ARBUSCULAR MYCORRHIZAL FUNGI AND THE OCCURRENCE OF FLAVONOIDS IN ROOTS OF PASSION FRUIT SEEDLINGS

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ABSTRACT: Composition and the role of root flavonoids in the regulation of mycorrhizal symbiosis are still poorly understood. Several flavonoids stimulate spore germination, mycelia growth and root colonization by arbuscular mycorrhizal fungi (AMF), and both root colonization and flavonoid composition are affected by plant nutritional status. Effects of AMF on the occurrence and content of aromatic secondary metabolites in the roots of passion fruit seedlings grown under two levels of phosphorus (P) fertilization (10 and 50 mg kg^{-1} of phosphorus) was studied. Seedlings were inoculated with *Glomus clarum* and a population of native fungi from a passion fruit plantation. Methanolic extracts of passion fruit seedlings roots were analyzed by high performance liquid chromatography (HPLC). It was recorded the occurrence of several compounds, possibly flavonoids, with seven major peaks. The root contents of the compound with a retention time of 4.5 minutes, varied in response to the root colonization by different mycorrhizal fungi, and the contents of two compounds with retention times of 3.4 and 18.9 minutes varied due to the poor plant growth and nutritional status. Passion fruit seedlings have several aromatic compounds, and their contents were correlated with root colonization by different mycorrhizal fungi, the reduced seedling growth due to nutritional stress, and/or the plant defense responses to the fungi.
Key words: phenolic compounds, mycorrhizal plants, phosphorus nutrition

FUNGOS MICORRÍZICOS ARBUSCULARES E A OCORRÊNCIA DE FLAVONÓIDES EM RAÍZES DE MUDAS DE MARACUJAZEIRO AMARELO

RESUMO: Os flavonóides nas raízes e seu papel na regulação da simbiose com fungos micorrízicos não são bem conhecidos. Vários flavonóides estimulam a germinação de esporos, crescimento micelial e colonização micorrízica. Ambos, a colonização micorrízica e a composição de flavonóides nas raízes são afetados pelo estado nutricional da planta. Avaliou-se o efeito de fungos micorrízicos arbusculares sobre a ocorrência e concentração de substâncias, possivelmente metabólitos aromáticos secundários pertencentes à classe dos flavonóides, em raízes de mudas de maracujazeiro amarelo, crescidas sob dois níveis de fósforo (10 e 50 mg kg^{-1}). As mudas foram inoculadas com *Glomus clarum* e uma população nativa de fungos micorrízicos de um plantio de maracujá do município de São João da Barra, RJ. Extratos de raízes em metanol foram analisados por cromatografia líquida de alta eficiência. Os cromatogramas mostraram a ocorrência de diversos compostos, possivelmente da classe dos flavonóides, diferenciados no cromatograma em sete picos principais. Foi observado que os conteúdos nas raízes do composto com tempo de retenção de 4,5 minutos, variou em função da colonização micorrízica pelas diferentes espécies de fungos e o conteúdo dos dois compostos com tempo de retenção de 3,4 e 18,9 minutos, variou com o baixo crescimento da planta e o estresse nutricional. Mudas de maracujazeiro apresentam diversos compostos aromáticos, possivelmente da classe dos flavonóides, e o seu conteúdo varia com a colonização radicular por diferentes espécies de fungos micorrízicos, o baixo crescimento da planta devido a estresse nutricional de fósforo e/ou a resposta de defesa da planta ao fungo.
Palavras-chave: compostos fenólicos, plantas micorrizadas, fertilização fosfatada

INTRODUCTION

Plants can synthesize several phenolic compounds in their tissues during growth. Flavonoids are phenolic compounds with important role in plant pigmentation, the structure of the cell wall, protection against ultra-violet radiation, symbiotic or pathogenic plant-microorganism interactions and in the interaction

between parasitic plants (Vierheilig & Piché, 2002; Koes et al., 1994).

Certain phenolic compounds induce the expression of genes and act as signals for the interaction *Rhizobium, Bradyrhizobium* and legumes (Lum & Hirsch, 2003; Hirsch & Kapulnik, 1998; Koes et al., 1994). However, the role of these secondary metabolites in mycorrhizal symbioses and tolerance of mycorrhizal plants to pathogens and other environmental stresses is poorly understood (Vierheilig & Piché, 2002; Gadkar et al., 2001; Siqueira et al., 1991). Nevertheless, it has been demonstrated that phenolic compounds, especially the flavonoids, stimulate spore germination, micelial growth and ramification of several arbuscular mycorrhizal fungi-AMF (Aikawa et al., 2000; Romero & Siqueira, 1996; Batista & Siqueira, 1994; Bécard et al., 1992), as well as the development of root colonization (Silva-Júnior & Siqueira, 1998; Kaminski et al., 1994).

Variations in flavonoid contents of plants also occur in response to nutritional status (Coronado et al., 1995). Most phenolic compounds are considered stress metabolites and their accumulation in plant parts is affected by nutritional deficiencies, carbon and nutrient balance, hormonal changes, abiotic stresses, plant age and development (Siqueira et al., 1991). The relationship between plant host nutrition, especially phosphorus and nitrogen, accumulation of phenolic compounds and the formation of mycorrhizal associations, demand additional, in-depth work.

Therefore, studying the occurrence and contents of phenolic compounds in roots of passion fruit seedlings inoculated with AMF, under different levels of phosphorus fertilization, is the objective of this paper.

MATERIAL AND METHODS

The experiment was carried out under greenhouse conditions, in Campos dos Goitacazes, RJ, Brazil (21°25' S, 40°55' W). Growing substrate was a 1:2 (v/v) mixture of sieved soil and washed sand fumigated with methyl bromide (Bromex), with the following chemical and textural characteristics: sand- 76%; fine sand- 6%; silt- 10%; clay- 8%; organic carbon- 8.0 g kg^{-1}; pH in water (1:2,5)- 5.6; P- 6 mg kg^{-1} (Melhich); K- 0.87 mmolc kg^{-1}; Ca- 16 mmolc kg^{-1}; Mg- 11 mmolc kg^{-1}; Al- 1 mmolc kg^{-1}; H-total 18 mmolc kg^{-1}; Na- 0.8 mmolc kg^{-1}. Phosphorus was added (10 and 50 mg kg^{-1} of P) as K_2HPO_4 or KH_2PO_4, in an aqueous solution and the potassium (100 mg kg^{-1} of K) as KCl, in an aqueous solution. Planting bags (0.5 kg substrate) were fertilized individually and each one considered an experimental plot.

Passion fruit seeds were surface sterilized with a 0.5% sodium hypochloride solution for 15 minutes, washed three times with sterile water and set to germinate in Petri plates with humidified and sterilized filter paper, in germination chambers with day and night temperatures alternating between 20 and 30°C, respectively, for seven to ten days. Three pre-germinated seeds were planted per seedling bag. One week after planting, only one plant was left per seedling bag.

The mycorrhizal inoculation was performed at planting. Forty cm^3 of a mixture of soil with spores and colonized roots of the AMF inoculum (*Glomus clarum and* the native population of mycorrhizal fungi) was placed 2-3 cm bellow the seeds. The inoculum was produced in sterile substrate having *Brachiaria brizantha* as host plant, for a period of five months. The native inoculum was produced with soil samples collected at 0-15 depth, from a passion-fruit plantation in the region of São João da Barra, in the State of Rio de Janeiro, Brazil, and maintained in plastic pots with *Brachiaria brizantha,* also for five months. Dr. Rosilaine Carrenho and Dr. Sandra Truffen, from the Botanical Institute of São Paulo, Mycology Department, identified the native population of AMF, after its multiplication in pots with *B. brizantha*. The following species were present in the native population: *Glomus clarum*, *Glomus spurcum*, *Scutellospora fulgida*, *Glomus macrocarpum*, *Glomus invermaium*, *Entrophospora colombiana*, *Scutellospora pellucida*, *Acaulospora appendiculata,* and *Scutellospora heterogama*, *Glomus clarum and Glomus spurcum* being predominant species.

A completely randomized, factorial design was used with two factors, considering three and two levels of inoculation and P fertilization, respectively (*Glomus clarum*, native AMF, nonmycorrhizal; two levels P: 10 and 50 mg kg^{-1}), with three replicates for each treatment. Plants were irrigated daily with distilled water and at every 15 days, 10 mL of the following nutrient solution without phosphorus was added: 2 mM of $Ca(NO_3)_2\ 4H_2O$; 2.5 mM of KNO_3; 1 mM of NH_4NO_3; 1 mM of $MgSO_4\ 7H_2O$; 40 µM of FeEDTA; 25 µM of H_3BO_3; 0.5 mM of KCl, and 1 mL of a micronutrient solution without Fe (Johnson et al., 1957). Plants were harvested 80 days after planting. The following variables were measured: dry weight of shoot and roots per plant, percentage of mycorrhizal colonization of roots and content of several secondary phenolic metabolites in the roots. The mycorrhizal colonization was determined after root clarification with 10% KOH at 90ºC in water bath for 90 minutes, followed by acidification in HCl (5%) for one minute and staining with Methylene Blue at 90ºC in water bath for ten minutes. Root colonization was counted using the grid line intersect method (Giovannetti & Mosse, 1980), under stereoscope microscope (40X). The contents of N, P, and K of the plant aerial parts were determined. The shoot plant parts, after being dried (70°C for 48h), were submitted to nitro-perchloric digestion (for determination of P, and K) and sulfur digestion (for determination of N). The N was determined by the method described by Nessler

(Jackson, 1958). The P was determined colorimetrically by the molybdate method (Embrapa, 1999), and the K was determined by flame photometry (Malavolta et al., 1989).

To prepare root methanolic extracts, roots were dried in a drying oven at 65°C for three days and weighed. After that, the primary root was removed and the secondary and fine roots were macerated in a porcelain pot and weighed. For each treatment, three cold extractions were done with 25 to 30 mL of concentrated methanol (HPLC quality), with the supernatant (methanol aliquot) being removed and saved every week, and another 25 to 30 mL of methanol added to the sample, for a period of three weeks. After the cold extractions, the three methanol extraction aliquots were mixed, filtered with cotton and filter paper (0.45 µm; Sartorius, Minisart RC25) and let to dry, in a chamber. The evaporated extract was weighed, diluted in methanol and kept in refrigerator until it was analyzed by reverse phase HPLC.

The chromatography analysis of the methanolic extracts was performed on HPLC equipment (Shimadzu LC-10AD), a chromatograph equipped with reverse phase column C18 ODS II (4.6 × 250 mm) and a Photo Diode Array Detector. Injection was done through a loop of 20 µL. The following solvent system was used: a gradient of acetonitril in water, both with 2% of orthophosphoric acid, for 40 minutes. The gradient of water with 2% orthophosphoric acid (solvent A) and acetonitril with 2% of orthophosphoric acid (solvent B) was used as follows: 0 to 2 min (7% of A in B); 2 to 8 min (7 to 15% of A in B); 8 to 15 min (15 to 75% of A in B); 15 to 27 min (75 to 80% of A in B), and 27 to 35 min (80% of A in B). This gradient of acetonitril in water was utilized for yielding best results for peak resolutions and reproducibility, after having tested several combinations of the solvents A and B, as described by Graham (1991) and Slacanin et al. (1991). Flavonoids analyzed as authentic standards, diluted in methanol, were: rutin and quercetin (Sigma Chemicals), formononetin (RhizoTech, Inc; gently donated by Dr. J.O. Siqueira), vitexin and campferol (Sigma Chemicals), which presented the following retention times (Rt; min.) rutin=2.72, quercetin=28.83, vitexin=22.23, canferol=28.52, and formononetin=29.15.

To quantify the peaks eluted under the conditions described above, quercetin and vitexin were used as external authentic standards (Lough & Wainer, 1996). Three replicates of a known concentration of the standards diluted in methanol (1 mg mL^{1}) were analyzed and the response factor was calculated with the following formula: Fr = Cp/AP (Fr = response factor; Cp = standard concentration; Ap = area of the standard peak). The concentration of the compound (Cc) is equal to the peak area times the response factor (Cc = Ap × Fr). Using the volume of the root ethanol extract injected (20 µL), the dilution of the methanol extract and the sample weight, the quantity of each detected compound was calculated per milligram of root dry weight. Each sample extract was injected three times in order to obtain the average values for the compound concentrations. Results were submitted to the analysis of variance, followed by the comparison of means by the test of Tukey, at the level of 5% of probability, with the statistical software SAEG-UFV.

RESULTS AND DISCUSSION

The chromatographs obtained presented several peaks, demonstrating the occurrence of different compounds (Figures 1 and 2). Compounds were represented by seven major peaks, and quantified and compared among the treatments (Figures 1 and 2). It was not possible doing structural identification of compounds by peaks. Yet, the methodology used to separate these compounds in the methanol extracts and the analysis of the authentic standard compounds yielded good peak resolution, and, suggests that these compounds are secondary metabolites of the flavonoid class. Graham (1991) described this protocol for HPLC analysis as suitable for the study of secondary metabolites bearing aromatic ring, in a study about the distribution of flavonoids and isoflavonoids in leguminous plants.

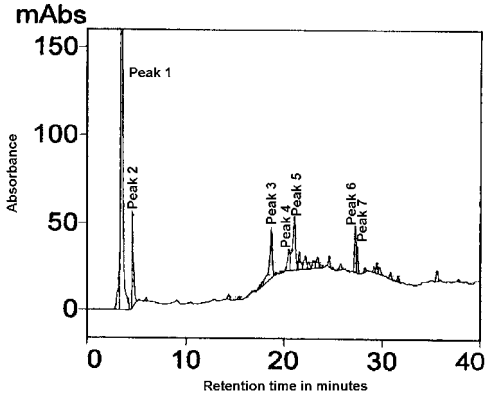

Figure 1 - HPLC chromatogram of the methanolic extracts of passion fruit seedling roots, not inoculated with AMF, fertilized with 10 mg kg^{-1} P of growth substrate.

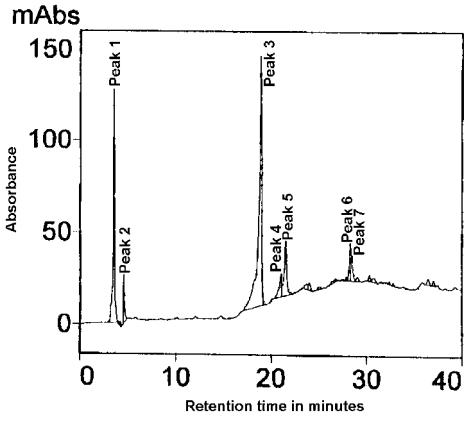

Figure 2 - HPLC chromatogram of the methanolic extracts of passion fruit seedling roots, inoculated with *Glomus clarum*, and fertilized with 10 mg kg^{-1} P of growth substrate.

Ponce et al. (2004) identified several flavonoids from cold ethanolic extracts of dried shoots and roots of *Trifolium repens* (white clover), grown with and without *Glomus intraradices*, and concluded that the metabolism of these compounds is strongly affected by the AMF colonization. The concentration of flavonoids in leguminous roots varies with root colonization with mycorrhizal fungi, the stages of interaction between the plant and its symbiont (Lum & Hirsch, 2003; Hirsch & Kapulnik, 1998; Volpin et al., 1994), and with deficiency of nitrogen and/or phosphorus (Coronado et al., 1995; Nair et al., 1991; Murali & Teramura, 1985). Table 1 shows the concentrations of the compounds represented by the seven major chromatograph peaks, as a result of treatments (seedling inoculation with AMF, and phosphorus levels). Comparisons of the concentration of each differentiated compound are made among the treatments, for the same chromatograph peak.

Compounds represented by peaks 1, 2 and 3 were the ones that presented the greatest variation in their concentration in the roots, as a result of the different treatments (Table 1). The major differences were found for the treatment with 10 mg kg^{-1} of phosphorus, for the uninoculated roots, when compared to the roots inoculated with the native mycorrhizal population (IN) or with *Glomus clarum* (Gc).

For the level of 10 mg kg^{-1} of P fertilization, the compound represented by peak 1 (retention time 3.40 min.), had a significantly higher concentration in the uninoculated roots, than in the inoculated roots (Table 1). In addition, for the uninoculated treatment, it can be observed that for the plants treated with the lower level of phosphorus fertilization, the root concentration of this compound was significantly higher than in the plants treated with higher level of phosphorus fertilization.

When not inoculated, and supplemented with only 10 mg kg^{-1} of phosphorus, seedlings had poorly developed root system (Table 2) and symptoms of phosphorus deficiency. Therefore, variations observed in the concentration of the compound represented in peak 1 could be a result of the reduced plant growth due to nutritional stress (phosphorus deficiency). Besides the nutritional stress, variations in the synthesis of secondary metabolites may result from plant defense response induced by presence of the mycorrhizal fungi, as demonstrated in roots of leguminous plants inoculated with AMF (Volpin et al., 1994; Morandi & Bailey, 1984), even though it seems that mycorrhizal fungi do not elicit typical plant defense response, or such response is rapidly suppressed by yet unknown mechanisms (Gadkar et al., 2001).

The compound represented by peak 2, (retention time 4.49 min.), was present in significantly higher concentration in the uninoculated roots, for both phosphorus fertilization levels. The concentration of this compound varied with the inoculation treatment, independent of the phosphorus level, which suggests that it is affected by the colonization of the mycorrhizal fungi, and not by the nutritional status of the plant, but probably by the defense response of the host plant.

Table 1 - Concentrations of the aromatic compounds detected in the roots of passion fruit seedlings.

Peaks (Retention time)	Levels of P	AMF[1]		
		SI	IN	Gc
	mg kg^{-1}	µg mg^{-1} dry root		
1 (3.40 min.)	10	1.549 Aa	0.542 Ba	0.667 Ba
	50	0.993 Ab	0.681 Aa	0.793 Aa
2 (4.49 min.)	10	0.232 Aa	0.064 Ba	0.104 Ba
	50	0.203 Aa	0.084 Ba	0.137 ABa
3 (18.92 min.)	10	0.167 Bb	2.211 Aa	2.938 Aa
	50	1.381 Aa	1.741 Aa	2.279 Aa
4 (20.88 min.)	10	0.088 Aa	0.175 Ab	0.204 Aa
	50	0.122 Ba	0.487 Aa	0.249 ABa
5 (21.51 min.)	10	0.277 Aa	0.247 Aa	0.292 Aa
	50	0.317 Aa	0.428 Aa	0.492 Aa
6 (27.65 min.)	10	0.173 Aa	0.112 Aa	0.207 A
	50	0.108 Aa	0.180 Aa	ND[2]
7 (27.98 min.)	10	0.057 Aa	0.043 Ab	0.091 Ab
	50	0.067 Ca	0.129 Ba	0.343 Aa

[1]SI= without inoculation; IN= native population; Gc= *Glomus clarum*
[2]ND= not detected
Means followed by same capital letter in the same line, and small letter in the same column, within the same peak, do not differ by the Tukey test ($\alpha < 0.05$).

Table 2 - Dry weight matter of shoots (SDW) and roots (RDW), mycorrhizal colonization, and contents of nitrogen (N), phosphorus (P), and potassium (K) in the shoots of passion fruit seedlings, 80 days after planting in sterile substrate.

Variables	Levels of P	AMF[1]		
		SI	IN	Gc
	mg kg^{-1}			
SDW (g plant^{-1})	10	0.30 Cb	1.25 Bb	1.49 Aa
	50	1.25 Ba	1.37 Aa	1.39 Aa
RDW	10	0.07 Bb	0.51 Ab	0.52 Aa
	50	0.32 Ba	0.55 Aa	0.51 Aa
Colonization (%)	10	NC[2]	87.23 Aa	56.93 Ba
	50	NC[2]	84.67 Aa	53.32 Ba
N (mg plant^{-1})	10	5.41 Cb	7.67 Bb	9.15 Aa
	50	10.18 Aa	8.74 Ba	8.71 Ba
P (mg plant^{-1})	10	0.11 Cb	1.81 Ab	1.45 Bb
	50	0.75 Ca	2.29 Aa	1.88 Ba
K (mg plant^{-1})	10	4.68 Cb	21.36 Ba	23.84 Aa
	50	21.21 Ba	22.99 Aa	22.69 Aa

[1]Fungus: SI = not inoculated; IN = native population; Gc = *Glomus clarum*
[2]NC= not colonized
Means followed by same capital letter in the same line, and small letter in the same column, for the same variable, do not differ by the Tukey test ($\alpha < 0.05$).

In contrast to the effects observed for the compounds represented by peaks 1 and 2, the compound represented by peak 3 (retention time 18.95 min.), had very low concentration (0.167 µg mg^{-1} of dry root) for the treatment with 10 mg kg^{-1} of phosphorus fertilization, in the uninoculated roots. The highest values for concentration of this compound occurred also for the uninoculated roots, at phosphorus level of 50 mg kg^{-1} (Table 1), suggesting that it was not a result of a plant response to the fungal colonization (possible plant defense response), but a result of a plant response to its nutritional status.

For compounds differentiated in the chromatograms, by peaks 5 and 6, there were no differences among the treatments ($P < 0.05$) (Table 1). The compounds represented by peaks 4 and 7, level of 10 mg P kg^{-1} of substrate, did not differ among the treatments with AMF ($P < 0.05$). On the other hand, for the level of 50 mg P kg^{-1} of substrate, the concentrations of these compounds were significantly higher for the roots inoculated with the native population of AMF, for peak 4, and with the native population and *Glomus clarum* for peak 7, suggesting that the concentration of these compounds also varied with the fungal species and the level of phosphorus. Flavonoids are fundamental regulating factors for AMF establishment, and studies on the concentration and variation of specific phenolic compounds as a function of environmental factors and root colonization are necessary to understand the plant-AMF symbiosis (Rhlid et al., 1993).

The production of flavonoids, which act as molecular signals for the nitrogen fixation process, is regulated by the level of nitrogen in the plant and induced in conditions of nitrogen deficiency in the plant.

The accumulation of flavonoids due to nutritional stresses of nitrogen and phosphorus has been demonstrated in leguminous plants (Coronado et al., 1995; Nair et al., 1991; Murali & Teramura, 1985). High levels of formononetin in roots of *Trifolium* can be induced by phosphorus stress in the plant (Nair et al., 1991). Phosphorus deficiency and irradiation with UV-B rays increase significantly the contents of flavonoids in the soybean leaves (Murali & Teramura, 1985). In soybeans, nitrogen fertilization caused a decrease in the concentration of isoflavonoids in the roots, and in *Lupinus albus,* the nitrate reduced the quantity of the present flavonoids in the root extracts (Coronado et al., 1995).

The concentration of flavonoids in roots of leguminous plants also varies with the root colonization by AMF (Volpin et al., 1994; Morandi & Bailey, 1984). Alfalfa plants inoculated with *Glomus intraradices* have higher concentrations of formononetin, and the variations observed resulted from the presence of the AMF (plant defense response) and were not due to the effect of the fungus in the nutritional status of the plant, since the inoculated plants and the uninoculated plants had the same growth (Volpin et al., 1994). Analyzing the contents of stress metabolites in soybean roots inoculated with *Glomus mosseae* or *Glomus fasciculatum* or a mixed inoculum of the two species, Morandi & Bailey (1984) observed a greater accumulation of gliceolin, cumestrol, and daidzein in the colonized roots, when compared to the

uncolonized roots and that soybean roots colonized with *G. mosseae,* and *G. fasciculatum* had higher levels of coumestrol than the roots colonized by only *G. mosseae*.

Morandi & Bailey (1984) observed also an increase in the contents of daidzein in soybean roots after four weeks of colonization with *G. mosseae* or *G. fasciculatum*, and after six weeks of colonization with a mixed inoculum of the two species; after eight weeks, the high levels of this compound were maintained only in the roots colonized with *G. fasciculatum*. The work of Morandi & Bailey (1984) showed that root colonization with AMF affects the root production of flavonoid compounds and this effect can vary among the different compounds synthesized by the plant and can also vary with the fungal species and the time period of colonization by the AMF.

Passion fruit seedlings have high mycorrhizal dependency in substrates with low levels of phosphorus (Soares & Martins, 2000). Further studies are necessary to separate and identify the secondary metabolites produced in the roots of passion fruit seedlings and to evaluate the role of these compounds in the plant-AMF symbiosis.

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