

The reduction in proline buildup in mycorrhizal plants affected by nematodes

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Abstract

Plants stressed by pathogens activate a variety of defense mechanisms to survive. The osmoprotector amino acids, including proline, are among these defense mechanisms. In this work, the effects of arbuscular mycorrhizal fungi on plants infested by root-knot nematodes were evaluated with regard to the accumulation of the osmoprotectant proline. A 2x3 factorial design was established with 8 treatments – with and without nematodes, with and without mycorrhizae, and with and without fertilizer application – with 4 replicates. Two weeks after inoculation with arbuscular mycorrhizal fungi, the plants were infested with 4 nematode egg masses, and 8 weeks later, the plants were harvested. The inoculation with the arbuscular mycorrhizal fungi significantly reduced the proline content, with the non-inoculated plants exhibiting a higher concentration. Neither the infestation of the nematodes nor the addition of fertilizer significantly affected the proline content. Plant height, stem diameter, leaf area, number of leaves, and fresh weight were significantly improved by the presence of the arbuscular mycorrhizal fungi. The interaction of the fungi and the fertilizer did have a significant effect for height and leaf area. The nematode infestation and the fertilization did not affect mycorrhizal colonization.

Keywords: mycorrhizae, proline, *Meloidogyne incognita*, *Impatiens balsamina*, stress.

1. Introduction

Arbuscular mycorrhizal fungi (AMF) play a significant role in plant physiology because they increase nutrient uptake and modify plant metabolism, which leads to a reduced response to stress and increased resistance to pathogen attacks (García-Rodríguez *et al.*, 2005; Hause *et al.*, 2007).

Phytoparasitic nematodes and AMF frequently colonize root tissues, and both types of organisms display the same seasonal dynamics; this spatial and temporal coincidence increases the likelihood of interaction (Ingham, 1988). Both organisms affect the host's physiology; however, nematodes cause a pathogenic stress to the plant (Fatemy *et al.*, 1985), whereas AMF may improve stress tolerance (Beltrano and Ronco, 2008).

A number of metabolites and survival defense mechanisms are activated in plants subjected to environmental stresses such as drought, salinity, or pathogen attack (Shulaev *et al.*, 2008). Previous research has revealed that mycorrhizal plants show a higher tolerance to environmental stress (Porcel *et al.*, 2007; Ruiz-Lozano and Azcón, 1995). As a stress response, some substances are synthesized by the plant in response to stress conditions, including osmoprotector amino acids (Hassan *et al.*, 1994) such as proline, which may increase stress tolerance to the plant (Shulaev *et al.*, 2008). Proline content has been shown to vary between mycorrhizal and non-mycorrhizal plants (Ruiz-Lozano and Azcón, 1995; Saglam *et al.*, 2008); thus, proline content may serve as an interesting parameter by which to evaluate the effect of microorganisms on plants.

Given that mycorrhizal symbiosis could represent a mechanism by which stress tolerance is increased in plants. The influence that the fungal symbiosis may have on a plant's defense strategies in response to pathogenic attack may provide more detailed infor-

mation related to the mechanisms involved in the interaction among mycorrhizal fungi, the pathogen and the plant stress responses this tripartite interaction.

2. Materials and methods

2.1 Experimental design and statistical analysis

This experiment included a 2x2x2 factorial design; each factor included two levels as follows: with/without nematodes, with/without mycorrhizae (MTZ-UV consortium), and with/without fertilizer. These conditions resulted in 8 treatments: control (C), with mycorrhizae (M), nematode plus mycorrhizae (MN), fertilizer plus mycorrhizae (MF), mycorrhizae plus nematode plus fertilizer (MNF), nematode (N), nematode plus fertilizer (NF) and fertilizer (F). Each treatment included four replicates. Data were subjected to a factorial analysis of variance (Kavanova *et al.*, 2006) with 8 treatments followed by Fisher's least significant difference (LSD) test.

2.2 Substrate

A mixture of soil, sand, red volcanic stone and peat moss (2:1:1:1 v/v) was sterilized with 0.38 g/L dazomet. This mixture was characterized by a pH of 5.4 (10 g soil in 25 mL water) and with N, P and K levels of 45.38, 5.3 and 40 mg kg⁻¹, respectively.

2.3 Nutrient addition

A nutrient solution including N, P and K was used in the fertilized treatment to achieve levels of 150 mg kg⁻¹ N, 32.23 mg kg⁻¹ P and 40.67 mg kg⁻¹ K (using a triple-17 formulation including NH₄NO₃, NHPO₄ and KNO₃ as a nutrient source).

2.4 Plant selection

Impatiens balsamina L. was used as model plant because its positive response to AMF inoculation has been previously demonstrated. Additionally, despite being a host of the gall-forming nematode, this plant resists infestations without dying, a tolerance that enables it to complete its life cycle. The roots of this species are scarcely pigmented, enabling AMF and nematode galls to be easily observed.

2.5 AMF inoculation

An inoculum (MTZ-UV) was used that consisted of 8 AMF species of the genera *Glomus*, *Acaulospora*, *Gigaspora* and *Scutellospora* propagated through the modified Sieverding technique (Sieverding, 1991).

2.6 Nematode inoculum

Meloidogyne incognita was used for these experiments. The inoculum was obtained from wild *I. balsamina* plants (McSorley and McGovern, 2001). Plants were inoculated with nematode eggs 15 days after AMF inoculation. Four egg masses were applied per sprout into pits in the substrate near the shoot base (0.5 cm from the shoot and 1.5 cm deep) (Sunil *et al.*, 2007). This four egg masses gave approximately 500 juvenile (J2) nematodes, and according to Zahid *et al.* (2001) that is the minimum number of juveniles for a potential infection

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2.7 The assessment of variables

Plants were harvested 10 weeks after AMF inoculation. Physical variables and mycorrhizal colonization were recorded. Proline was measured in the shoot 2 days after harvesting using the Bates (1973) technique; this amino acid was not analyzed in roots because the presence of nematodes and fungi may affect proline concentrations (Verbruggen *et al.*, 1993).

3. Results

The effects on growth promoted by inoculation with the MTZ-UV consortium were evident 30 days after AMF inoculation (Table 1), with colonization ranging between 25% and 65%. All *I. balsamina* plants infested with *M. incognita* exhibited galls in the root system. However, only those with no mycorrhizae showed noticeable symptoms of disease, including wilting, leaf yellowing, severe root damage and the presence of galls; in contrast, non-infested plants displayed no symptoms of disease (Table 1).

Table 1. The mean values of the measured parameters in *Impatiens balsamina* plants inoculated with *Meloidogyne incognita* (N), AM fungus (M) and fertilization (F) as the main factors and their interactions.

Variable	C	F	N	NF	M	MF	MN	MNF
Leaf area (cm ²)	0.6c	0.46c	0.56c	0.43c	13.04b	27.51a	9.2b	25.88a
Leaf number	3.25d	3.33d	2.33d	2.33d	6.67c	12.0b	7.0c	16.0a
Leaf fresh weight (g)	0.04d	0.07d	0.04c	0.04d	0.67c	1.03b	0.46c	1.53a
Height (cm)	1.16c	1.56c	1.23c	1.26c	2.96b	5.56a	2.43b	5.33a
Diameter (cm)	1.2d	1.4cd	1.5d	1.49d	2.8b	2.75b	2.03c	3.4a
AM fungal colonization [~]	-	-	-	-	49.4b	24.47d	38.19c	66.31a
Proline (µg mL ⁻¹)	3.42a	2.21ab	3.59a	2.19ab	0.69b	1.15b	0.72b	0.69b

Different letters denote significant differences of a multiple-range test. Treatments without AMF were not included in the analysis of variance. C= Control plants.

3.1 The response of growth variables

The AMF had a significant effect on all the variables measured (Table 2). Inoculation with the nematodes did not lead to any significant differences in growth variables (Table 2). With regard to the interaction between factors, the MN and NF treatments caused no noticeable differences in growth variables (Table 2).

The presence of the nematodes affected only the root colonization percentage (Table 2). The addition of the fertilizer had a significant effect on all the variables except for the diameter (Table 2). The proline content was significantly affected by the M treatment, whereas neither the fertilizer nor the presence of nematodes affected the proline content. The interaction of M and F had a significant effect on the variable.

Table 2. The probability (P) values from analyses of variance of the measured parameters in *Impatiens balsamina* plants inoculated with *Meloidoyne incognita* (N), AM fungus (M) and fertilization (F) as the main factors and their interactions.

Variable	M	N	F	NF	MN	MF	MNF
Leaf area (cm ²)	***	NS	***	NS	NS	***	***
Leaf number	***	NS	***	NS	NS	***	***
Leaf fresh weight (g)	***	NS	***	NS	NS	**	***
Height (cm)	***	NS	***	NS	NS	***	***
Diameter (cm)	***	NS	***	NS	NS	NS	***
AM fungus colonization [~]	-	-	-	-	**	***	**
Proline (µg mL ⁻¹)	***	NS	NS	NS	NS	**	NS

*: P < 0.05; **: P < 0.01; ***: P < 0.001. Treatments without AMF were not included in the analysis of variance. AMF: arbuscular mycorrhizal fungi; NS: no significance.

4. Discussion

AMF inoculation improves plant development, as shown in several reports in which a great variety of species have responded favorably to mycorrhizae (Jaizme-Vega and Rodríguez-Romero, 2004; Talavera et al., 2001).

In the present investigation, mycorrhizal inoculation resulted in an increase in plant tolerance to nematode attacks in agreement with the findings of other reports (Talavera et al., 2001). Plants inoculated with AMF displayed good development and, hence, were more resistant to that stress because vascular cylinders are clogged and the root area decreases in plants infested by nematodes (Agris, 1989). AMF provide ben-

efits to the plant, likely through root hydration mechanisms strengthened or mediated by fungal hyphae (Hardie, 1985; Marulanda et al., 2003; Ruiz-Lozano and Azcón, 1995), facilitating mineral nutrient absorption (Varma, 1995) or through morphological changes in roots caused by AMF (Kothari et al., 1990). Such effects of mycorrhizae enable plants to maintain a better hydration and nutrition status at all times, even in the presence of the parasite (Augé, 2001). This result demonstrates the ability of AMF to offset the effects of nematodes when a certain mycorrhizal inoculation level is reached. Saleh and Sikora (1984) demonstrated that to achieve some degree of pathogen control, the mycorrhizal colonization of 38% of the root system is required, although this percentage may vary

depending on the host species, the symbiont species and other biotic and abiotic conditions. The percent colonization observed in the present work (Table 1) was higher than those reported by Saleh and Sikora (1984); hence, the level of colonization here can be deemed sufficient to offset the damage caused by the pathogen. Although AMF-inoculated plants displayed galls, these showed no signs of damage in the shoot.

This investigation assessed biochemical aspects on plant tolerance to a pathogen as a result of AMF inoculation. Mycorrhized plants displayed a rise in biomass production (586%) over the course of 50 days following nematode inoculation. However, Melakeberhan and Webster (1993) note that biomass loss due to nematode infestation depends on the infestation level, larval stage, reproductive potential and duration of the infestation.

In this study, a considerable reduction in proline content was observed in AMF-inoculated plants. Similar results have been reported in the case of water stress, with mycorrhizal plants showing lower proline levels in the shoot (Ruiz-Lozano and Azcón, 1995; Saglam *et al.*, 2008). Although the stress derived from nematode attacks involves mechanisms that differ from those associated with water deficit, since plant vascular cylinders are clogged when nematodes infest the root (Agrios, 1989), thereby altering root functioning and reducing water uptake, which leads to water stress and nutrient deficit.

Although proline is accumulated in stressed plants, it is normally present at certain levels (Grote *et al.*, 2006; Masadeh, 2005), either because of a nutrient deficit, light intensity, shifts in temperature, salinity, anaerobioses, air pollution or UV radiation (Deuschle *et al.*, 2004; Hare and Cress, 1997).

The decrease in proline content in mycorrhizal plants, even in the presence of the pathogen, may be related to the mycorrhizal function of exerting a qualitative and quantitative influence on flavonoid content

and metabolism (Harrison and Dixon, 1993), thereby reducing proline synthesis and use, although this parameter was not measured. Plants inoculated with the AMF displayed a lower concentration of proline, which may indicate a lower stress level in the plant under normal conditions, as observed by Hare and Cress (1997). This lower stress stage may be related to a better nutritional status (Cantrell and Linderman, 2001).

The findings reported here may indicate that the presence of inorganic nutrients without mycorrhizae failed to produce better results in terms of growth variables as reported previously (Endlweber and Scheu, 2006; Rodríguez-Romero *et al.*, 2005). An increase in nutrient intake by the plant is likely when it is colonized by AMF, even in the presence of the nematode, a condition that results in an impaired root system. The proper development of plants in this experiment suggests that the mycorrhizae promoted an adequate nutrient intake, given that AMF-inoculated plants grew even under nematode infestation.

Impatiens balsamina is a highly mycotrophic species. Because its roots are scarcely pigmented, they are readily stained, enabling AMF structures to be easily observed. Additionally, the tolerance of this plant to gall-nematode attacks, its rapid growth and high reproductive effort jointly suggest a high capacity to withstand stress (because it survives in heavily disturbed environments). Therefore, this species represents an attractive model for investigating nematode-mycorrhiza interactions.

Impatiens balsamina has been proposed by McAbee *et al.* (2005) as a suitable species for studying the diversification in the integument morphology within the genus *Impatiens*. Furthermore, this species has been used as a model for assessing the flowering process and floral reversion (Battley and Lyndon, 1986, 1988, 1990; Pouteau *et al.*, 1995, 1997, 1998), all of which support the suitability of this species for a number of studies.

5. Conclusions

Overall, the mycorrhizal plants improved the growth variables, despite the presence of nematodes, when the fertilizer was present. Mycorrhizal plants produced less proline. In contrast, the concentration of the proline the amino acid increased in plants infested by nematodes. These results support the hypothesis that mycorrhizae contribute to both the nutrition, expressed in growth, and the production of secondary compounds, associated with the decreased effect of the pathogen. *Impatiens balsamina* was successfully used as a model plant to study the interaction between AMF and nematodes because it exhibited a good response to the mycorrhizal effect in 20-30 days; a short life cycle; low pigmentation in the roots, which aids in observing the microorganisms; and the ability to tolerate nematode infestation enough to complete its life cycle.

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