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ORIGINAL PAPER

Response of mycorrhizal periwinkle plants to aster yellows phytoplasma infection

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Abstract The objective of our research was to assess if arbuscular mycorrhizal (AM) fungal colonization can modify the effect of infection by two aster yellows phytoplasma strains (AY1, AYSim) in *Catharanthus roseus* plants. Both phytoplasma strains had a negative effect on the root fresh weight, but they differed in symptoms appearance and in their influence on photosynthetic and transpiration rates of the periwinkle plants. AM plants showed significantly reduced shoot fresh weight, while the transpiration rate was significantly increased. AM fungal colonization significantly affected shoot height and fresh weight of the plants infected by each phytoplasma strains as well as the root system of plants infected with the more aggressive AYSim phytoplasma strain. Double inoculation did not reduce the negative effects induced with phytoplasma alone on the photosynthetic activity of phytoplasma-infected plants.

Keywords Arbuscular mycorrhiza · Aster yellows phytoplasma · *Catharanthus roseus*

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Introduction

Phytoplasmas are plant pathogenic, uncultured prokaryotes (class Mollicutes). They usually inhabit phloem sieve tubes and are transmitted from plant to plant by phloem-feeding insects, primarily leafhoppers. Phytoplasmas are associated with diseases of several hundred species of higher plants and are recognized as economically important pathogens. Phytoplasmas were first recognized as agents of plant diseases in 1967 (Doi et al. 1967), although many of the diseases they cause have been known much earlier. Aster yellows phytoplasma group (AY, 16SrI), now reclassified as the species "*Candidatus* Phytoplasma asteris", seems to be most widespread in Europe and North America. Phytoplasmas of 16SrI group have a very wide host range, and they have been detected in several crops (Seemüller et al. 1998; Lee et al. 2000; Marcone et al. 2000). Very recently, aster yellows has become the most devastating and commonly present phytoplasma disease in Poland (Kamińska et al. 2001, 2003; Kamińska and Dziekanowska 2002; Kamińska 2006). Phytoplasma infection of susceptible plants frequently induces symptoms of leaf yellowing, chlorosis, and other pathological changes closely related to infection stress.

Arbuscular mycorrhizal (AM) fungi are obligate symbionts that colonize the roots of most crop plants and play a key role in nutrient cycling in the ecosystem and also protect plants against environmental and cultural stress. Previous works have shown the positive effect of AM fungi in increasing tolerance to damage caused by soil-borne pathogens (Lindermann, 1994; Lingua et al. 2001), enhancing plant nutrition (Azcon-Aguilar and Barea 1997), and conferring protection against root lesion nematodes (López et al. 1997). AM fungi reduced the deleterious effect of *Verticillium dahliae* on pepper growth and yield (Idoia et al. 2004) but bioprotection against *Verticillium* wilt was

conditioned by plant phenology at the moment of pathogen attack. Experimental results evaluating the effect of AM fungi on diseases caused by other pathogens are not conclusive (Dehne 1982; Gernns et al. 2001).

The effect of AM fungi on diseases caused by phytoplasmas has been reported previously. Lingua et al. (2002) reported protective effects of *Glomus mosseae* against a stolbur phytoplasma disease of tomato. According to D'Amelio et al. (2007), *G. mosseae* slightly reduced the number of plants infected with chrysanthemum yellows phytoplasma and extended the life span of the affected plants. Beneficial effects of mycorrhiza on the shoot length of pear decline (PD) phytoplasma-infected pear rootstock as well non-PD-infected plants has been recorded by García-Chapa et al. (2004). Very recently, Musetti et al. (2007) indicated that two endophytic fungi, *Aureobasidium pullulans* and *Epicoccum nigrum*, reported as antibiotic producers and antagonists against different phytophatogenic microorganisms, induced cytological effects in phytoplasma-infected *Catharanthus roseus* plants, preventing phytoplasma movement in the host. In the present study, we have analyzed whether AM can modify periwinkle plant response to infection with aster yellows phytoplasma in *C. roseus*.

Materials and methods

Plant material, mycorrhiza, and phytoplasma inoculation

Seeds of *C. roseus* Don L. G. were germinated in washed sand. Three-week-old seedlings were transplanted to 11 pots containing a sterilized mixture of peat–sand–perlite (3:2:1). Periwinkle plants were inoculated with *G. mosseae* BEG 12 under the root when about 20 cm high (after 8 weeks of growth) using 0.25 g of commercial inoculum (Biorize, Dijon, France) per plant. All nonmycorrhizal and mycorrhizal plants were fertilized weekly with 200 ml of 5% Universal Green–Scotts Nutrient Solution during the first 3 weeks after inoculation. In addition, plants received water three times a week to prevent wilting.

Three weeks after AM fungal inoculation, the plants were graft-inoculated with phytoplasma (Kamińska and Śliwa 2005) using scions of infected periwinkle plants with one of the following phytoplasma strain of "*Candidatus* Phytoplasma asteris": (1) the Polish strain AYSim associated with lily leaf scorch disease (Kamińska and Śliwa 2008), and (2) Maryland aster yellows AY1 (Lee et al. 1998) associated with periwinkle virescence, kindly provided by Dr. I.-M. Lee (Beltsville, USA). Both phytoplasma strains had the same restriction profiles of 16S ribosomal RNA (rRNA) gene fragment, as checked by RFLP analysis. These strains had been previously transmitted from their original plants to *C. roseus* by grafting. The control plants were grafted in the same way using scions of healthy periwinkle plants.

Six treatments were set up: control plants (C) without any inoculation, mycorrhizal plants (AM-inoculated with the mycorrhizal fungus), phytoplasma-infected plants (PHY) with strain AY1 or AYSim, and phytoplasma-infected and mycorrhiza-colonized plants (AM PHY AY1 or AM PHY AYSim).

All AM and PHY plants and corresponding healthy controls were maintained in an insect-proof greenhouse at 25/15°C day per night and received natural daylight supplemented with irradiation from fluorescent lamps Son0T-Agro (Philips Netherland B.V., Eindhoven) that provided minimum photosynthetic photon flux of 300 µmol m^{-2} s^{-1} during a 14-h photoperiod.

Root staining for the evaluation of AM root colonization

The presence of AM fungi in five inoculated and five noninoculated *C. roseus* plants was demonstrated by root staining using a modified method of Koske and Gemma (1989), 75 days after inoculation. Live healthy roots from the central part of the pot were selected, washed thoroughly in distilled water, and cut into 0.5 cm long segments. Then, the roots were bleached in hot (90–100°C) 10% KOH and rinsed three times with distilled water. Next, roots were immersed in 10% HCl for 20 min and then stained in 0.05% trypan blue in acid glycerol solution at 90–100°C for 3 min. Roots were destained in acidic glycerol. Samples of roots were observed with a microscope (Nikon Eclipse 80i).

Phytoplasma detection and identification

Samples of *C. roseus* inoculated by grafting were tested for the presence of phytoplasma 16S rDNA by polymerase chain reaction (PCR). Total nucleic acids were extracted from periwinkles leaves using DNeasy Plant Mini Kit according to manufacturer's instruction (Qiagen, Syngen Biotech, Wrocław, Poland). Extracted nucleic acids were used as templates for direct PCR with universal primers P1/P7 (Deng and Hiruki 1991; Schneider et al. 1995). Products from the first PCR were diluted approximately 50 times and then used as templates in nested amplification with universal primer pairs R16F2n/R16R2 (Lee et al. 1998) and fA/rA (Ahrens and Seemüller 1992), as well as group 16SrI-specific primer pair R16(I)F1/R16(I)R1 (Lee et al. 1994). All the PCR assays were run under previously described parameters (Kamińska and Śliwa 2005). PCR products (5 µl) were analyzed by 1% agarose gel electrophoresis in 0.5 × TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.3) buffer followed by staining with ethidium bromide (0.5 µg·ml^{-1}) and visualized with UV transilluminator (Syngen Biotech, Wrocław, Poland).

Morphometry

Plant morphometry involved measurement of shoot height and shoot fresh weight, as well as root fresh weight and length. Measurements were performed at the end of the experiment on six plants per treatment. Root samples from each plant were collected and washed. The total root length was measured using an image analysis system WinRhizo (Regent Instrument, Canada).

Physiological status of plants

The physiological status of plants was assessed by measurement of photosynthetic activity (gas exchange method) of six plants per treatment. The gas exchange rates (net photosynthesis and transpiration) were measured on two young, fully expanded leaves from each plant using an LI-6400 portable photosynthesis system (LI-COR, USA). Temperature, CO_2 concentration, and irradiance in the leaf chamber during analysis were set to approximate ambient conditions. Measurements were performed 75 days after the commencement of the experiment.

Ca^{2+} concentration

Ca^{2+} concentration was assessed by measurement of six plants per treatment. Leaves and stems were rinsed with 0.01 M HCl, then with double-deionized water, dried at 60°C in a forced-draft oven, and ground in a Wiley stainless steel mill. In order to determine calcium, tissue samples were microwave digested (in microwave oven, model MLS 1200, Milistone Inc., Monroe, CT, USA) in HNO_3, using closed Teflon vessels. Calcium concentration was analyzed by atomic emission spectrometry with inductively coupled plasma at a wavelength of 317.9 nm in Central Analytical Laboratory of Research Institute of Pomology and Floriculture in Skierniewice.

Statistical analysis

Data were statistically analyzed using analysis of variance (ANOVA), followed by means separation using Duncan's multiple range *t* test. Calculations were performed with the Statistica 7.0 software package (StatSoft, USA).

Results

Mycorrhizal colonization

Microscopic observations of stained roots of *C. roseus* plants inoculated with *G. mosseae* indicated that all AM-inoculated periwinkles were successfully colonized by the mycorrhizal fungus. Typical fungal structures (mycelium, numerous arbuscules, and vesicles on longitudinal hyphae) were observed in samples of all AM-inoculated plants but not in the control ones.

Disease symptoms

Experimental inoculation of *C. roseus* plants with aster yellows phytoplasma Polish strain AYSim induced in 2–3 weeks after grafting leaf chlorosis and vein clearing. They followed leaf blade reduction and yellowing and flower discoloration. Periwinkle plants inoculated with the strain AY1 from the USA were infected, but they did not develop visible disease symptoms. The presence of phytoplasma strain in symptomatic periwinkle plants was indicated by direct PCR, while in asymptomatic periwinkle plants—by nested PCR.

Phytoplasma infection did not influence the shoot fresh weight of periwinkle plants. The shoot height was slightly reduced only of the plants infected with AY1 strain. Mycorrhiza formation did not influence shoot height of the healthy plants but had a negative effect on the shoot height of phytoplasma infected plants. Mycorrhiza formation significantly reduced the shoot weight of the control as well as phytoplasma-infected *C. roseus* plants (Table 1).

Root system

Our results showed that root fresh weight of phytoplasma-infected periwinkles was significantly reduced in comparison to the control plants (Table 1). Mycorrhiza formation did not influence root fresh weight of the healthy plants and had a negative effect on the root weight of phytoplasma-infected periwinkles, however, significant differences were found only for AYSim infected plants which showed disease symptoms. Infection with phytoplasma and/or colonization with the mycorrhizal fungus had no significant effect on the total length of roots of all tested periwinkle plants (Table 1).

Physiological status

Photosynthetic activity of *C. roseus* plants, symptomlessly infected with phytoplasma strain AY1, was at the same level as the control plants and significantly higher than that of plants infected with strain AYSim and showing pronounced disease symptoms (Table 2). Mycorrhiza formation did not influence photosynthetic activity of control or phytoplasma-infected periwinkle plants.

The transpiration rate of *C. roseus* plants infected with phytoplasma strain AY1 was significantly increased and higher than that of healthy plants or plants infected with strain AYSim (Table 2). Mycorrhiza formation significantly

Table 1 Effect of arbuscular mycorrhizal colonization (M) on growth-related parameters of *C. roseus* test plants infected with aster yellows phytoplasma strain AY1 or AYSim

Treatment	Shoot height (cm)	Shoot fresh weight (g)	Root fresh weight (g)	Root total length (cm)
Control	35.20 d	46.94 d	3.41 c	827.84 a
AY1	29.80 abc	40.45 cd	2.80 b	869.68 a
AYSim	31.20 bcd	45.20 d	2.73 b	859.26 a
M-control	32.40 cd	36.99 bc	3.32 bc	701.46 a
M-AY1	27.40 ab	31.02 ab	2.91 bc	705.84 a
M-AYSim	26.20 a	28.14 a	2.04 a	684.90 a
Phytoplasma	–[b]	–[a]	–[b]	NS
Mycorrhiza	–[b]	–[b]	NS	NS
Interaction	NS	NS	NS	NS

Within each column values were analyzed with two-way ANOVA with phytoplasma inoculation and mycorrhizal colonization as the main effects. Values followed by the same letter are not significantly different ($P \leq 0.05$)

NS Nonsignificant

[a] Significant at 5% level

[b] Significant at 1% level

influenced transpiration rate of periwinkle plants; it was increased in the case of healthy *C. roseus* and decreased in plants infected with phytoplasma strain AY1. Mycorrhiza did not change transpiration rate of symptomatic *C. roseus* plants infected with phytoplasma strain AYSim.

Calcium concentration

Periwinkle plants symptomlessly infected with phytoplasma strain AY1 had significantly higher concentration of calcium than the control plants or plants severely infected with strain AYSim (Table 2). Arbuscular mycorrhizal colonization had no significant effect on calcium concentration in phytoplasma infected or in healthy periwinkle plants.

Discussion

In this preliminary study, we have analyzed if arbuscular mycorrhizal fungi (AM) colonization can modify the effect of infection by two aster yellows phytoplasma strains in periwinkle plants. Both phytoplasma strains, the Polish strain AYSim from lily and the reference strain AY1 from periwinkle, USA, showed high 16S rRNA gene sequence homology with the "*Candidatus* Phytoplasma asteris"

Table 2 Effect of arbuscular mycorrhizal colonization (M) on rates of gas exchange and calcium content of *C. roseus* test plants infected with aster yellows phytoplasma strain AY1 or AYSim

Treatment	Photosynthetic rate (μmol CO_2 $m^{-2}s^{-1}$)	Transpiration rate (mmol H_2O $m^{-2}s^{-1}$)	Ca concentration (% in dry matter basis)
Control	10.85 b	2.29 b	1.82 a
AY1	12.13 b	3.37 c	2.35 b
AYSim	2.37 a	0.74 a	1.97 a
M-control	11.40 b	2.93 c	2.14 ab
M-AY1	11.11 b	2.10 b	2.12 ab
M-AYSim	3.35 a	0.89 a	1.96 a
Phytoplasma	–[b]	–[b]	–[a]
Mycorrhiza	NS	NS	NS
Interaction	NS	–[b]	NS

Within each column values were analyzed with two-way ANOVA with phytoplasma inoculation and mycorrhizal colonization as the main effects. Values followed by the same letter are not significantly different ($P \leq 0.05$)

NS Nonsignificant

[a] Significant differences at 5% level

[b] Significant differences at 1% level

(Kamińska and Śliwa 2005, 2008). However, in spite of the same results obtained in RFLP analysis of PCR-amplified fragments of the two phytoplasma strains, their biological properties were different. The reference strain of AY1 which previously was stable (Kamińska and Śliwa 2005) in the condition of our experiment in *C. roseus* plants induced symptomless infection. In these symptom-free plants, phytoplasma could be detected in the second but not in the first round of PCR. The second phytoplasma strain, AYSim, induced pronounced disease symptoms in grafted periwinkles, and it could be detected in the direct PCR.

The effect of phytoplasma infection on the growth of infected periwinkle plants depended on the pathogen strain. Both phytoplasma strains had similar negative effect on the root fresh weight and little or no effect on shoot and root growth of the test plants. However, the two phytoplasma strains used in the experiment differed mainly in symptom appearance and their effects on leaf gas exchange rate (net photosynthesis and transpiration rate). The more aggressive strain AYSim induced pronounced disease symptoms and affected photosynthesis and transpiration very severely. Simultaneously, AY1 phytoplasma strain enhanced transpiration rate of the symptomlessly infected plants. We speculate that symptom expression in phytoplasma-infected plants could contribute to the gas exchange rates.

The response of periwinkle to colonization by *G. mosseae* was generally less marked than in other species, and differences between AM-inoculated and noninoculated plants were less evident. In the conditions used in our experiments, AM-inoculated plants showed slightly modified growth. However, symptoms induced by the phytoplasma were less severe when the plants also harbored the AM fungus, and morphological parameters were closer to those of healthy plants. Lingua et al. (2002) indicated protective effects induced by AM fungi against a phytoplasma of the stolbur group in tomato. Beneficial effects of mycorrhiza on the growth of pear decline phytoplasma-infected pears were recorded by Garcia-Chapa et al. (2004), and some positive effect by AM fungi on the number of plants infected with chrysanthemum yellows phytoplasma and their life span was indicated by D'Amelio et al. (2007). In contrast to these studies, other reports have indicated an absence of AM typical growth effects and reduction (Trotta et al. 1996) or lack of modification (Vigo et al. 2000) in branching of colonized root systems. In our study, one of the parameters—shoot fresh weight—was significantly reduced, while the other—transpiration rate—was significantly increased by AM formation. The other evaluated parameters were not significantly modified in AM-inoculated plants when compared with the control plants.

The study indicated that some of the plant growth or physiological parameters were strongly and significantly modified by phytoplasma infection and AM formation in periwinkle plants. Double inoculation increased the negative effects induced by the pathogen alone, leading to plants significantly different from PHY plants. It was evident from the fresh weight of the tested plants infected by each phytoplasma strains as well as from their root system which was strongly reduced by the presence of the AM fungus and the more aggressive phytoplasma strain AYSim. Double inoculation did not reduce the negative effects on the photosynthetic activity induced by phytoplasma alone.

The present study provides evidence that periwinkle plants infected with the phytoplasma strain AY1 had higher calcium content when compared to noninfected ones or plants severely infected with strain AYSim. The higher calcium concentration in phytoplasma-infected periwinkles could be due to the activation of the defense mechanisms against pathogen. Many studies in the last two decades have clearly established that Ca^{2+} may be a second messenger in many developmental processes in plants, among which in stress adaptation to pathogens invasion (Rudd and Franklin-Tong 1999; Blume et al. 2000) and regulation of enzymes activity (Avdiushko et al. 1997). The results of our experiment confirm earlier studies with *C. roseus* and *Gladiolus* x *hybridus*, which showed that phytoplasma infection lead to the accumulation of calcium (Musetti and Favali 2003; Rudzińska-Langwald and Kamińska 2003). However, in our experiment, double inoculation with phytoplasma and the AM fungus had no effect on calcium concentration in periwinkle plants.

The decrease in shoot and root growths as well as photosynthetic activity in phytoplasma and AM fungal inoculated plants is possibly caused by hormone factors, which may explain many other symptoms such as virescence, phyllody, witches' broom, and stunting (Lee et al. 2000). AM fungi too have been shown to produce hormones, for instance cytokinin (Torelli et al. 2001), that could be responsible for several plant processes. Previous data have offered contrasting information concerning the bioprotective effect of AM fungi towards pathogens of the aerial part of plants (Dehne 1982; Lindermann 1994; Dugassa et al. 1996). Very recently, Musetti et al. (2007) demonstrated that fungal leaf endophytes induce ultrastructural changes associated with increases in host defense responses, such as formation of phloem protein plugs, callose occlusions, and presence of vacuolar phenolic depositions in the lumen of sieve elements. These modifications lead to the enhancement of physical barriers preventing phytoplasma movements in the host. Our results do not correspond with them, and they suggest that AM formation can decrease plant tolerance of phytoplasma infection, since plant growth as well as photosynthetic activity was negatively modified. Further studies are needed to determine the mechanisms responsible for decreased tolerance of AM plants to phytoplasma infection.

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