Inoculation of cyclamen (*Cyclamen persicum*) and poinsettia (*Euphorbia pulcherrima*) with arbuscular mycorrhizal fungi and *Trichoderma harzianum*

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ABSTRACT

Dual inoculation of peat based horticulture substrate with a mixture of four species of arbuscular mycorrhizal fungi and fungal biocontrol agent *Trichoderma harzianum* showed a significant positive effect on the growth and flowering of cyclamen plants. Inoculation substantially decreased plant mortality caused by spontaneous infection by the fungal pathogen *Cryptocline cyclaminis*. Plant mortality was also reduced by separate inoculation with arbuscular mycorrhizal fungi. Both separately inoculated agents positively affected the plant growth, although to a lesser extent. Very few significant effects of inoculation were observed on the growth of poinsettia plants cultivated from cuttings. Use of arbuscular mycorrhizal fungi together with the introduction of *Trichoderma* for inoculation of horticultural substrates is suggested to alleviate the inevitable effects of various stresses during the cultivation of horticultural crops.

Keywords: *Cyclamen persicum*; *Euphorbia pulcherrima*; inoculation; arbuscular mycorrhizal fungi; mycorrhizal growth response; *Trichoderma harzianum*

Horticultural crops and flowers have been used as host plants in several experimental tests as potential target plants for practical use of mycorrhizal inoculation (Chang 1994, Lovato et al. 1995, Šrámek et al. 2000). The arbuscular mycorrhizal fungi (AMF) can stimulate plant growth especially in soils or substrates with lower fertility and the effect of AMF on plant nutrition mainly due to improved phosphorus nutrition has been documented (Johnson et al. 1982, Smith et al. 1986). The effect of AMF inoculation is generally more pronounced in plants growing under stress conditions, e.g. nutritional deficiency and water stress. Promotion of mycorrhizal colonisation of plant roots by inoculation facilitates adequate P nutrition, higher resistance to some root pathogens and environmental stresses (Smith and Read 1997). Inoculation of plants may increase crop uniformity and reduce transplant mortality (Waterer and Coltman 1988). It has been accepted that an appropriate management of mycorrhizal symbiosis allows reduction of chemical fertilizers and pesticides input (Azcón-Aguilar and Barea 1997).

Since in most soils the indigenous populations of AMF are present, the preinoculation of seedlings in AMF free substrates gives the introduced fungal strain a spatial advantage over the indigenous fungi after transplanting (Powell 1981). In soilless substrates lacking the indigenous AMF or under the conditions where soils are fumigated and most of the indigenous AMF are eliminated, mycorrhizal inoculation is often successful (Nelsen et al. 1981, Vosatka et al. 1992, Vosatka 1995). Tissue cultured plants or plants grown from cuttings or small seedlings can be inoculated with pure strains of highly effective AMF with rapid colonisation rates, which allow a successful colonisation of newly formed roots after transplanting (Vosatka and Gryndler 1999).

Several strains of *T. harzianum* have been found to exhibit a positive growth effect on the plants probably due to their antagonism to various root pathogens such as *Pythium* or *Rhizoctonia* (Windham et al. 1986, Chet 1987, Baker 1989). The positive effects of combinations of the AMF and *Trichoderma aureoviride* were previously reported for *Tagetes erecta* grown in a peat-perlite mixture (Calvet et al. 1993) and for *Citrus reshni* grown in two horticulture substrates (Camprubí et al. 1995). Some studies showed that *T. harzianum* exhibits a negative effect on the development of AMF (Green et al. 1999), however, Šrámek et al. (2000) showed the same positive effects of AMF inoculation on the growth of three balcony plant species (*Verbena*, *Torenia*, *Diascia*) when inoculated separately or together with *Trichoderma harzianum* application.

Two experiments were conducted to investigate the effects of dual inoculation of peat based horticulture cultivation substrate with a mixture of four AMF and *T. harzianum* on the survival and growth of cyclamen and poinsettia in horticulture practice.

MATERIAL AND METHODS

Chemical analyses of substrates. Chemical analyses of dry samples were carried out before and after the cultivation period. Electric conductivity and pH values were

estimated in a water extract 1w:10v, the content of available nutrients was estimated in Göhler leaching extract (0.52 M CH_3COOH, 0.05 M CH_3COONa) 1w:10v.

Experiments design. Both experiments involved four treatments: control uninoculated plants, plants inoculated with AMF (AMF), plants inoculated with *Trichoderma* (Trich), and plants inoculated with both inocula, (AMF/Trich). The AMF inoculum was a mixture of four AMF isolates, three were from the Bank of European Glomales – BEG: *Glomus claroideum* BEG23, *G. mosseae* BEG91, *G. intraradices* BEG93 and *G. geosporum* isolate 24 from the Institute of Microbiology, Prague, all maintained in a greenhouse in open pot cultures with maize grown in sand: Vermiculite mixture (5:1, v:v) for 4 months. The dose of mycorrhizal inoculum containing spores, mycelium and colonized root fragments of pure AMF cultures was 0.1 L per 10 L of substrate. The inoculum was mixed before use with 0.9 L of Vermiculite for better homogenisation to the whole volume of substrate. *Trichoderma harzianum* inoculum was introduced as spores of fungal culture (concentration $1.4.10^{10}$ of spores per one gram) in a commercial product Supresivit used as a biocontrol agent against the complex of root pathogens (manufactured by Fytovita Ltd. Praha, Czech Republic). *Trichoderma harzianum* inoculum (0.002 g per one L of substrate) was also mixed with 0.9 L of Vermiculite for better homogenisation to the whole volume of substrate. The same amount of Vermiculite as in inoculated treatments was added to the substrate in uninoculated controls.

Cyclamen experiment. Seeds of *Cyclamen persicum* var. Rosa mit Auge were sown in the third week of February in plastic boxes 30 × 20 × 10 cm filled with a peat-based medium. Substrate for young seedlings in three treated variants was inoculated before potting (May 5) into 6 cm plastic pots (volume 0.15 L). Seedlings were regularly watered and fertilised and after 8 weeks (July 7) they were transplanted into 12 cm plastic pots (volume 0.8 L) filled with a Stender's substrate C for pot plants (manufactured by Stender GmbH, Schermbeck, Germany). The substrate in the three treated variants was inoculated again in the same way as in previous stage. Each treatment consisted of 5 replicates with 10 plants in each replicate. Replicates were randomly placed in the temperate greenhouse and plants were grown for 6 months. Plants were watered daily with tap water and fertilised weekly with 0.2% solution of Kristalon Blue fertilizer (19% N, 6% P_2O_5, 20% K_2O) and from 11 weeks after potting (September 29) with 0.2% solution of Kristalon White fertilizer (15% N, 5% P_2O_5, 30% K_2O). After cultivation (November 24) all the plants were subjected to a non-destructive measurement, where the following parameters were estimated: plant mortality, plant height and width, and the number of leaves. The number of flowers was counted twice (November 10 and 24). Three plants from each replicate block, in total 12 plants per treatment were harvested and the number and the total area of leaves, the number of flowers and buds, and the dry weights of leaves and flowers were evaluated.

Poinsettia experiment. *Euphorbia pulcherrima* Peter Star plants were grown from cuttings rooted in rockwool. The plants were transplanted in August to 12 cm plastic pots filled with Stender's substrate for *Ericaceae*. The substrate in three treated variants was inoculated before potting as in cyclamen experiment. Plants were fertilised weekly with 0.2% solution of Kristalon Blue. Each treatment consisted of 4 replicates with 12 plants in each replicate. Replicates were randomly placed in the temperate greenhouse and plants were grown for 6 months (until December 5). After cultivation, all plants were subjected to a non-destructive measurement, where the following parameters were estimated: plant mortality, plant height and width, number of leaves, red bracts and stems. Three plants from each replicate block, in total 12 plants per treatment were harvested destructiving and the number of leaves, the total area of leaves and bracts, the number and total length of stems, dry weights of leaves, bracts and stems were evaluated.

The root subsamples from destructive harvests of both experiments were sampled and stained according to Phillips and Hayman (1970). Mycorrhizal colonisation (ie.percentage of root length colonised by AMF) was determined microscopically at a magnification of 120× using the grid-line intersect method (Giovannetti and Mosse 1980). The whole surface of the root samples was observed for the presence of *T. harzianum* hyphae and graded into four classes according to percentage proportion of root segments with occurrence of hyphae (class 0 = no hyphae, 1 = scarce presence of hyphae up to 30% of root surface, 2 = medium abundance of hyphae 31–60%, 3 = abundant hyphae 61–100%). The roots of plants from the uninoculated treatments were checked for the presence of mycorrhizal and *Trichoderma* hyphae and the values found were subtracted from the values obtained in the inoculated treatments.

All the data sets were tested for normality and analysed by a two-way analysis of variance. The non-normal distributed data sets were transformed logarithmically and the analysis of variance was repeated for the transformed data. The significance level $p < 0.05$ was used, and the significant differences between means were evaluated using Duncan's Multiple Range Test.

RESULTS AND DISCUSSION

In general, nutrients in all substrates were sufficient for appropriate plant growth. The only substantial change of substrate in both experiments caused by experimental treatments was detected in the poinsettia experiment, where the total content of calcium was increased in both treatments inoculated with AMF (Table 1).

The most apparent result of the cyclamen experiment was a decrease of mortality observed in inoculated treatment compared with uninoculated control treatment, where more than one third of the plants died (Table 2). The reason for the high mortality in the experiment was

Table 1. pH value, electric conductivity (EC) and content of available nutrients in cultivation substrate at the beginning and at the end of the experiments

Substrate / Treatment	pH	EC mS/cm	$N\text{-}NH_4^+$	$N\text{-}NO_3^-$	P	K	Ca
			mg/L				
			Cyclamen persicum				
Beginning	4.2	0.7	115	85	57	147	1170
Control	6.6	0.8	20	55	47	58	2020
AMF	6.8	0.7	10	35	37	42	2120
Trich	6.6	0.7	15	25	45	41	1850
AMF/Trich	6.9	0.7	8	20	44	48	2070
			Euphorbia pulcherrima				
Beginning	3.2	1.9	16	12	8	21	220
Control	4.0	1.2	12	12	1	92	270
AMF	4.4	1.2	10	21	3	85	450
Trich	4.0	1.0	14	15	1	114	250
AMF/Trich	4.3	1.4	15	27	5	100	430

Control = uninoculated substrate, inoculation with arbuscular mycorrhizal fungi (AMF) and *Trichoderma harzianum* (Trich), AMF/Trich = dually inoculated plants

a spontaneous infection by the fungal pathogen *Cryptocline cyclaminis*. It is spread by seeds, inhibits leaf and flower development and causes plant dying. Because of application of fungal inoculants, plants in the experiment were not treated by fungicides. The decrease of mortality should be attributed mainly to AMF inoculation. The AMF inoculation itself decreased the mortality of cyclamen compared with uninoculated control plants; however, dual inoculation with AMF and *Trichoderma* eliminated the mortality. AMF inoculation as a single factor evaluated by two-way ANOVA positively influenced the growth of plants, increased significantly the plant height and number of leaves (Table 2). Inoculation with *Trichoderma* as a single factor increased significantly number of flowers at the end of the experiment. At destructive harvest AMF inoculation was found to increase the dry weight of leaves and significantly increase the total leaf area (Table 3). Plants inoculated with AMF only had lower flower number, especially at the first evaluation. The AMF inoculation slightly delayed flowering but on the other hand it increased the number of flower buds. Destructive analysis also showed the positive effects of separate inoculation with *Trichoderma* on number of flowers and total leaf area. Dual inoculation with AMF and *Trichoderma* shortened time necessary to achieve the market ripeness (when in each replicate at least 80% of plants had one or more flowers) by one week compared with control, whereas separated inoculation with *Trichoderma* or AMF prolonged this time by one or two weeks respectively (data not shown). The AMF inoculation delayed the beginning of flowering but it did not influence the total number of flowers and it did not change the quality of plants and moreover it increased the number of flower buds. Dual inoculation generally

Table 2. The effect of inoculation with arbuscular mycorrhizal fungi (AMF) and *Trichoderma harzianum* (Trich) on *Cyclamen persicum* – non-destructive analysis, results of two-way and one-way ANOVA

Inoculation		Plant mortality (%)	Plant height (cm)	Plant width (cm)	No. of leaves 1st term	No. of flowers 2nd term	No. of flowers
AMF	0	28 a	13.9 b	33.0 a	49.6 b	8.3 a	9.8 a
	1	8 b	15.4 a	35.4 a	55.3 a	6.5 b	8.4 a
Trichoderma	0	25 a	14.1 b	33.7 a	51.5 a	5.7 b	7.2 b
	1	11 b	15.2 a	34.8 a	53.4 a	9.3 a	11.0 a
AMF × Trich	00	33 a	12.8 b	32.1 a	47.6 a	6.7 bc	8.1 bc
	01	23 ab	14.9 a	33.9 a	51.6 a	9.9 a	11.5 a
	10	16 b	15.3 a	35.2 a	55.4 a	4.7 c	6.3 c
	11	0 c	15.4 a	35.7 a	55.2 a	8.4 ab	10.5 ab

Means followed by the same letter are not significantly different within one factor and parameter according to Duncan's Multiple Range test, $p < 0.05$, $n = 40$ per treatment
0 = without inoculation, 1 = with inoculation

Table 3. The effect of inoculation with arbuscular mycorrhizal fungi (AMF) and *Trichoderma harzianum* (Trich) on *Cyclamen persicum* – destructive analysis, results of two-way and one-way ANOVA

Inoculation		No. of flowers	No. of leaves	Total leaf area (cm^2)	No. of flower buds	DW of leaves (g)	DW of flowers (g)
AMF	0	14.0 a	59.1 a	2477 b	22.0 b	20.0 a	5.7 a
	1	14.8 a	64.7 a	2750 a	27.6 a	22.0 a	6.3 a
Trichoderma	0	11.1 b	60.3 a	2461 b	23.9 a	20.0 a	5.1 a
	1	17.8 a	63.5 a	2767 a	25.6 a	22.0 a	6.9 a
AMF × Trich	00	11.3 b	56.3 a	2292 b	20.3 b	19.1 b	5.0 b
	01	16.8 a	61.9 a	2661 a	23.6 ab	20.8 a	6.4 a
	10	10.8 b	64.3 a	2629 a	27.5 a	20.9 a	5.2 b
	11	18.8 a	65.1 a	2872 a	27.8 a	23.2 a	7.4 a

Means followed by the same letter are not significantly different within one factor and parameter according to Duncan's Multiple Range test, $p < 0.05$, $n = 12$ per treatment
0 = without inoculation, 1 = with inoculation

increased the size and quality of plants and it improved their market value.

Both microbial partners were developed in plant cultures after inoculation. Mycorrhizal colonisation of cyclamen roots was 75.1 (*SD* 8.1)% of the root length colonized in mycorrhizal treatment (AMF) and 83.2 (*SD* 9.6)% in dually inoculated (AMF/Trich) treatment. Relatively extensive development of mycorrhizal symbiosis found in the cyclamen roots might be caused by the longer cultivation period compared with poinsettia. The root surface was colonised also by hyphae of *Trichoderma*, evaluation showed that in *Trichoderma* treatment (Trich) was 26.1 (*SD* 4.8)% of the root length colonised, and in dually inoculated treatment the colonisation was 24.3 (*SD* 7.9)%. Visual evaluation of the root systems development showed that the roots were fairly and sufficiently developed in all treatments of both plants, the whole volume of the pots was fully filled with roots and no differences between the treatments were found.

There were very few significant effects of inoculations on the growth of poinsettia (Tables 4 and 5). The mycorrhizal colonisation of poinsettia roots was relatively low, about 34% of the root length in the mycorrhizal treatment and 39% of the root length in dually inoculated plants were found to be colonised by AMF and the majority of the fungal structures were vesicles, which indicates rather symbiosis dormancy and less active AMF, probably due to the inoculation at late stages of plant development. Colonisation of the roots surface by hyphae of *Trichoderma* was 23.8 (*SD* 3.5)% in *Trichoderma* treatment in dually inoculated treatment the colonisation was 30.2 (*SD* 5.6)%.

Dual inoculation of cyclamen with AMF and *T. harzianum* inoculum is recommended. Adoption of this practice decreases the eventual mortality and loss of plants caused by the attack of pathogens and the negative influence of other stresses and in general improves the quality and market value of the plants. Only few significant effects of inoculation observed on poinsettia plants could be caused by later inoculation of cuttings, therefore the cuttings should be inoculated earlier to ensure more extensive development of mycorrhizal symbiosis. Potential of inoculation for poinsettia should be tested in further experiments where the plant cuttings will be inoculated before rooting.

Table 4. The effect of inoculation with arbuscular mycorrhizal fungi (AMF) and *Trichoderma harzianum* (Trich) on *Euphorbia pulcherrima* – non-destructive analysis, results of two-way and one-way ANOVA

Factor		Height (cm)	Width (cm)	No. of stems
AMF	0	26.1 a	44.6 a	4.0 a
	1	26.2 a	43.8 a	4.0 a
Trichoderma	0	26.6 a	43.8 a	4.0 a
	1	25.6 a	44.6 a	4.0 a
AMF × Trich	00	26.4 a	43.9 a	4.2 a
	01	25.7 a	45.3 a	3.8 a
	10	26.9 a	43.6 a	3.8 a
	11	25.6 a	43.9 a	4.3 a

Means followed by the same letter are not significantly different within one factor and parameter according to Duncan's Multiple Range test, $p < 0.05$, $n = 40$ per treatment
0 = without inoculation, 1 = with inoculation

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Table 5. The effect of inoculation with arbuscular mycorrhizal fungi (AMF) and *Trichoderma harzianum* (Trich) on *Euphorbia pulcherrima* – destructive analysis, results of two-way and one-way ANOVA

Factor		No. of bracts	No. of leaves	Total bract area (cm^2)	Total leaf area (cm^2)	DW of leaves	No. of stems (g)	Length of stems (cm)	DW of stems (g)
AMF	0	47.3 a	49.8 a	1273 a	1832 a	7.2 a	5.3 a	75 a	3.0 a
	1	49.7 a	55.8 a	1205 a	2255 b	7.6 a	5.9 a	82 a	3.0 a
Trichoderma	0	53.1 a	53.3 a	1425 a	2144 a	7.7 a	5.8 a	82 a	3.3 a
	1	43.9 b	51.4 a	1054 b	1943 b	7.1 a	5.3 a	75 a	2.8 a
AMF × Trich	00	51.5 a	51.5 a	1455 a	1977 ab	7.7 a	5.3 a	80 b	3.4 a
	01	43.2 a	48.0 a	1102 b	1688 b	6.7 a	5.2 a	70 b	2.7 a
	10	54.7 a	55.2 a	1404 a	2311 a	7.7 a	6.3 a	84 a	3.2 a
	11	44.7 a	54.8 a	1006 b	2199 ab	7.4 a	5.5 a	80 b	2.9 a

Means followed by the same letter are not significantly different within one factor and parameter according to Duncan's Multiple Range test, $p < 0.05$, $n = 40$ per treatment
0 = without inoculation, 1 = with inoculation

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ABSTRAKT

Inokulace brambořiku (*Cyclamen persicum*) a poinsetie (*Euphorbia pulcherrima*) arbuskulárními mykorrhizními houbami a *Trichoderma harzianum*

Kombinovaná inokulace zahradnického substrátu směsí čtyř arbuskulárních mykorrhizních hub a přípravku biologické kontroly na bázi houbové kultury *Trichoderma harzianum* pozitivně ovlivnila růst a kvetení brambořiku a podstatně snížila mortalitu rostlin způsobenou spontánní infekcí houbovou chorobou *Cryptocline cyclaminis*. Mortalitu rostlin snižovala i samostatná aplikace arbuskulárních mykorrhizních hub, oba přípravky aplikované samostatně ovlivnily růst rostlin pozitivně, i když méně výrazně. Podstatně menší vliv měla inokulace na růst poinsetií pěstovaných z řízků. Inokulaci mykorrhizními houbami, resp. duální inokulace v kombinaci s *Trichoderma harzianum* lze doporučit k omezení nepříznivých vlivů stresů životního prostředí při kultivaci některých zahradnických kultur.

Klíčová slova: *Cyclamen persicum*; *Euphorbia pulcherrima*; inokulace; arbuskulární mykorrhizní houby; růstová reakce na inokulaci; *Trichoderma harzianum*

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