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Mycorrhiza-induced differential response to a yellows disease in tomato

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Abstract The protective effects induced by arbuscular mycorrhizal (AM) fungi against a phytoplasma of the Stolbur group have been investigated in tomato by morphometry and flow cytometry. Symptoms induced by the phytoplasma were less severe when the plants also harboured AM fungi. Morphological parameters such as shoot and root fresh weight, shoot height, internode length, leaf number and adventitious root diameter were closer to those of healthy plants when arbuscular mycorrhiza were present. Reduced nuclear senescence was observed in AM plants infected with phytoplasmas; the percentages of nuclear populations with different ploidy levels were intermediate between AM and phytoplasma-infected plants. The mechanisms underlying these interactions are discussed and a direct action of the AM fungus is hypothesized.

Keywords Arbuscular mycorrhiza · Phytoplasma · Yellows disease · Tomato

Introduction

Arbuscular mycorrhiza can induce resistance or increase tolerance to some root pathogens, such as nematodes or fungi (Azcon-Aguilar and Barea 1996, 1997; Trotta et al. 1996; Cordier et al. 1997). Contrasting results have been reported concerning the contribution of arbuscular mycorrhiza in plants infected by pathogens of the aerial part, where arbuscular mycorrhiza can be harmful or beneficial according to the various situations and different pathogens involved (Dehne 1982; Gange and West 1994; Lindermann 1994; Dugassa et al. 1996; Shaul et al. 1999).

Tomato is susceptible to yellows diseases, caused by phytoplasmas. These are wall-less prokaryotes and obligate parasites belonging to the order *Mollicutes*. They are naturally transmitted by insects and spread in the phloem of the host plant, where they take up nutrients and eventually cause plant death (Hiruki 1988; McCoy et al. 1989; Agrios 1997).

We investigated earlier the changes induced in tomato roots infected by the soil-borne fungus *Phytophthora nicotianae* var. *parasitica* when the arbuscular mycorrhizal (AM) fungus *Glomus mosseae* was also present. AM colonization induced a significant increase in the percentage of 8C nuclei, the population with the highest DNA content. Roots infected by *P. parasitica* showed a significant reduction in fresh weight and size (and of many other morphological parameters), together with a significant increase in the percentage of 2C nuclei. AM colonization can moderate these symptoms, leading to the development of plants similar to those not infected by the pathogen (Trotta et al. 1996; Lingua et al. 1996; Fusconi et al. 1997). Similar results concerning balance of the nuclear population (i.e. an increase in 2C nuclei) were found for phytoplasma-infected tomato (Lingua et al. 2001).

In the present work, we have analysed the effects of infection with a phytoplasma on mycorrhizal and non-mycorrhizal tomato plants. The aim was to verify, by means of morphometry and flow cytometry, whether AM colonization can also modify plant response to infection by phytoplasmas.

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Materials and methods

Plant growth and infection

Seeds of *Lycopersicon esculentum* Mill. cv Early Mech were germinated in Petri dishes on moist quartz sand for 3–4 days at 24°C.

The seedlings were then transferred to quartz sand (0.5 mm coarse grade) in 3-l nylon containers. The latter were placed in a glasshouse (min-max temperature 25–30°C, light/dark photoperiod 16/8 h, average light irradiation 300 µE m^2 s^{-1}) and watered on alternate days with 100 ml of half strength Long Ashton nutrient solution, according to Trotta et al. (1996). About 500 ml of sand was removed from half of the containers and replaced with 162 g inoculum of *Glomus mosseae* BEG 12 (Biorize, Dijon, France) mixed with the amount of quartz sand necessary to fill the gap. The inoculum was guaranteed by the producer to contain at least 60,000 propagules per kg of carrier (assessed by the MPN method).

Thirty days after sowing, 32 plants were side-grafted with scions of tomato infected with STOL-S1 as described by Minucci and Boccardo (1997), a phytoplasma of the 16Sr XII-A subgroup (Lee et al. 1998; Marzachì et al. 2000) isolated from Sardinian tomato (Lovisolo et al. 1982); 32 plants were grafted with healthy scions as controls. Four treatments were set up: control plants (C) without any inoculation; mycorrhizal plants (AM) inoculated with the mycorrhizal fungus; phytoplasma-infected plants (*Phy*); phytoplasma-infected and mycorrhiza-colonized plants (AMP).

The root systems were processed for morphometry, light and electron microscopy and flow cytometry 60 days after grafting.

Evaluation of mycorrhizal colonization

The degree of AM colonization was evaluated in terms of the M% and A% indices, according to Trouvelot et al. (1986), after random sampling 30 pieces of root 1 cm long.

Morphometry

Several morphological parameters were measured in eight plants per treatment. These were: shoot height, fresh weight and stem diameter, leaf number, internode number and length and root fresh weight. Ten adventitious roots per plant (a total of 80 adventitious roots) were analysed by morphometry. Digital images of the roots were acquired and the following parameters measured by the software MacRhizo 3.9 (Regent Instruments, Canada): total length, diameter and degree of branching.

Flow cytometry

Five plants per treatment were processed for flow cytometry. Nuclei were extracted from whole root systems, after removal of apices. The roots were chopped with a razor blade in a few drops of extraction buffer (0.1 M citric acid, 0.5% Tween 20). Nuclei were extracted after 30 min incubation in this buffer by filtering through a 20-µm nylon mesh. All steps were carried out on ice. The samples were fixed by adding two volumes of ice-cold ethanol-acetic acid (3:1) to one volume of nuclei suspension and stored at –20°C.

Aliquots (2 ml) of the fixed samples were centrifuged at 500 *g* on a 1 M sucrose cushion to eliminate the fixative and reduce the debris. The pellet was resuspended in 500 µl of PBS buffer (phosphate-buffered saline) prepared according to Sambrook et al. (1989).The nuclei were counted and diluted with PBS to a concentration of 5×10^5 ml^{-1} and then stained with saturating (5.6 µm) 4', 6-diamidino-2-phenylindole (DAPI, Sigma), a DNA-specific fluorochrome.

Polyscience Fluorsbryte beads (4.2 µm) were used as a standard to calibrate the instrument and to check instrumental stability. The beads were run before and after each sample in order to compare the intensities of fluorescence of the various samples.

A mercury arc lamp was used in conjunction with BG1 and UG1 filters and a TK420 dichroic mirror to select the exciting wavelength (UV) of a Partec PAS-IIIi flow cytometer (Partec GmbH, Münster, Germany). DAPI fluorescence was detected using a GG415 barrier filter. Data were acquired and analysed with Parteclist, the software included in the instrument package. All data were compared with a Student *t*-test (significance 95%).

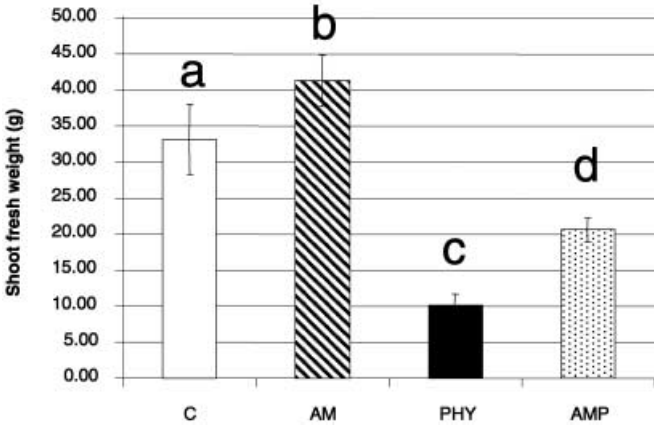

Fig. 1 Mean shoot fresh weight (g) in the four treatments. The absence of letters in common indicate statistically significant differences ($P \leq 0.05$). (*C* control, *AM Glomus mosseae* colonized, *Phy* phytoplasma, *AMP Glomus mosseae* + phytoplasma)

Light and electron microscopy

Two-hundred small segments were sampled from the adventitious roots of two plants per treatment. The segments were cut at 1 and 2 cm from the apex, at 1 and 2 cm from the root base and in the middle of the root. They were then fixed in 3% glutaraldehyde in 0.15 M phosphate buffer (pH 7.2) for 3 h at 4°C, post-fixed in 1% OsO_4 in the same buffer for 2 h at room temperature, stained in 1% uranyl acetate in water for 12 h at 4°C, dehydrated and embedded in Epon-araldite resin at 60°C. Semi-thin and thin sections were cut with a Reichert Ultracut E ultramicrotome. Semi-thin sections were stained with 1% toluidine blue in 1% sodium tetraborate and examined under a light microscope. Thin sections were examined under a Philips EM 201 electron microscope.

Results

Mycorrhizal colonization

The degree of mycorrhizal colonization (M%) and the relative abundance of arbuscules (A%) were 25.4%±5.2 (M%) and 20.3%±4.3 (A%) in AM plants, 23.7%±3.2 (M%) and 22.4%±2.7 (A%) in AMP plants. None of these differences were significant.

Morphometry

Shoot fresh weight was strongly and significantly reduced in *Phy* plants in comparison with C and AM. AMP plants were also reduced in weight, but their weights were significantly higher than those of *Phy* plants (Fig. 1). A similar pattern was observed for a number of parameters: shoot height, internode length, leaf number and adventitious root diameter (Fig. 2). The number of internodes and the root fresh weight were both reduced significantly in AMP and *Phy* plants compared with AM and C plants (Fig. 3A, B). Double infection (AMP) reduced adventitious root length compared with AM, while the pathogen alone did not (Fig. 3C).

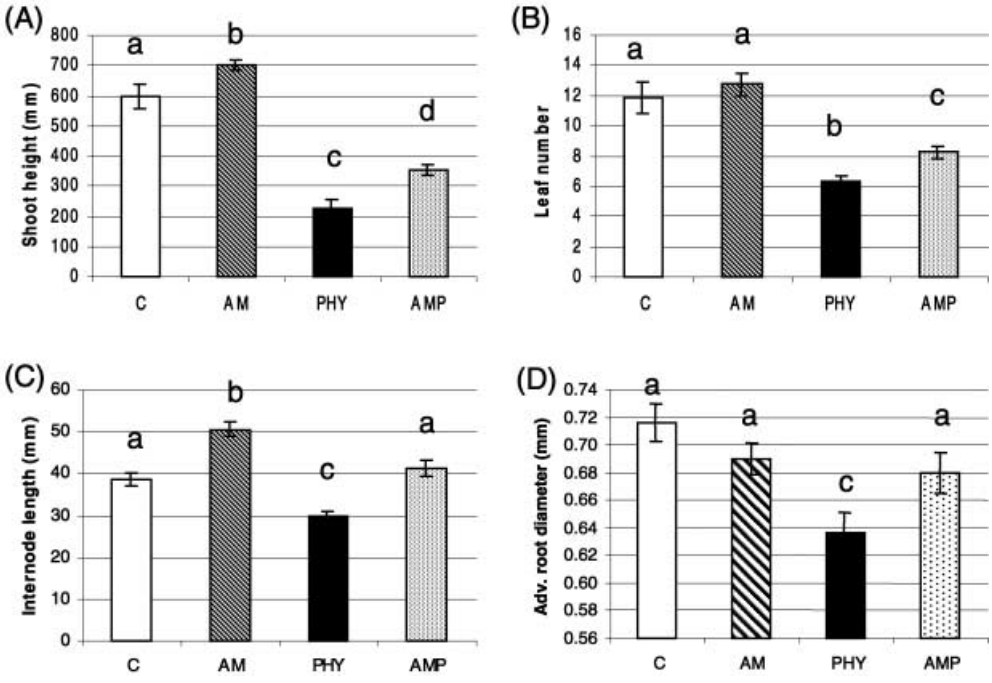

Fig. 2 Four different morphological parameters: **A** shoot height, **B** leaf number, **C** internode length and **D** adventitious root mean diameter in plants. The absence of letters in common indicate statistically significant differences ($P \leq 0.05$). Abbreviations as in Figure 1

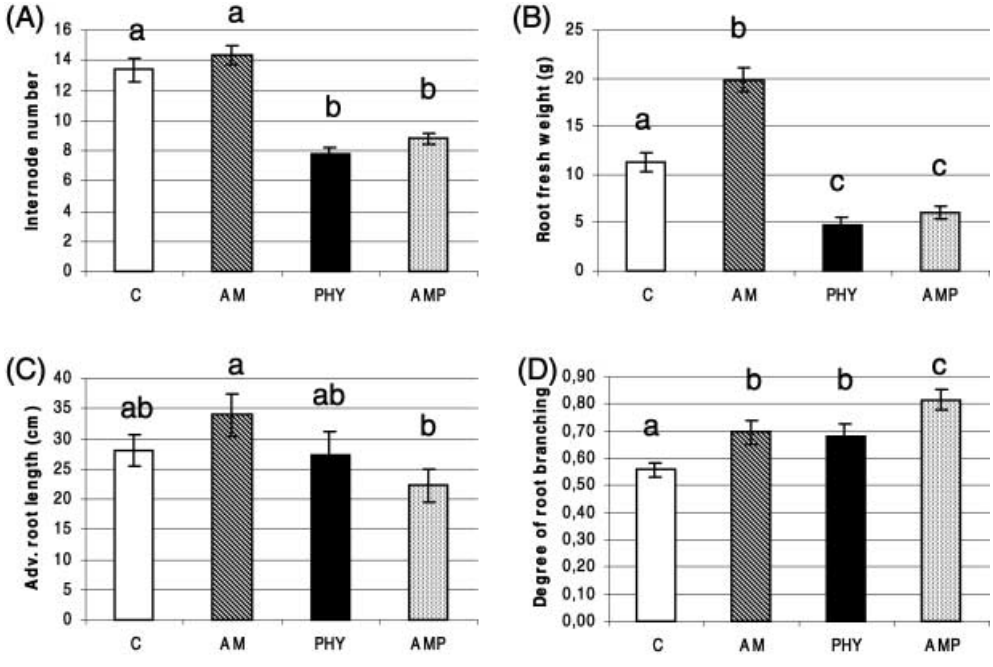

Fig. 3 **A** Internode number, **B** root fresh weight, **C** adventitious root length and **D** degree of branching in plants. The absence of letters in common indicate statistically significant differences ($P \leq 0.05$). Abbreviations as in Figure 1

Both AM and *Phy* induced an increase in the degree of branching, and double infection produced an additive effect (Fig. 3D).

Flow cytometry

Tomato is a multiploid species with three levels of ploidy 2C, 4C and 8C (Fusconi et al. 1997). About 20,000 nuclei for each treatment were analysed. In all populations, the intensity of fluorescence was maximal in the AM nuclei and minimal in the *Phy* nuclei. No significant differences were observed between C and AMP treatments (Fig. 4).

The highest percentage of 8C nuclei and the lowest percentage of 4C nuclei were found in AM roots. Infection with the pathogen (*Phy*) induced a significant reduction in 8C and increase in 2C nuclei relative to AM and C plants. Both these effects were reduced in AMP roots and nuclei extracted from AMP roots showed percentages intermediate between C and *Phy*. Differences in the proportions of the three nucleus populations between *Phy*, on the one hand, and C and AM, on the other, were significant (Table 1).

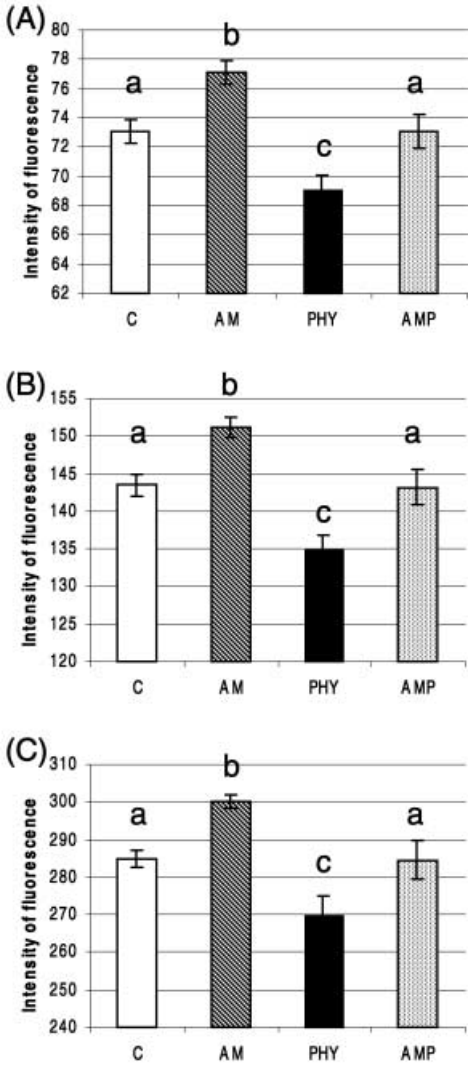

Fig. 4 Fluorescence intensity of nuclei extracted from roots, analysed by flow cytometry: **A** 2C nuclei, **B** 4C nuclei, **C** 8C nuclei, in plants. The absence of letters in common indicate statistically significant differences ($P \leq 0.05$). Abbreviations as in Figure 1

Table 1 Percentage and relative standard errors of the three nuclear populations (corresponding to 2C, 4C and 8C DNA content) in differentiated roots of tomato plants after four different treatments (*C*, *AM*, *AMP* and *Phy*). The absence of common letters indicate statistically significant differences among percentages in the same column ($P \leq 0.05$)

Treatment	2C nuclei	4C nuclei	8C nuclei
C	18.79±1.02 a	69.30±0.75 a	11.91±0.70 a
AM	18.11±1.07 a	65.80±1.68 b	16.09±1.85 b
Phy	23.88±1.61 b	67.90±1.26 ab	8.21±0.84 c
AMP	20.99±0.95 ab	69.62±0.70 a	9.35±0.75 ac

Light and electron microscopy

Phytoplasmas were observed in the sections from each sampling zone of both AMP and *Phy* roots (Fig. 5). The pathogens often filled the entire cell lumen and young elementary bodies of the phytoplasma were more common towards the apex of the root. The young elementary bodies were especially abundant in *Phy* roots (Fig. 6A), while they were rare in AMP roots (Fig. 6C).

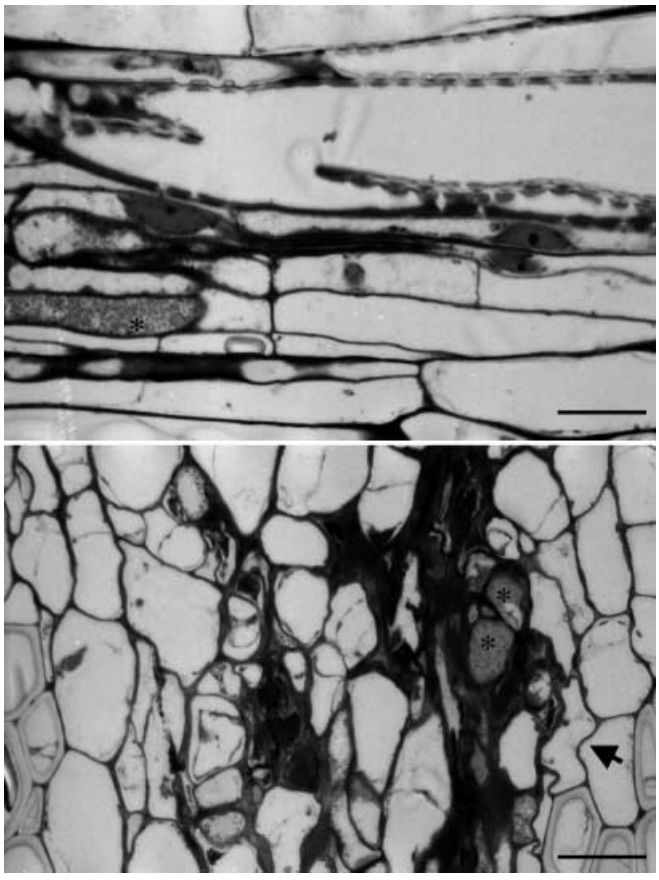

Fig. 5 Phytoplasmas in the adventitious roots of tomato plants, light microscopy. The upper section is from an AMP root. A sieve element is filled with phytoplasmas (*asterisk*); *bar* 20 µm. The lower section is from a *Phy* root. Some sieve elements are filled with phytoplasmas (*asterisk*). Note the distorted, anomalous cell wall of a cell of the parenchyma associated to the sieve elements (*arrow*); *bar* 20 µm

The host cells often showed symptoms not seen in the controls, such as distorted cell walls (Fig. 5B), deposition of callose near the sieve pores (Fig. 7), and pyknotic or chromatolitic nuclei with broken and condensed chromatin (Fig. 8).

In the AMP roots, phytoplasmas often showed features of degeneration, like agglutination (Fig. 6B), cytoplasm confined to the periphery of the cell and reduced number of ribosomes (Fig. 6C). Degenerated forms of phytoplasma were also observed in *Phy* roots. Nevertheless, they were constantly present in AMP roots, while they were not always observed in *Phy* roots. No phytoplasmas were detected in comparable tissue of C and AM roots.

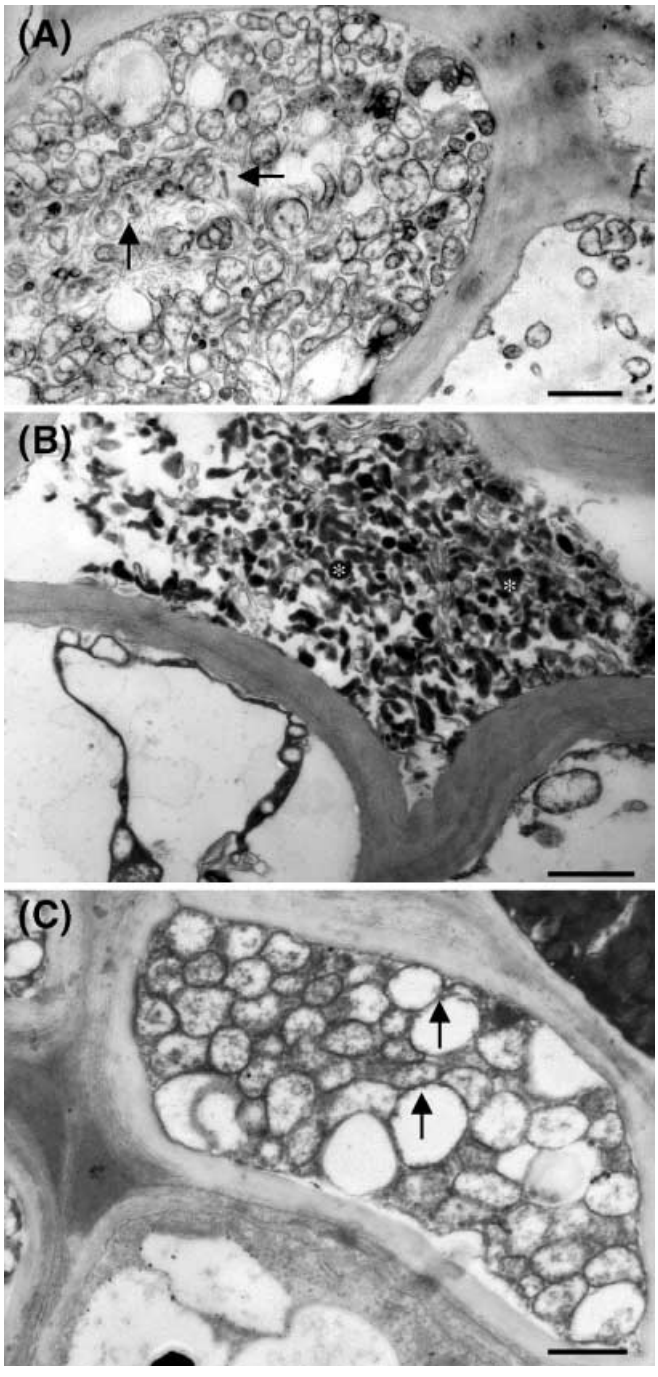

Fig. 6A–C Phytoplasmas in the adventitious roots of tomato plants, electron microscopy. **A** Section 2 cm from the root apex in a *Phy* plant. A sieve element filled with phytoplasmas in different developmental stages. Note some young elementary bodies (*arrows*); *bar* 1 µm. **B** Section 2 cm from the root apex in an AMP plant. Note the strongly osmiophilic and alterated phytoplasmas in the degenerated sieve cell (white asterisks); *bar* 1 µm. **C** Sections 2 cm from the root base in an AMP plant. Numerous mature phytoplasmas are seen in the phloem cells. Note cytoplasm confined at the periphery of the phytoplasma cells (*arrows*); *bar* 1 µm

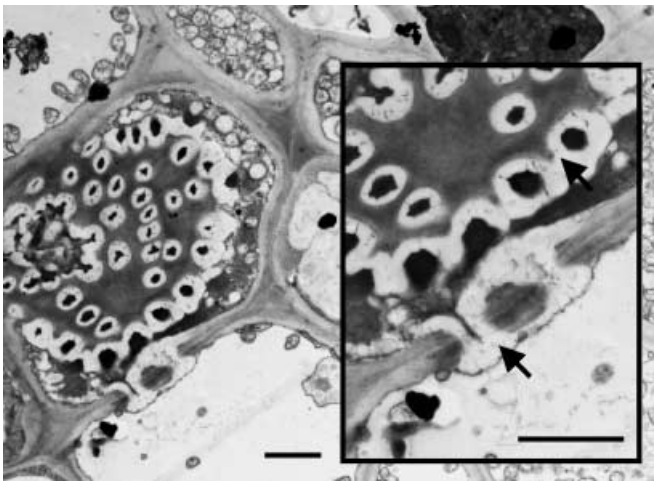

Fig. 7 The presence of callose at the sieve pores in *Phy* roots. *Inset* Note the accumulation of callose at the sieve pores (*arrows*); *bar* 2 µm

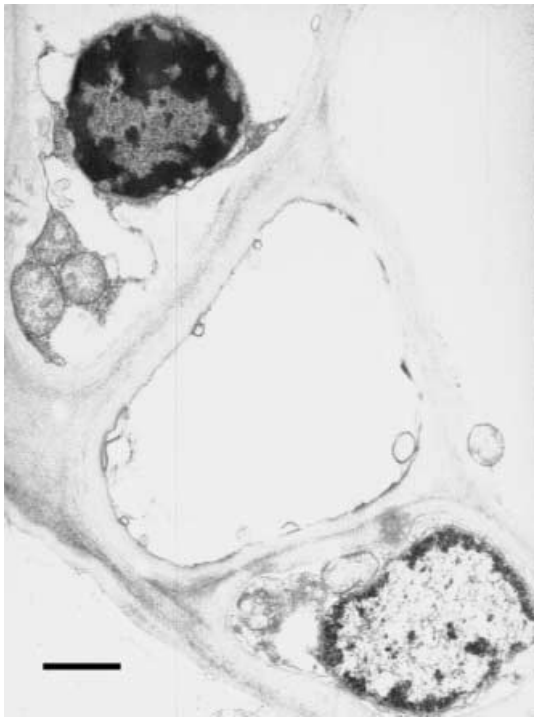

Fig. 8 Nuclei in *Phy* root cells. Note a pyknotic (*upper left corner*) and a chromatolitic nucleus (*lower right corner*); *bar* 10 µm

Discussion

For a long time, AM fungi were considered unable to affect development of the root system (Harley and Smith 1983). However, in the last 12 years it has been shown that colonization by AM fungi can induce several changes in root organization and longevity (Berta et al. 1990a; Schellenbaum et al. 1991; Hooker and Atkinson 1992; Hooker et al. 1995; Citernesi et al. 1998; Espeleta et al.

1999; Lingua et al. 1999). Modifications often resulted in a more-branched root system, with a larger proportion of smaller diameter, higher order roots (Hooker et al. 1995).

Results concerning the effect of pathogens on the root system are less abundant and the available literature is conflicting. It has been shown that *Cochioliobolus* and *Fusarium* reduce root branching in wheat (Kokko et al. 1995), while *Phytophthora nicotianae* var. *parasitica* (Trotta et al. 1996) and *Rhizoctonia solani* (Sampò et al. unpublished results) increased it in tomato. Hence, variation in degree of branching cannot be considered a reliable marker of stress or pathogenic infection.

Macroscopically, the response of tomato to AM colonization is generally less marked than in other species and differences between mycorrhizal and non-mycorrhizal plants are less evident. In the growth conditions used in our experiments, AM tomato plants showed improved growth. Previous reports indicated absence of an AM-typical growth effect and reduction (Trotta et al. 1996) or lack of modification (Vigo et al. 2000) in branching of colonized root systems. Our results only partly confirm published data. Shoot height, shoot and root fresh weight and degree of root branching were increased by AM colonization. Leaf number, internode number and adventitious root diameter were not significantly modified in AM plants compared with C plants. Many of these parameters were strongly and significantly affected by phytoplasma infection. The increase in degree of root branching observed in *Phy* plants cannot be related to an increased efficiency of nutrient absorption, as it was accompanied by a large reduction in root biomass. Double inoculation reduced the effects induced by the pathogen alone on several parameters, leading to plants more similar to *C* plants and significantly different from *Phy* plants. However, the root system was strongly affected by the presence of both microorganisms: the degree of branching was significantly increased and reached the highest level; but, again, there was a strong reduction in root mass. This increase in root branching in both *Phy* and AMP plants is possibly caused by hormonal factors, which may also explain many other symptoms such as virescence, phyllody, witches' broom and stunting (see Lee et al. 2000). AM fungi too have been shown to produce hormones that could be responsible for morphogenetic modifications. For instance, they can alter proton extrusion by plant cells and ethylene release (Vierheilig et al. 1994). Altered levels of IAA (Kaldorf and Ludwig-Muller 2000; Torelli et al. 2001), cytokinin (Torelli et al. 2001) and abscisic acid (Dannenberg et al. 1992; Esch et al. 1994) have also been reported in AM-colonized plants.

Young phytoplasma elementary bodies, similar to those reported in the literature (Hiruki 1988; D'Agostino 1991; Lherminier at al. 1994), were more common towards the apex of the root, but this morphotype was less frequent in AMP plants. Patterns of phytoplasma cell degeneration similar to those presented in this paper have been reported in non-mycorrhizal plants (Hirumi and Maramorosch 1972; Hiruki and Dijkstra 1973; Conti et al. 1988). Quantitative analyses could confirm that arbuscular mycorrhiza increase the frequency of phytoplasma degeneration and this would suggest that the improvement in the morphology and flow cytometry parameters of AMP plants over *Phy* plants is due to degeneration of the phytoplasmas.

Musetti et al. (1999) have shown in periwinkle that exogenous polyamine treatment is associated with agglutination and degeneration of phytoplasma cells in leaves. Polyamines and ethylene have a common precursor (S-adenosylmethionine) and AM fungi, as reported above, are known to affect the production of some hormones. Therefore, involvement of phytohormones in the degeneration of phytoplasmas and in the ability of AM plants to tolerate the infection seems possible.

Flow cytometry of extracted nuclei has been shown to be an effective tool for evaluating the physiological condition of a root system (Lingua et al. 1996, 2001). Measurements in this present work showed an increase in fluorescence intensity of the nuclei of AM plants over C plants at each ploidy level. This is consistent with previously published data from various mycorrhizal systems and is due to reduced nuclear senescence and reduced chromatin condensation in AM roots (Berta et al. 1990b, 1996; Sgorbati et al. 1993; Lingua et al. 1999). Chromatin decondensation is positively correlated with increased transcriptional activity (Berta et al. 1996). The pathogen significantly reduced the intensity of fluorescence, in agreement with previous results with the same experimental system (i.e. tomato infected with phytoplasmas) and also tomato infected by the fungal root pathogen *Phytophthora nicotianae* var. *parasitica* (Lingua et al. 1996, 2001). The reduced intensity of fluorescence is correlated with DNA loss, increased chromatin condensation and other phenomena depending on nuclear senescence (like chromatolysis and pyknosis), which are more frequent in pathogen-infected cells (Lingua et al. 1996, 2001) and significantly reduced in mycorrhizal roots (Lingua et al. 1999). Mycorrhizal colonization completely relieved this symptom, as AMP plant nuclei showed fluorescence values identical to those of *C* nuclei.

The proportion of the various nuclear populations is a marker of pathogenic attack in the roots of tomato. Different pathogens may cause an identical effect, i.e. increase in nuclear populations with the lowest DNA content (2C) (Lingua et al. 1996, 2001). This result might be due to a block of endoreduplication. In the present work, we report similar results for *Phy* roots, where the block occurred not only at the transition from 2C to 4C DNA content, but also at the transition from 4C to 8C, as shown by reduction of the percentage of 8C nuclei in *Phy* roots over C and AM. Again, mycorrhizal colonization was able to restore a condition in *AMP* roots nearer to that of the C roots. However, AMP roots failed to reach the high percentage of 8C typical of AM roots. Polyploidization is common in various symbiotic systems (Williamson and Hadley 1969; Hadley and

Williamson 1972; Meijer 1982) and should be related to increased cell size (Meleragno et al. 1993), high metabolic activities (Berta et al. 2001), rearrangement of DNA organization (Wolffe 1991; Baluska et al. 1995) and silencing or overexpression of some genes (Mittelsten Scheid et al. 1996; Galitski et al. 1999).

Previous data have offered contrasting information concerning the bioprotective effect of AM towards pathogens of the aerial part of the plants (Dehne 1982; Gange and West 1994; Lindermann 1994; Dugassa et al. 1996). Our results suggest that AM colonization can increase plant tolerance of phytoplasma infection, as the severity of several symptoms was reduced. However, the mechanisms responsible for improved tolerance have to be elucidated. Further investigations are needed also to find out whether and how fruit yield is affected.

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