Seed exudates of *Sesbania virgata* (Cav.) Pers. stimulate the asymbiotic phase of the arbuscular mycorrhizal fungus *Gigaspora albida* Becker & Hall

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ABSTRACT - (Seed exudates of *Sesbania virgata* (Cav.) Pers. stimulate the asymbiotic phase of the arbuscular mycorrhizal fungus *Gigaspora albida* Becker & Hall). *Sesbania virgata* is a legume used in the restoration of degraded areas and forms a symbiosis with arbuscular mycorrhizal fungi (AMF). Its seeds exude secondary metabolites that may influence the colonization by AMF. In this work, we studied the effects of seed (SE) and root exudates (RE) of *S. virgata* on the asymbiotic phase of *Gigaspora albida.* Spores of *G. albida* were germinated in medium supplemented with different concentrations of SE or RE. After seven days, spore germination was stimulated (46.6%) in the medium supplemented with the highest concentration of SE, while the mycelial growth was stimulated with the lowest SE concentration. In turn, RE had no effect on the fungal asymbiotic phase. We concluded that SE exert a positive effect on the asymbiotic phase of *G. albida* and that the different effects between SE and RE of *S. virgata* can be explained by their distinct content of secondary metabolites.
Keywords: germination, *Glomeromycota*, mycelial growth, plant exudation

RESUMO - (Exsudatos de sementes de *Sesbania virgata* (Cav.) Pers. estimulam a fase assimbiótica do fungo micorrízico arbuscular *Gigaspora albida* Becker & Hall). *Sesbania virgata* é uma leguminosa utilizada na restauração de áreas degradadas e que forma simbiose com fungos micorrízicos arbusculares (AMF). Suas sementes exudam metabólitos secundários que podem influenciar a colonização pelos AMF. Neste trabalho, estudamos os efeitos de exsudatos de sementes (SE) e radiculares (RE) de *S. virgata* na fase assimbiótica de *Gigaspora albida.* Esporos de *G. albida* foram germinados em meio suplementado com diferentes concentrações de SE ou RE. Após sete dias, a germinação dos esporos foi estimulada (46,6%) com a maior concentração de SE, enquanto o crescimento micelial foi estimulado com a menor concentração de SE. Por sua vez, RE não teve efeito na fase assimbiótica de *G. albida*. Conluiu-se que SE exercem efeitos positivos na fase assimbiótica de *G. albida* e que os efeitos diferenciais entre SE e RE de *S. virgata* na fase de assimbiótica fúngica podem ser explicados pelo seu conteúdo distinto de metabólitos secundários.
Palavras-chave: crescimento micelial, exudação de plantas, germinação, *Glomeromycota*

Introduction

Arbuscular mycorrhizal fungi (AMF), soil fungi of the sub-phylum Glomeromycotina (phylum Mucoromycota) (Spatafora *et al*. 2016), associate with plant roots and promote the growth of the host and the exchange of nutrients that are fundamental for the symbiotic association. Through interaction with the fungus, not only phosphorus (P) and nitrogen (N) but also micronutrients such as zinc (Zn), copper (Cu) and iron (Fe) are supplied to the plant. In turn, the AMF benefits from the plant carbohydrates (Bucher *et al*. 2009, Garg & Chandel 2010).

The establishment of symbiosis in the compatible host plants allows AMF to complete their life cycle. Such fungi are obligatory biotrophs, which life cycle starts with the germination of spores, which are formed in the soil or within the roots (Maia & Yano-

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Melo 2001, Souza *et al*. 2010). Studies have shown that the molecular dialogue between the symbionts precedes the physical contact and the molecules, which are produced by plants and fungi, are involved in the communication process (Kiriachek *et al*. 2009, Bonfante & Requena 2011).

Before establishing symbiosis, the AMF develop the asymbiotic phase (or pre-symbiotic), which consists of germination, formation of germination tubes and limited production of asymbiotic mycelium. This phase can be affected by physical, chemical and biological factors (Maia *et al*. 2010). During this phase, the fungus uses triglyceride reserves, one of the main forms of AMF carbon storage (Bago *et al*. 2000, Requena *et al*. 2007).

Each spore formed by the Glomeromycotina constitutes one unique, multinucleated cell with a diameter varying from 22 to 1050 µm (Souza *et al*. 2010), which shows a particular physiology. The spore is able to germinate and interrupt growth in the soil many times, even in the absence of the host signals (Requena *et al*. 2007, Willis *et al*. 2013). Even though the AMF spores have the metabolic capacity for germination and limited growth of the hyphae, such events are maximized when environmental and endogenous inducing factors are favorable (Nagahashi & Douds 2000).

Compounds that are present in the root exudates of the host plants stimulate spore germination, hyphal growth and ramification, and its nuclear division (Buee *et al*. 2000, Bücking *et al*. 2008, Ellouze *et al*. 2012). Akiyama *et al*. (2005) isolated an inducing ramification factor of root exudates of *Lotus japonicus* and identified it as a strigolactone (5-deoxy-strigol), responsible for morphogenetic changes that convert germination tubes with limited growth into pre-symbiotic mycelium with the capacity to initiate colonization of susceptible roots.

The majority of studies concerning the asymbiotic phase of AMF have focused on the effects of root exudates; however, there are only a few reports on the effects of seed exudates (Tsai & Phillips 1991). Souza Filho *et al*. (2011) documented the importance of seeds as an alternative source of chemical compounds with allelopathic activity, considering that they represent positive signals for symbiosis with microorganisms in the soil. Secondary metabolites that are produced by seeds also play a role in the symbiotic association of certain legumes and nitrogen-fixing bacteria (Ndakidemi & Dakora 2003).

Sesbania virgata (Fabaceae) is a shrubby legume that produces a large number of seeds, which are viable for a long time and exudate secondary metabolites at the beginning of the imbibition process. The main metabolite exuded by *S. virgata* seeds is the flavonoid (+)-catechin, found in the testa and released in high concentrations on the first day of imbibition (Simões *et al*. 2008). *S. virgata* is used for restoration of degraded areas due to its rusticity, availability of seeds and the capacity to establish symbiosis with rhizobia and AMF, which favors nutrient absorption in soils with low fertility (Potomati & Buckeridge 2002, Araújo *et al*. 2004, Schiavo *et al*. 2009, Bomfeti *et al*. 2013). However, the effects of its crude seed (SE) and root exudates (RE) on the asymbiotic phase of AMF have not been evaluated.

Studies have been conducted aiming evaluate the effects of different compounds on the asymbiotic growth stages of AMF with a focus on species of the genera *Gigaspora* and *Glomus* (Tawaraya *et al*. 1996, Oba *et al*. 2002, Scervino *et al*. 2009, Cantor *et al*. 2011, De Jaeger *et al*. 2011). In this study the hypothesis that SE and RE of *S. virgata* can exert an influence on the asymbiotic phase of *Gigaspora albida* was tested. For that, we examined the effects of crude SE and RE of *Sesbania virgata* on spore germination and *in vitro* mycelial growth of *G. albida*.

Material and methods

Crude seed exudates (SE) of *Sesbania virgata* - *S. virgata* seeds (1000 seeds) were selected based on coat color (light brown) and size (about 5 mm long) and manually scarified with the use of sanding paper (P80 3M). Seeds were then superficially disinfected four times by immersion in a 10% sodium hypochlorite solution (Super Cândida®, 2% active chlorine) for 20 min and washed with sterile distilled water. In a laminar flow chamber, seeds were distributed in 150 mm glass Petri dishes (50 seeds/dish) containing qualitative filter paper (Qualy®), soaked in sterile distilled water (35 mL). The dishes were transferred to a germination chamber (BOD model 347 FG, Fanem®) at 25ºC and 12 h photoperiod. After 48 h, the crude SE of *S. virgata* were collected and aliquoted in different volumes for posterior lyophilization.

Crude root exudates (RE) of *Sesbania virgata* - Seeds of *S. virgata* were germinated as described above and, after 6 days in the germination chamber, 300 plantlets were transferred to polyethylene pots (5 per pot) filled with 500 g of autoclaved quartz as a substrate. The pots

were maintained in a greenhouse and watered daily with distilled water (50 mL), a volume below to field capacity. Nutritive Hoagland solution (50 mL) was given to the plantlets at the first day of the experiment. After 5 days, each pot was watered with a fixed volume of distilled water (100 mL), superior to field capacity, and the leachates were collected to obtain the crude RE. The exudates were aliquoted in different volumes for posterior lyophilization.

Analysis of *Sesbania virgata* exudates - Soluble carbohydrates, total proteins, total phenols and total flavonoids were determined in the crude SE and RE of *S. virgata*. The soluble carbohydrates were quantified by the phenol-sulfuric method (Dubois *et al*. 1956), using glucose (Vetec®) as a standard. Total protein was quantified using the Bradford colorimetric method (1976), using a Bovine Serum Albumin (Sigma®) as standard and total phenols were quantified with the Folin-Ciocalteau (Merck®) method using tannic acid (Sigma®) as the standard (Monteiro *et al*. 2006). Total flavonoids were quantified according to Araújo *et al*. (2008) using rutin (Sigma®) as standard.

Aliquots of the crude aqueous SE of *S. virgata* were also fractionated with ethyl acetate (2:1 v/v). The remaining water in the collected organic fraction was removed by drying with anhydrous sodium sulfate (Sigma®). Subsequently, the organic solution was filtered, concentrated by evaporation and dissolved in 1 mL of methanol for analysis by liquid chromatography in combination with mass spectrometry (LC/MS) as described by Simões *et al*. (2008). The peaks were compared with (+)-commercial catechin (Sigma®) (figure 1).

In vitro germination assay of *Gigaspora albida* - Different volumes of crude SE and RE of *S. virgata* were lyophilized and then resuspended in 3 mL of distilled water to obtain different concentrations: 1 (2.8 µg catechin mL^{-1}), 2 (8.4 µg catechin mL^{-1}), 3 (13.9 µg catechin mL^{-1}), 4 (19.5 µg catechin mL^{-1}) and 5 (27.9 µg catechin mL^{-1}) of SE and 1 (0.1 µg catechin mL^{-1}), 2 (0.4 µg catechin mL^{-1}), 3 (0.7 µg catechin mL^{-1}), 4 (1.0 µg catechin mL^{-1}) and 5 (1.4 µg catechin mL^{-1}) for RE. All solutions were filtered under sterile conditions using a 0.22 µm membrane, before being aseptically incorporated into the medium.

The control was prepared with the aseptic incorporation of the same quantity of distilled water to the water agar medium. *G. albida* spores isolated from the soil, according to Gerdemann & Nicolson (1963) and Jenkins (1964), were used for inoculation. The spores were obtained from the AMF inoculum bank of Laboratory of Enzymology and Phytochemistry Applied to Mycology, University of Pernambuco, Petrolina, Brazil. In a laminar flow chamber, the selected AMF spores were disinfected in a sodium hypochlorite solution (0.05%/2 min) and washed four times in sterile distilled water (Nuutila *et al*. 1995). Groups of five superficially disinfected spores were transferred individually to Petri dishes containing 8 mL of water agar medium (2.0%) autoclaved at 121 ºC, 15 min and supplemented with the *S. virgata* exudates (SE and RE). The dishes were sealed with Parafilm M®, wrapped in aluminum foil and incubated at room temperature (28 ± 2 ºC) and relative air humidity of 60-80%. At 7 and 14 days after inoculation, the percentage of spore germination was examined and the mycelial length (mm/germinated spore) was determined according to Newman (1966), under a stereomicroscope (40 ×), after staining of the hyphae with an aqueous solution of Trypan blue (0.05%). For each type of crude exudates (SE and RE) of *S. virgata*, the experimental design was entirely randomized with six concentrations of exudates of *S. virgata* in 4 repetitions, totalizing 24 experimental plots per evaluation time (7 and 14 days).

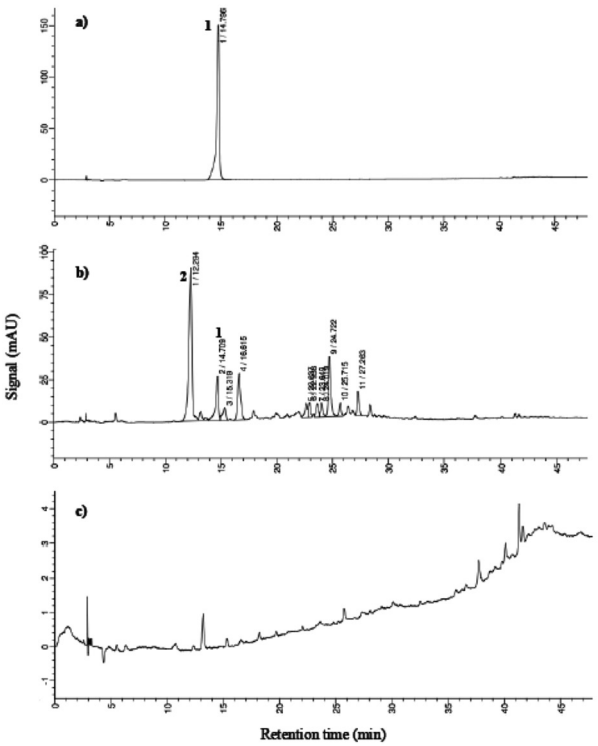

Figure 1. LC/MS profile of commercial catechin (a), seed exudates (b) and root exudates (c) of *Sesbania virgata*. Peaks: 1: catechin (retention time 14.7 min); 2: catechin dimer (retention time 12.3 min).

Under the same experimental conditions mentioned above, an assay was carried out with a stock solution of (+)-commercial catechin (Sigma®), in a concentration of 280 µg mL^{-1}, based on the (+)-catechin concentration of that was detected in the crude SE of *S. virgata*, a value approximately 95% higher compared to the crude RE (data not shown). To determine if such compound exerts effects on the asymbiotic phase of *G. albida*, the experimental design was entirely randomized with six treatments (0, 2.8, 8.4, 14.0, 19.5, and 28.0 µg catechin mL^{-1}), in four repetitions.

Experiment and analysis sites - Department of Plant Physiology and Biochemistry, Institute of Botany, São Paulo, Brazil (crude exudates of *S. virgata* production); Laboratory of Enzymology and Phytochemistry Applied to Mycology, University of Pernambuco, Petrolina, Brazil (exudates of *S. virgata* analysis and spore germination/hyphal growth of *G. albida*); Chemical Institute, University of São Paulo, São Paulo, Brazil (LC/MS profile of commercial catechin and exudates of *S. virgata*).

Statistical analysis - The data were submitted to ANOVA and when appropriate the averages were compared by the Tukey-test ($P < 0.05$) using the ASSISTAT program, version 7.6 beta (Silva 2008).

Results

In the presence of crude SE of *S. virgata*, germination of *G. albida* spores varied from 20 to 46.6% after 7 days and from 20 to 60% on the 14th day of incubation (table 1), which indicates the viability of the spores. The medium supplemented with the highest concentration of exudates (27.9 µg catechin mL^{-1}) stimulated spore germination (46.6%) after 7 days, but his stimulation was not observed on the 14th day. High mycelial growth (0.69 mm/germinated spore) was observed in the medium supplemented with the lowest exudates concentration (2.8 µg catechin mL^{-1}), after 7 days; however, on the 14th day, the presence of crude SE did not interfere in the production of asymbiotic mycelium of *G. albida* (table 2).

There was no significant effect of the crude RE on spore germination of and mycelial growth of *G. albida*, independently of the time of examination (tables 1 and 2).

Higher concentrations of soluble carbohydrates, proteins, phenols, and flavonoids were found in the crude SE of *S. virgata* corresponding to 298.6, 28.4, 104.8 and 0.012 mg mL^{-1}, respectively, when compared to the crude RE (33.9, 4.2, 0 and 0.004 mg mL^{-1}, respectively).

The presence of catechin and a catechin dimer was confirmed in SE and RE, although in lower amounts in RE (figure 1). Commercial catechin used as a control in the assays had no effects on the asymbiotic phase of *G. albida* (data not shown).

Discussion

Compounds present in the SE of *S. virgata* favored the germination of *G. albida* spores. The characterization of the exudates confirmed that different compounds were leaked by seeds, mainly sugars, probably as result of the mobilization of the storage carbohydrates that occur at the beginning of the imbibition process as reported by Buckeridge & Dietrich (1996). Besides sugars, phenolic compounds present in the exudates can also have promoted spore germination. Simões *et al*. (2008) observed that

Table 1. Percentage of *in vitro* germination of *Gigaspora albida* spores in water agar medium supplemented with different concentrations of crude seed (SE) and root exudates (RE) of *Sesbania virgata* at seven and 14 days after inoculation.

	Treatments					
	SE of *S. virgata* (µg catechin mL^{-1})					
Time (days)	0	2.8	8.4	13.9	19.5	27.9
7	20.0 b	20.0 b	33.3 ab	33.3 ab	30.0 ab	46.7 a
14	46.7 ab	60.0 a	45.0 ab	53.3 ab	20.0 c	33.3 bc
	RE of *S. virgata* (µg catechin mL^{-1})					
Time (days)	0	0.1	0.4	0.7	1.0	1.4
7 days	15.0 a	10.0 a	15.0 a	5.0 a	10.0 a	0 a
14 days	20.0 a	35.0 a	10.0 a	10.0 a	5.0 a	0 a

Averages followed by the same letter in lines do not differ by the Tukey test ($P < 0.05$).

Table 2. *In vitro* mycelial growth (mm/germinated spore) of *Gigaspora albida* spores in water agar medium supplemented with different concentrations of crude seed (SE) and root exudates (RE) of *Sesbania virgata* at seven and 14 days after inoculation.

	Treatments					
	SE of *S. virgata* (μg catechin mL^{-1})					
Time (days)	0	2.8	8.4	13.9	19.5	27.9
7	0 b	0.69 a	0.18 b	0 b	0 b	0 b
14	0.93 a	0.49 a	0.66 a	0.41 a	1.28 a	0.58 a
	RE of *S. virgata* (μg catechin mL^{-1})					
Time (days)	0	0.1	0.4	0.7	1.0	1.4
7	0.03 a	0 a	0.08 a	0 a	0 a	0 a
14	0.05 a	0.07 a	0.05 a	0.23 a	0.11 a	0 a

Averages followed by the same letter in lines do not differ by the Tukey test ($P < 0.05$).

(+)-catechin was quickly released from the seed coat of *S. virgata* on the first day of imbibition at higher levels than those documented for other species of *Sesbania* (Ceballos *et al*. 1998).

In our study, we used the direct collection method of crude exudates without the conventional extraction with organic solvents or even fractionation. Bücking *et al*. (2008) concluded that the crude root exudates differ in their effect on the pre-symbiotic growth of AMF from those that are partially purified. The authors showed that crude exudates accelerate spore germination of *Glomus intraradices* and also stimulated the ramification of the germination tubes; when exudates from partially purified roots were added, the spores germinated lately but the number of ramifications after 14 days did not differ from the control.

Similar to the results present in this work (table 1), Tahat *et al*. (2010) found differences in the germination of *Glomus mosseae* supplied with different root exudates. The authors suggested that these differences were due to the quality and the source of the exudate. Spore germination was stimulated by increasing amounts of non-mycorrhizal root exudates of tomato and corn.

Present findings show that only SE were efficient in stimulating the mycelial growth of *G. albida* (table 2), which suggests the existence of signal substances that stimulate the hyphal ramification in the leachates of *S. virgata* seeds.

There are situations in which root exudates stimulate the hyphal ramification of germinated spores of *Gigaspora* species (Buee *et al*. 2000, Nagahashi & Douds 2000, Akiyama *et al*. 2005), which diverges from the results obtained in our experiments. In these studies, the root exudates of all examined plant species showed an inductive activity of the hyphal ramification, with the exception of exudates from non-host roots. However, generalizations about the effects of exudates should be avoided and the type, the dosage and the stage of the asymbiotic phase (germination or mycelial growth) must be taken into consideration.

Studies have shown that flavonoids are components of root exudates capable of stimulating spore germination, mycelial growth, and ramification of various AMF. In many cases, they are also responsible for mycorrhizal colonization, for active molecular mechanism of the root colonization and for establishing symbiosis (Gianinazzi-Pearson *et al*. 1989, Nair *et al*. 1991, Siqueira *et al*. 1991, Romero & Siqueira 1996, Soares *et al*. 2005, Scervino *et al*. 2009). However, it was found that, by using chalcone synthase-deficient corn mutants, a key-enzyme in the biosynthesis of flavonoids, these compounds are not essential for establishing arbuscular mycorrhizal symbiosis (Bécard *et al*. 1995, Buee *et al*. 2000, Bécard *et al*. 2004). The characterization of the exudates that were used in the present study points to small, available concentrations of these compounds, which suggests the presence of other substances than flavonoids are responsible for activating the asymbiotic phase of *G. albida*. The complex composition of SE, verified by LC/MS analysis (figure 1), suggests that multiple compounds that are exuded by the seeds could be responsible for the stimulating effect that was observed in the asymbiotic phase of *G. albida*, as was documented by Siqueira *et al*. (1991) for *G. margarita*. It is reasonable to

suppose that such molecules can act in a synergic way resulting in the asymbiotic mycelial production observed (table 2). In this regard, Tsai & Phillips (1991) reported that naturally exuded flavonoids from alfalfa seeds (*Medicago sativa* L.) promoted the *in vitro* germination of *Glomus etunicatum* and *Glomus macrocarpum* spores.

The flavonoid (+)-catechin is the main metabolite exuded by *S. virgata* seeds. It is found in the seed coat and liberated in high concentrations (235 µg of (+)-catechin/seed), after 24 h of imbibition (Simões *et al*. 2008). However, such molecule did not stimulate the asymbiotic phase of *G. albida* (data not shown), indicating that probably other exuded molecules are involved in the stimulation of the fungal asymbiotic phase. Studies with other AMF isolates are still necessary to determine the beneficial effect of *S. virgata* exudates. Only the isolation, chemical characterization and assays using isolated compounds will help to identify whether the effect of *S. virgata* exudates is due specifically to one substance or to a combination of several substances.

In conclusion, our results show that SE and RE of *S. virgata* exert a distinct influence on the asymbiotic phase of *G. albida*, stimulating spore germination when the medium is supplemented with SE. This effect could not be attributed to catechin, which is exuded by seeds of *S. virgata* at the beginning of the imbibition process, but is rather related to other compounds found in SE.

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