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CHANGES IN ANTIOXIDANT ENZYME ACTIVITIES, HORMONE LEVELS AND GROWTH TRAITS OF ROSE INDUCED BY THREE NATIVE STRAINS OF *TRICHODERMA HARZIANUM*

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Abstract

Roses (*Rosa* spp.) are considered as poplar ornamental plants for urban landscape with their pretty petals and used for beautification in public gardens and improving the environment. *Trichoderma harzianum* was applied to various plants as a potential bio-control agent helping plants to resist pathogens. In our study, three native strains of *T. harzianum* were inoculated together on the soil in the pots of planting *R. chinensis* 'Shi-Jie-Mei' (the Ten-sisters Roses) and compared with that of no-inoculated to find their changes in growth traits. Analysis results showed that many traits, including the number of branches, the height of stems, fresh weight, dry weight, the water content in leaves and stems, were significantly increased. It was found that antioxidant enzyme activities-catalase, peroxidase, and superoxide dismutase in leaves were also significantly increased compared to the CK. In addition, hormone levels, such as salicylic acid, indole-3-acetic acid, gibberellins, jasmonic acid, zeatin, and abscisic acid in leaves were enhanced significantly induced by *T. harzianum*. Our results indicated that *T. harzianum* could improve the growth traits of the rose by regulating antioxidant enzyme activities and phytohormones level.

Key words: *Rosa chinensis, Trichoderma harzianum,* Antioxidant enzyme, Hormone.

Introduction

Roses (*Rosa* spp.) are world-wide popular ornamental plants that are loved, whether in landscape architecture or cut flowers. With the global development of the economy and the increase of population, sustainable floriculture is in great demand just as sustainable agriculture. *Trichoderma* spp. fungi of the plant rhizosphere are well-known eco-friendly biofertilizers and biological control agents for plant pathogens (Topolovec-Pintarić, 2019). The application of *Trichoderma* spp. can bring multidirectional beneficial effects on agricultural and horticultural practices, including substantially increasing of crop yield, improvement of the crop nutritional quality, and protections from both abiotic and biotic stresses to an extent (Fiorentino *et al*., 2018; Ghazanfar *et al*., 2018; Szczałba *et al*., 2019). *Trichoderma* has been reported as mycofungicides for roses, with emphases on *T. harzianum* strains. Foliar application of *T. harzianum* liquid formulations could control rose black spot disease (Oros & Naár, 2017). *T. harzianum* inoculation could reduce the disease occurrence and yield loss caused by rose Fusarium root rot in soilless culture systems (Nosir, 2016). *T. harzianum*-based commercial biocontrol agent was effective in managing rose powdery mildew (Kumar and Chandel, 2018). Vegetative propagation using stem cuttings is an important method for reproducing woody plants such as roses in horticultural practices. However, the application and effect of *Trichoderma* on the propagation and growth in roses are rarely reported.

Trichoderma promotes plant growth and defense through multiple mechanisms (Martinez-Medina *et al*., 2016; Ortíz-Castro *et al*., 2009). Most significantly, *Trichoderma* can produce phytohormone-like compounds in vitro or alter the hormone levels in the plant to modify the growth and defense abilities of plants (Ortíz-Castro *et al*., 2009). Phytohormones modulate the growth, development, immunity, and defense of plants through an interconnected signal network (Yang *et al*., 2019). Some *Trichoderma* strains have been reported to produce exogenous growth-promoting hormones, including gibberellin-related molecules (GAs), auxin-related molecules, and zeatin to improve plant growth (Contreras-Cornejo *et al*., 2009; Gravel *et al*., 2007; Hermosa *et al*., 2012). Colonization of the rhizospheres or roots of plants by *Trichoderma* can induce both local and systemic resistance through protein elicitor *Sm1* (Small protein 1). As a result, the production of reactive oxygen species (ROS) is triggered in plants, which subsequently induces an increase in the gene expression levels and enzyme activity of ROS scavenging proteins. Meanwhile, *Trichoderma* can induce the systemic resistance, sometimes also the systemic acquired resistance (SAR), through enhancing the signaling or metabolism of the phytohormones salicylic acid (SA) and/or jasmonate acid (JA) in plants (Hermosa *et al*., 2012; Singh *et al*., 2018). An efficient ROS-scavenging system and highly alert SA, JA signaling pathways in plants are crucial for resistance and defense (Hung *et al*., 2005; Yang *et al*., 2019). In this study, we report the promoted propagation efficiency and growth of a popular landscaping rose cultivar in China, *Rosa chinesis* 'Shi-Jie-Mei' (the Ten-sisters Roses), resulted from inoculation of *Trichoderma*.

Materials and Methods

***Trichoderma* strains:** The *Trichoderma* strains used in this study were isolated from the rhizosphere soil of *Quercus Mongolica* growing in the lava plateau area of Ning'an City, Mudanjiang, Heilongjiang province, China, in our previous study, which were preserved by the garden plant application laboratory of Northeast Forestry University (Chang *et al*., 2020). Three *T. harizanum* strains named as T_8, T_{13}, and T_{20}, respectively, were used in the experiment.

***Trichoderma* cultivation experiment:** The activated three native strains of *Trichoderma* were inoculated separately at a diameter (5 mm) in Petri dish with 9 cm diameter, which was individual, pairs, and three together of *Trichoderma*. *Trichoderma* was inoculated at the size of 1 cm from the edge of each dish, and the dishes were placed in an incubator at 26°C for six days, and the state of colonies was observed.

***Trichoderma* preparation:** Activated *T. harizanum* strains T_8, T_{13}, and T_{20} were inoculated in the M1 medium (The medium was established by our laboratory, named as M1, which comprised of 3 gram of barley bran, 1 gram of glucose and 100 mL tape water, sterilized at 121°C under the condition of moist heat sterilization for 30 min, and at 28°C, and were operated at 200 rpm/min for seven days. *T. harizanum* conidia and the fermentation broth were obtained. Thereafter, mixed conidia with the concentration of 1×10^8 cfu/ml and the fermentation both of 200 mL were applied on 15th June, 15th July, and 15th August in each pot of Rose, while in control equal amount of tap water was applied.

***Rosa chinensis* 'Shi-Jie-Mei' reproduction:** Semi-hardwood cuttings of *R. chinesis* 'Shi-Jie-Mei' were purchased from Nangang nursery breeding center of Jiamusi city in Northern Heilongjiang province of China, at the end of Dec. 2016. Sterilized soil and sand were kept as 4:1 for each pot. Prepared mixed conidia and the fermentation broth of T_8, T_{13}, and T_{20} were poured separately in the pots (diameter × height as 35 cm × 35 cm), and control pots prepared with equal quantity soil and water. Approximately 20 cm long, 20 semi-hardwood cuttings of rose were placed in pots and kept in the dark for fifteen days in the chamber at 24°C with a relative air humidity of 80%. Watering was done on a daily basis in order to keep moist soil, and buds sprouting number was observed of CK and T.

Field experimentation: Field experiments were carried out at the research station of the College of Landscape Architecture, Northeast Forestry University, Harbin, China. During spring season May, pots planting rose seedlings were moved from the laboratory to the research station under natural condition. A complete randomized block (CRB) was set as the experimental design. Pot to pot distance of 80 cm was kept, row to row distance was 60 cm from each other. There were ten pots (35 cm × 35 cm) in each row. Watering and management of weeds were followed according to the method of Afroz *et al*. (2015).

Biomass measurement and sample collection of *R. chinensis* 'Shi-Jie-Mei': On the 15th September, record the number of branches per rose seedling and measure the plant height with a tape measure. The leaves of the third node at the top of the stem of each rose were collected, weighed, and put in liquid nitrogen immediately, subsequently employed for defensive enzymes activation and hormone levels estimation. At a distance of 20 cm from the soil surface, all branches of the rose plants were cut off and brought back to the laboratory in an icebox. The harvested branches and leaves were weighed separately with an electronic scale to obtain the fresh weight. Then the branches and leaves were dried according to Cornelissen *et al.* (2003) for the dry weight. The water content of branches and leaves was assessed, followed as: [(fresh weight – dry weight)/fresh weight] × 100%.

Determinations of defense enzymes activity in *R. chinensis* 'Shi-Jie-Mei': About 0.500 gram rose leaves were used for analyzing the activity of the defensive enzyme including CAT (catalase activity), POD (peroxidase) and SOD (superoxide dismutase) according to the methods of Shahzad *et al*., (2018). Each sample had three biological duplicates.

Extraction and detection of hormones by LC-MS/MS: About 0.0500 gram rose leaves were used for estimation hormone levels. All reagents in this extraction experiment, including in IAA, GA_3, ABA, ZT, SA, JA, Indole-3-butyric acid (IBA) and (±)-hydrojasmonic acid (H_2JA) were purchased from Sigma-Aldrich (Mainland, China), and HPLC-grade methanol and formic acid were from Merck (Darmstadt, Germany). The standard addition method (Zhai *et al*., 2019) was performed to quantify the hormone of rose leaves. Indole-3-butyric acid (IBA) and H_2JA ((±)-hydrojasmonic acid) as double internal standard were prepared in methanol solution and stored at 4°C for extraction experiments. LC column system and gradient elution profile were employed in accordance with Zhang *et al*., (2012a; 2012b) and Zhai *et al*., (2019) for detection hormones level of IAA, GA_3, ABA, ZT, SA, and JA. ZT was detected simultaneously with IAA, GA_3, ABA under the same LC condition and itself MS parameters in Table 1. API3000 Triple tandem quadrupole mass spectrometer (Applied Biosystems, USA) with a TurboIon-Spray interface in the negative electrospray ionization mode (ESI) was used. The analytes were ionized in ESI- conditions as shown in (Table 1). The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode to heighten the selectivity and specificity of testing the low-content hormone of rose leaves. All samples were detected in triplicates.

Table 1. Mass spectrometric parameters for hormones.

	MRM(amu)	IS(v)	DP(v)	FP(v)	EP(v)	CE(v)	CXP(v)	CUR(psi)	NEB(psi)	TEM(℃)
IAA	174.0→130.0	-4500	-75	-375	-10	-16	-7	12	10	300
GA3	345.2→239.0	-4500	-40	-375	-10	-25	-8	12	10	300
ABA	263.2→153.0	-4500	-90	-375	-10	-18	-8	12	10	300
ZT	218.2→133.9	-4500	-80	-375	-10	-25	-6	12	10	300
SA	136.9→93.0	-4500	-35	-375	-10	-20	-6	12	10	300
JA	209.3→59	-4500	-37	-375	-10	-25	-23	12	10	300
IBA	202.1→116.0	-4500	-105	-375	-10	-25	-6	12	10	300
H_2JA	211.1→59.2	-4500	-90	-375	-10	-24	-7	12	10	300

Abbreviations: IS: In-spray voltage; DP: Declustering potential; FP: Focal potential; EP: Entrance potential; CE: Collision energy; CXP: Collision export potential; CUR: Curtain gas; NEB: Nebulize gas

Data analysis: For statistical analysis, we used Microsoft Excel 2007 (Microsoft Company; USA) and SPSS 22.0 (SPSS Inc., IBM Company, USA). Data presented in the figures and tables are means ± standard deviation (SD) of replications. Data of LC-MS/MS were processed through Analyst 1.4.0 software.

Results

***Trichoderma* cultivation:** After inoculation for six days, the morphology of the individual, pairs, and three together of *Trichoderma* were shown in Fig. 1. The appearance displayed that the three strains of *T. harzianum* had similar competitiveness, sporulation ability, and colonization space. Three strains were friendly symbiotic.

Changes in activities of antioxidant enzymes in rose leaves by applied *T. harzianum*: Results of this study showed that all SOD, POD and CAT activities in rose leaves treated with *Trichoderma* strains were increased significantly compared with CK (the un-inoculated group). SOD and POD activities in T group were 160.9 and 49062 U g^{-1} min^{-1}, respectively. They were enhanced separately 43.95% and 87.51% compared with CK (p<0.05) (Fig. 1A & B). The CAT activity was 1467.67 U g^{-1} min^{-1}, which was increased significantly by 97.23% compared to CK (p<0.05) (Fig. 1C).

Analysis of hormone levels in rose leaves by applied *T. harzianum*: The results of detected hormone levels displayed that IAA and ZT levels in the leaves applied *Trichoderma* strains were 201.9 and 0.7 ng g^{-1} FW, respectively, which enhanced separately 7.23% and 47.56% compared to CK, but showed non-significant differences (p>0.05) (Fig. 2A & B). On the other hand, the level of GA_3 was 19.0 ng g^{-1} FW, which increased significantly by 40.14% compared with CK (p<0.05) (Fig. 2C). In addition, the results showed that SA, JA and ABA levels in the leaves treated by *Trichoderma* were 4807.4, 1299.7 and 2428.7 ng g^{-1} FW, respectively, all of them were significantly increased compared to CK (p<0.05), reached separately to 1.06, 4.53 and 1.21 times of CK (Fig. 2D, E, and F).

Bud development in rose semi-hard cuttings by applied *T. harzianum*: The results revealed that application of *T. harzianum* increased significantly the buds sprouting from the semi-hardwood cuttings of rose in the pot experiment (Fig. 4) The average buds number of T_{13} group was 4.1 per semi-hard cutting, which was increased by 1.26, 0.48 and 0.30 times of CK, T_8, and T_{20} (p<0.05).

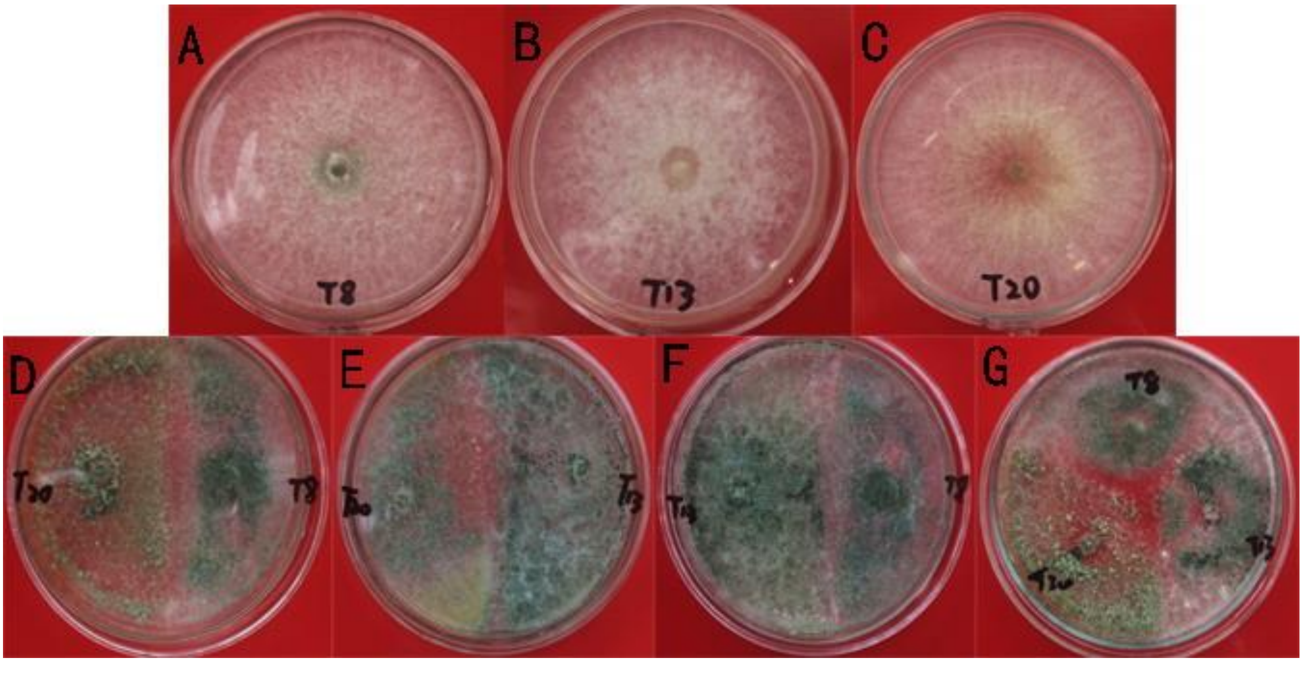

Fig. 1. Dual culture of *Trichoderma* A: T8; B: T_{13}; C: T_{20}; D: T_{20} & T_8; E: T_{20} & T_{13}; F: T_{13} & T_8; G: T_8 & T_{13} & T_{20}.

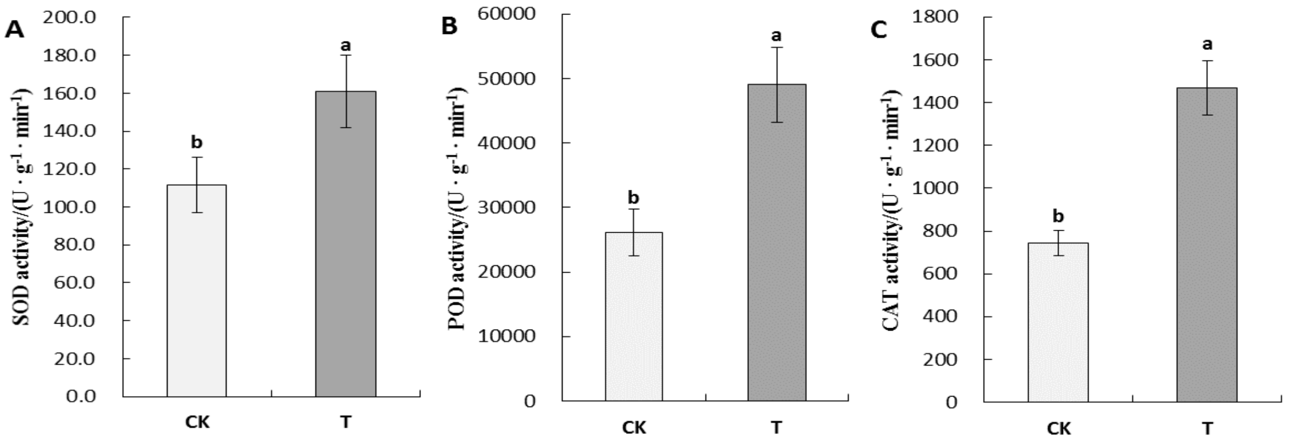

Fig. 2. Activities of antioxidant enzymes in rose leaves by applied *Trichoderma*. A: SOD; B: POD; and C: CAT.

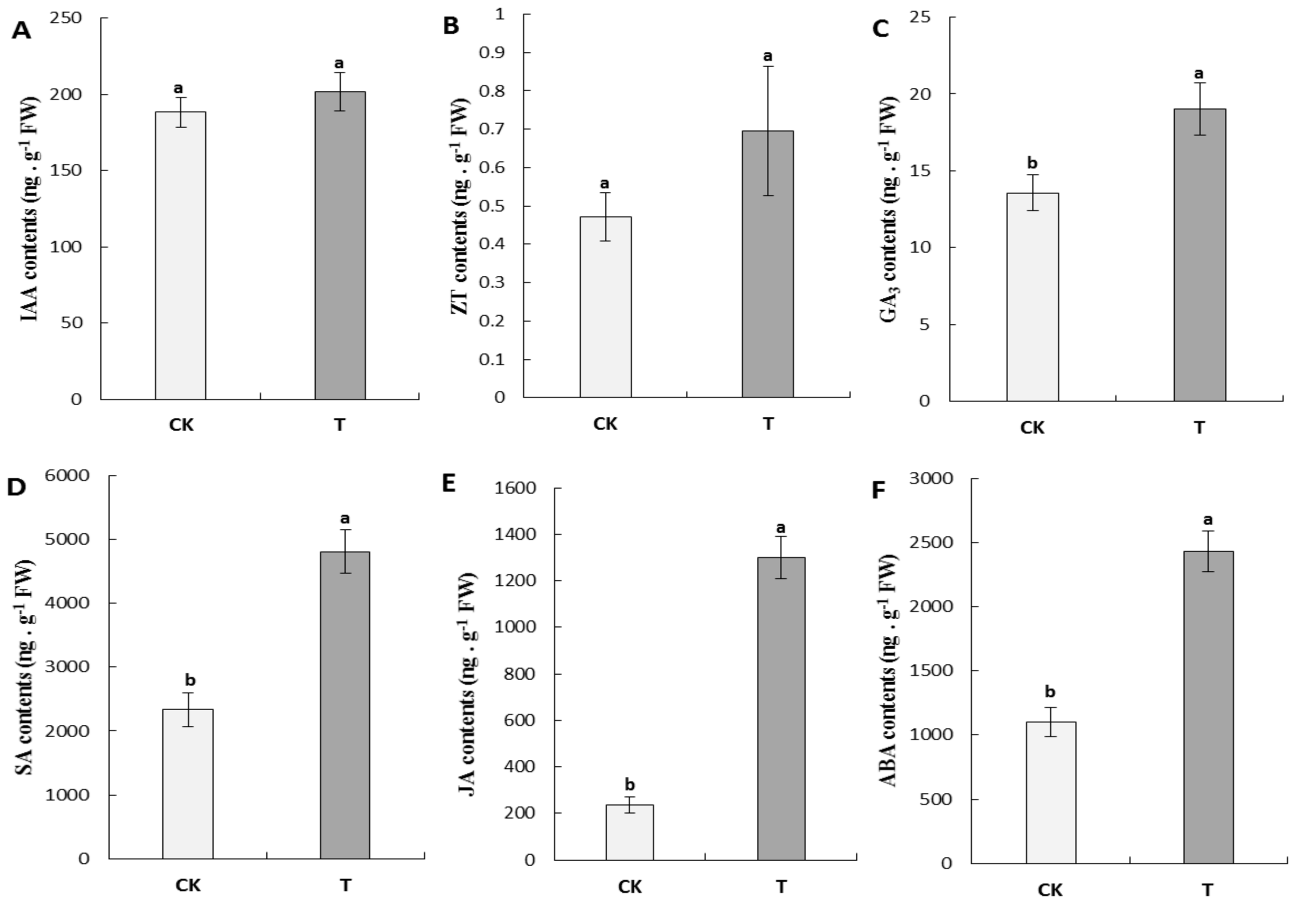

Fig. 3. Hormone levels in rose leaves by applicated *T. harzianum.* A: IAA; B: ZT; C: GA_3; D: SA; E: JA; and F: ABA.

Table 2. Growth improvement by *T. harzianum* in rose.

	Stem height	Branches number
CK	112.0 ± 47.2a	2.0 ± 1.3b
T	150.7 ± 22.9a	4.2 ± 1.5a

Growth promotion in rose by applied *T. harzianum*: Plant height and number of shoots are the main index in plant architecture and play the main role as associated in biomass production. In the current study, the results displayed that plant-growth-promoting fungi *T. harzianum* changed stem heights and branches number. The stem height reached up to 150.7 cm, which was higher 0.35 times of CK but had no significant difference ($p>0.05$) (Table 2). Besides, the branches number was increased significantly in 4.2 ($p<0.05$) (Table 2), which was 1.1 times compared to CK.

Biomass assessing in rose by applied *T. harzianum*: The production of biomass in stems, leaves such as fresh weight, dry weight, and water contents were determined in the present study. Fresh and dry weights in the stem were observed as 56.1 and 26.8 g, which were improved significantly compared with CK ($p<0.05$) (Table 3), increased 1.03, and 0.90 times of CK. The water contents in the stem of the CK and T group were 48.3 and 52.5%, respectively, which had no significant difference ($p>0.05$)

(Table 3). On the other hand, the fresh and dry weight of leaves was measured as 57.5 and 23.1 g, which was improved significantly compared with CK ($p<0.05$) (Table 3), increased 0.78, and 0.57 times of CK. The water contents in the leaves of CK and T groups were 54.4 and 60.0%, respectively, which also had a significant difference ($p<0.05$) (Table 3).

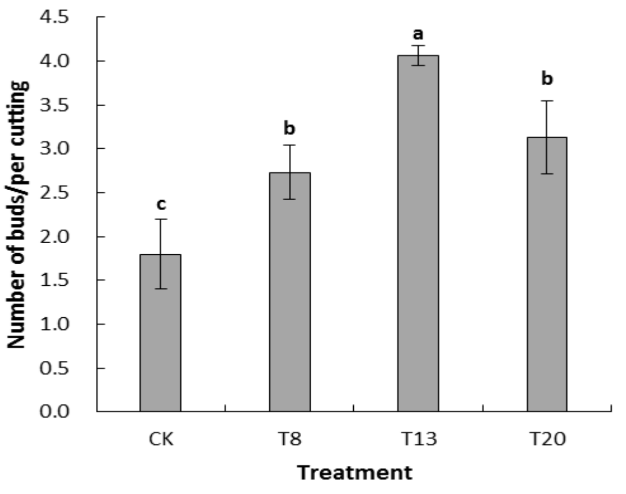

Fig. 4. Number of buds sprouting from per semi-hard cuttings of rose.

Discussion

Trichoderma spp. have been studied and applied as a bio-control agent globally. These species present in soil rhizosphere of farming fields and helps rapidly in increasing the crops yield, improvement of plant traits such as increase of number of branches, fruits per plant, fruits weights, stem height, prevent in number pathogens attack, and improvement of soil fertility (Brotman *et al*., 2013), and beneficial for microbial community (Doni *et al*., 2013). In this regard, in the present study screened isolated strains of *T. harzianum* as promoting fungi on growth improvement, which significantly increased the number of sprouting buds of semi-hardwood cuttings and increased in the production of leaves (Fig. 4). Additionally, isolates strains of *T. harzianum* caused an increase in stem heights and the maximal number of branches (Table 2). This study also confirmed that treatments with *T. harzianum* strains were significantly increased fresh weight, dry weight, and relative water content in rose leaves and stem, and whereas the decline was noticed in all these parameters in CK (Table. 3). The obtained results in agreement with previous research (Rubio *et al*., 2017), that *T. harzianum* resulted in increased plant height, biomass production in root as fresh, dry matter weight, moisture content, also increase the number of leaves in tomato plant when compared with untreated plants.

Catalase, peroxidase, and superoxide dismutase are key plant defense enzymes in several plant parts, which protect the plants against certain plant pathogens and protect them in nature (Muhammad *et al*., 2018). Previous studies reported that antioxidants enzymes activities were increased in rice (Yongendra *et al*., 2014). Fuli *et al*., (2017) reported that *T. harzianum* significantly increased enzymatic activities in soybean seedlings. This study also confirmed that strains of *T. harzianum were* a source of increase to enzyme activities of catalase, peroxidase, and superoxide dismutase significantly in mature rose leaves than that of untreated plants. It was presumed, according to our study, that rose leaves exposed treatment with *T. harzianum* could increase activities of antioxidant enzymes and protect them in nature stress (Fig. 2).

In our study, the level of phytohormones was significantly increased in leaves of the treatment group of *R. chinensis* 'Shi-Jie-Mei' when compared with CK (Fig. 3). The results were in accord with Gravel *et al*., (2007) and Contreras-Cornejo *et al*., (2009), that *T. virens* strains increased performance of Indole-3-acetic acid hormone in lateral growth roots of *Arabidopsis*, and inoculated *T. atroviride* improved in fruits yield of tomato by up-regulating indole-3-acetic acid level. In addition to this, the applications of *T. harzianum* increased all hormone levels with maximum hikes when compared with CK. In summary, the *T. harzianum* applications not only increased hormones level but also increased antioxidants enzymatic activities in rose leaves when compared with CK plants. Consequently, *T. harzianum* strains significantly increased biomass production, the number of branches, stem height, and buds number when these all parameters compared with CK. Therefore, these results suggested that applications of *T. harzianum* might be proved important for vegetative growth in rose.

Table 3. Improvement in biomass production in *R. chinensis* by *T. harzianum*.

	Fresh weight (g)		Dry weight (g)		Water content/%	
	Stem	Leaves	Stem	Leaves	Stem	Leaves
CK	27.7 ± 9.7b	32.3 ± 10.1b	14.1 ± 4.3b	14.7 ± 4.3b	48.3 ± 5.6a	54.4 ± 3.9b
T	56.1 ± 13.0a	57.5 ± 8.9a	26.8 ± 7.1a	23.1 ± 4.2a	52.5 ± 1.6a	60.0 ± 1.4a

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