses (Vinale et al., 2008a; Vinale et al., 2008b). Therefore, the optimum concentration of such compounds in seed inducing fast germination and/or in seedling development may depend on plant species as well as the type of compounds produced as secondary metabolites. These require further study to determine the aspects for each *Trichoderma*-plant interactions. Menzies (1993) also demonstrated that the inhibition factor/factors in the cultural filtrate are heat stable and can not be removed by autoclaving.

In our study, when maize seeds were exposed to the *Trichoderma* spore suspension (10^6 - 10^7 spores per ml), we observed another scenario in which the *Trichoderma* colonized the seed and/or emerged rootlet and shoot surface and inhibited the seed germination as well as rootlet and shoot development. Also, treated *Trichoderma* were re-isolated from injured seed and seedling. This colonization among *T. hamatum* T614, *G. virens* G525

and *Trichoderma* sp. T were found superficially and about *T. harzianum* T969, *T. harzianum* T 447 and *T. roseum* T678 were observed. Necrosis region in the inner layer of seed and/or emerged rootlet and shoot, indicates ability to hasten growth on seed and root exudates substrate, secretion of toxic metabolite(s) and/or penetrate the *Trichoderma* into the tissue as parasite function rather than symbiotic (Figure 1). Although *Trichoderma* spp. has rarely been regarded as a parasite, there are several papers demonstrating pathogenicity of *Trichoderma* spp. to germinating maize seed and seedlings (Mc-Fadden and Sutton, 1975; Sutton, 1972), as well as other seeds (Menzies, 1993). Yedidia et al. (1999) indicated that *Trichoderma* isolates are able to penetrate the root epidermis but without causing extensive damage. The hyphae are stopped probably by the deposition of callose by the surrounding cells. The results present here, indicated that the *Trichoderma* isolates are not able to stimulate the seedling defense response and/or maize seedling does not respond more rapidly and efficiently to pathogen attack leading to damaging effect on seedling. However, it does not only depend on *Trichoderma*-seedling interaction as maize genotype and genetic type of *Trichoderma* isolates between species in the same

Table 2. The effect of *Trichoderma* conidia on seedling growth parameters when added on the surface of soil near emerged seed.

Treatment	Chlorophyll content	Stomata conductivity (mM/m ² s)	Leaf area (mm ²)	Fresh root weight (g)	Fresh shoot weight (g)	Fresh root weight/ Fresh shoot weight	Root length (mm)	Shoot length (mm)	Root length/ Shoot length
Control	25.74 ± 1.18 ^{a*}	74.02 ± 9.94 ^{ab**}	298.16 ± 41.47 ^{a**}	9.34 ± 0.80 ^{a**}	11.87 ± 0.53 ^{a**}	0.79 ± 0.04 ^{b**}	40.63 ± 4.2 ^{a*}	25.04 ± 1.52 ^a	1.63 ± 0.93 ^{ab*}
<i>Trichoderma</i> sp T	23.66 ± 0.87 ^a	107.16 ± 10.77 ^a	273.09 ± 30.58 ^{ab}	7.47 ± 0.52 ^{ab}	11.07 ± 0.18 ^{ab}	0.68 ± 0.06 ^b	41.5 ± 2.32 ^a	28.1 ± 1.04 ^a	1.48 ± 0.05 ^b
<i>T. harzianum</i> T969	24.88 ± 3.22 ^a	72.13 ± 17.92 ^{ab}	285.49 ± 26.21 ^{ab}	9.36 ± 0.11 ^a	7.93 ± 0.71 ^c	1.21 ± 0.12 ^a	45.5 ± 1.6 ^a	26 ± 1.36 ^a	1.77 ± 0.12 ^a
<i>T. hamatum</i> T614	26.29 ± 2.04 ^a	58.29 ± 7.69 ^{ab}	206.37 ± 19.74 ^b	6.13 ± 0.32 ^b	7.26 ± 0.02 ^c	0.84 ± 0.4 ^b	37.88 ± 1.92 ^a	22.42 ± 1.4 ^b	1.70 ± 0.6 ^{ab}
<i>T. harzianum</i> T447	25.46 ± 2.01 ^a	83.99 ± 1.13 ^{ab}	231.26 ± 25.27 ^{ab}	5.94 ± 0.36 ^b	9.14 ± 0.75 ^{bc}	0.66 ± 0.06 ^b	41.42 ± 2.13 ^a	26.38 ± 1.6 ^{ab}	1.55 ± 0.09 ^{ab}
LSD	6.14	45.52	123.22	2	2.19	0.29	7.86	4.21	.26

Values with the same letter within the column are not significantly different (** P≤0.01, * P≤ 0.05) according to Fischer's protected LSD test; results are means of four replicates for each treatment; the value in parentheses is the standard error of the mean.

species, but also on factors such as the *Trichoderma* population threshold and environmental condition. For example, Mc-Fadden and Sutton (1975) reported that at least 10³-10⁴ *T. koningii*, propagules per gram of soil are required for infection and disease development. Therefore, further experiments should be performed for better understanding of efficient factors on the *Trichoderma*-maize interaction.

Evidences also suggested that the effect of *Trichoderma* isolates on seed germination rate and seedling vigor on the sterile blotting paper did not guarantee this effect in which it appears on the potted seed in the soil. In the present study, the maize seed potted in inoculated soil with the *Trichoderma* isolates did not emerge from the soil among all tested *Trichoderma* isolates except for non-inoculated soil 30 days after potting. This is due to parasitizing after seed germination which caused pre-emergence damping off. As opposed to when *Trichoderma* conidia were added on the surface of soil near the emerged seed, response of seedling to *Trichoderma* isolates application varied. A significant (p≤0.01) decrease of seedling treated separately with *T. hamatum* T614 isolate was observed on the leaves area, fresh root and

shoot weight compared to the non-inoculated seedling (Table 2), whereas *T. hamatum* T447 and *T. harzianum* T969 markedly reduced (p≤0.01) seedling fresh root weight and fresh shoot weight respectively, when compared to the control (Table 2). *Trichoderma* isolates had no influence on chlorophyll content in leaves of treated seedling as well as root length, than untreated seedling 30 days after inoculation. Meanwhile, *Trichoderma* isolates did not markedly affect stomata conductivity in treated seedling, except for *Trichoderma* sp. isolate T that increased stomata conductivity of seedling significantly (p≤0.01) as compared with untreated seedling. These obtained results demonstrated that the vigor of maize seedling decreased with *Trichoderma* conidia application and *Trichoderma* isolates act as pathogen among seedling, rather than improve the seedling vigor and development.

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