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Acclimatization of Micropropagated Orchid *Guarianthe skinnerii* Inoculated with *Trichoderma harzianum*

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Abstract: The objective of this study was to investigate survival rate and growth of the orchid *Guarianthe skinnerii* when inoculated with or without *T. harzianum* during acclimatizing of the seedlings obtained after micropropagation. The *G. skinnerii* seedlings were obtained by *in vitro* germination of seeds from the capsule. Seedlings were transferred to unicel pots contained peat moss and were inoculated with *T. harzianum*. A treatment without inoculation served as control. The seedlings were kept in climatic chamber at 20±2°C for 4 months (step one), in the laboratory at 25±8°C for four months (step two) and in the greenhouse at 30±12°C for four months (step three). The seedlings were distributed according to a completely aleatorized experimental design. After each step, the percentage of plants that survived, plant height, number of leaves and shoots were determined. Survival of *G. skinnerii* inoculated with *T. harzianum* was significantly 1.6 times higher than those non-inoculated seedlings. Plant height of inoculated plants was 3.6 times greater than control plants after the first step. The number of leaves was 2.5 times higher only after the step one. The amount of shoots was 1.4 times higher in the seedlings inoculated with *T. harzianum*. The results indicated that the survival and growth of seedlings inoculated with *T. harzianum* increased in extreme temperature conditions for acclimatization.

Key words: Acclimatization inoculation, orchid *Guarianthe skinnerii*, survival rate and plantlet growth, *Trichoderma harzianum*

INTRODUCTION

Orchidaceae are the largest family of flowering plants with between 20000 and 30000 different species (Chase *et al*., 2003). Although an orchid produces hundreds of thousands of seeds, it is exceptionally difficult to grow orchids from them. Each seed contains so little nutrients for the plantlet that few make it even to the seedling stage (Arditti and Ghani, 2000). A major breakthrough occurred in 1917 when Dr. Lewis Knudsen of Cornell University discovered that the seedlings could also be nurtured by growing them in sugar-based solid media and since then efficient micropropagation systems using tissue culture techniques have been reported for many Orchidaceae genera (Arditti and Ghani, 2000). Now, most commercially produced orchids are propagated using modifications of this method (Batty *et al*., 2006). However, low survival of the seedlings in the acclimatizing phases is still a major impediment (Pospíšilová *et al*., 1999). The special conditions during in vitro culture result in the formation of seedlings with abnormal morphology, anatomy and physiology. After ex vitro transfer, these seedlings are easily damaged by sudden changes in environmental conditions and they therefore need an acclimatization period to correct abnormalities (Pospíšilová *et al*., 1999).

Srinath *et al*. (2003) found that micropropagated *Ficus benjamina* seedlings co-inoculated with *G. mosseae*

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and *Trichoderma harzianum* were significantly higher with larger shoot, root and total plant biomass compared to non-inoculated seedlings. *Trichoderma* strains have long been known as effective antagonists against soil borne plant pathogenic fungi, for example *Fusarium moniliforme* (El-Hasan *et al*., 2008). It has also been reported that *T. harzianum* increases the solubility of phosphorus thereby promoting plant growth (Rudresh *et al*., 2005). Little or no work has been done on the effect of inoculation of orchid micropropagated seedlings with *Trichoderma* sp. The objective of this study was to investigate survival rate and growth of the orchid *Guarianthe skinnerii* when inoculated with or without *T. harzianum* during acclimatizing of the seedlings obtained after micropropagation.

MATERIALS AND METHODS

The *G. skinnerii* seedlings were obtained by *in vitro* germination of seeds from five green capsules. The intact capsules were washed with a soap solution, rinsed in water and immersed in 1% sodium hypochlorite (bleach solution) to which a drop a detergent has been added for 10 min. The capsules were cut open under sterile conditions and placed in culture flasks containing MS medium (Murashige and Skoog, 1962). The seeds were germinated for six weeks when protocorms were obtained. These protocorms were sub-cultivated in MS medium added with 1 g L^{-1} activated carbon. After 10 weeks, the seedlings obtained were transferred using forceps to unicel pots filled with peat moss.

T. harzianum, obtained from Cinvestav-Irapuato (Mexico), was added to 500 mL Erlenmeyer flasks containing potato dextrose broth and cultivated for 8 days. The cultures were filtered through Whatman filter paper® No 1. The mycelial material was macerated using a Waring blender for 1 min and mixed with 250 mL 0.1 M $MgSO_4.7H_2O$ solution.

A soil-agrolite mixture (3:1), previously sterilized in an autoclave at 1 kg cm^{-2} for 20 min, was added to 500 cm^3 unicel 8-vessels. A planting hole was prepared and 10 mL *T. harzianum* inoculum containing 5×10^4 cfu mL^{-1} was added to the planting hole. One *G. skinnerii* seedling was planted per pot and one hundred plants were used per treatment.

The seedlings were covered with a nylon mesh for two weeks to reduce water loss and kept in climatic chamber at 20±2°C for four months (step one), in the laboratory at 25±8°C for another four months (step two) and in the greenhouse at 30±12°C for 4 months (step three). The pots with the seedlings were distributed at random during each of the experimental phases. After each step, the percentage of plants that survived, plant height, number of leaves and shoots were determined. Significant differences between the treatments were analysed for by ANOVA using the Least Significant Difference (LSD) criteria with $p<0.05$ (PROC GLM, SAS Institute, 1989).

RESULTS AND DISCUSSION

The survival of *G. skinnerii* plantlets was not different between the treatments after the first and second acclimatization step (Table 1). After step three, however, survival of *G. skinnerii* plantlets was significantly different between the treatments ($p<0.05$). Survival of *G. skinnerii* seedlings inoculated with *T. harzianum* was 79% and significantly higher than those that were not inoculated ($p<0.05$) (Table 1). Plant height of inoculated plants was significantly greater than that of non-inoculated plants after the first step ($p<0.05$) (Table 2). Similar results were obtained after the second and third step.

The number of leaves was 2.5 times higher only after the step one for plantlets inoculated with *T. harzianum* than those without inoculum, but only in the first step ($p<0.05$) (Table 2). The number of shoots was 1.4 times higher in the seedlings inoculated with *T. harzianum* in inoculated plantlets compared to those without inoculum after the second and third step ($p<0.05$) (Table 2) (Fig. 1).

Srinath *et al*. (2003) found that the height of arbuscular micorrhizas-inoculated *Ficus benjamina* plantlets increased when they were co-inoculated with either *Bacillus coagulans* or *T. harzianum*. Ozbay and Newman (2004a) reported that shoot height, stem calliper and shoot fresh weight of tomato seedlings increased when inoculated with *T. harzianum*. In the study reported here, *T. harzianum* increased survival of *G. skinnerii* seedlings through the different acclimatization steps. We are not aware that this increased survival of orchidaceae using *T. harzianum* has been reported before. It is difficult to speculate why *T. harzianum* has a positive effect on survival of *G. skinnerii*, but it is known that *T. harzianum* increases the solubility of phosphorus thereby promoting plant growth. Additionally, *G. skinnerii* seedlings were exposed to pathogenic microorganisms in each acclimatization step and *T. harzianum* might be antagonistic towards these pathogens. *T. harzianum* is known to inhibit crown and root rot of tomatoes and was found that *T. harzianum* strains increased yield in the presence of measurable disease (Ozbay and Newman, 2004b). Reduction of disease by the use of *T. harzianum* strains had improved tomato yields between 6 and 37% in coir and between 2 and 25% in rockwool. This finding

Table 1: Survival of *Guarianthe skinnerii* treated with *Trichoderma harzianum* kept in climatic chamber at 20±2°C for 4 months, in the laboratory at 25±8°C for 4 months and in the greenhouse at 30±12°C for another 4 months

	Survival (%)		
Treatments	One[a]	Two[b]	Three[c]
Without inoculum	100a[d]	84a	50b
With *Trichoderma harzianum*	99a	87a	79a
Minimum significant difference (p<0.05)	4	6	7

[a]Survival of the plantlets in the climatic chamber at 20±2°C after four months, [b]Survival of the plantlets in the laboratory at 25±8°C after 4 months, [c]Survival of the plantlets in the greenhouse at 30±12°C after four months, [d]Values with a different letter are significantly different from each other (p<0.05)

Table 2: Increase in plant height of *Guarianthe skinnerii* (cm month^{-1}) treated with *Trichoderma harzianum* kept in climatic chamber at 20±2°C for 4 months, in the laboratory at 25±8°C for 4 months and in the greenhouse at 30±12°C for 4 months

	Increase in plant height (cm month^{-1})			No. of leaves			No. of shoots		
Treatments	One[a]	Two[b]	Three[c]	One	Two	Three	One	Two	Three
Without inoculum	0.20b[d]	0.55b	1.48b	0.6b	2.6a	4.1a	1.2a	1.9b	2.4b
With *Trichoderma harzianum*	0.72a	1.30a	2.32a	1.5a	2.3a	3.5a	1.3a	2.8a	3.5a
MSD (P<0.05)	0.13	0.43	0.47	0.1	0.4	0.6	0.6	0.8	0.9

[a]Survival of the plantlets in the climatic chamber at 20±2°C after 4 months, [b]Survival of the plantlets in the laboratory at 25±8°C after 4 months, [c]Survival of the plantlets in the greenhouse at 30±12°C after 4 months, [d]Values with a different letter are significantly different (p<0.05)

Fig. 1: Micropropagation of *Guarianthe skineerii*. (a) Micropropagated plantlets after four months, (b) Shoot proliferation, (c) rooting plantlets and (d) acclimatization after one year

suggests that *T. harzianum* strains used in this experiment act only as biocontrol agents (Ozbay and Newman, 2004 b). *T. harzianum* is an efficient biocontrol agent that is commercially produced to prevent development of several soil pathogenic fungi. Different mechanisms have been suggested as being responsible for their biocontrol activity, which include competition for space and nutrients, secretion of chitinolytic enzymes, mycoparasitism and production of inhibitory compounds (Roco and Pérez, 2001).

It was concluded that the inoculation with *T. harzianum* increased the survival and growth of *G. skinnerii* seedlings in extreme temperature conditions for acclimatization. We are not aware that this increased

survival of orchidaceae using *T. harzianum* has been reported before and therefore our results are important for possible implementation in orchid culture.

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