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Growth of *Lippia alba* (Mill.) N. E. Brown inoculated with arbuscular mycorrhizal fungi with different levels of humic substances and phosphorus in the soil

Caroline Lermen[a], Rayane Monique Sete da Cruz[a], Jean Silva de Souza[b], Bianca de Almeida Marchi[b], Odair Alberton[a,*]

[a] Postgraduate Program in Biotechnology Applied to Agriculture, Paranaense University, Praça Mascarenhas de Moraes, 4282, 87502-210, Umuarama, PR, Brazil
[b] Paranaense University, Praça Mascarenhas de Moraes, 4282, 87502-210, Umuarama, PR, Brazil

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ABSTRACT

Inoculation with arbuscular mycorrhizal fungi (AMF) and the addition of humic substances (HSs) might contribute to the growth and development of *Lippia alba* (Mill.) N. E. Brown. The objective of this study was to evaluate the effect of the AMF *Rhizophagus clarus* (Nicolson T.H. & Schenck N.C.), with low and high (20 and 200 mg dm^{-3} of soil, respectively) additions of phosphorus (P) and with or without HSs in the soil, on the growth and uptake of P and nitrogen (N) by *L. alba*, and microbial soil quality. The experiment was designed in a completely randomized factorial of 2 × 2 × 2 (with and without AMF, levels of P, and with or without HSs in the soil), with six replications, totaling 48 experimental units. Development of the plant was evaluated by measuring shoot dry matter (SDM), root dry matter (RDM), total dry matter (TDM), chlorophyll index (CI), N content in the shoots, and P in the plant. In addition, spore density and root colonization by AMF, as well as basal soil respiration (BSR), and the microbial biomass carbon (MB-C) and metabolic quotient (qCO_2) of the soil were measured. The RDM and TDM, CI, N, and P content in the plant, the spore density and root colonization by AMF, and MB-C and qCO_2 were significantly increased with the inoculation of AMF. The addition of P to the soil significantly increased the SDM (up to 74%) and TDM (up to 29%). Plant inoculation with AMF and the addition of HSs and P to the soil increased plant P content (up to 60%) and N in the shoot (up to 74%) compared with that of the control (uninoculated, without HSs and P). The increase in plant uptake of P and N after inoculation with AMF and addition of HSs and P to the soil stimulated growth of *L. alba*. It was concluded that inoculation with AMF, with the addition of HSs and P, increased growth and the content of N and P of *L. alba*.

1. Introduction

The plant *Lippia alba* (Mill.) N. E. Brown belongs to the family Verbenaceae. It is a shrub that grows essentially throughout Brazil and in several areas of North Africa (Hennebelle et al., 2008; Reis et al., 2010). This plant can be used as an aromatic and medicinal component, and in the perfume and agrochemical industries owing to its antifungal, antibacterial, antiviral, insecticidal, and repellent activities (Ospina et al., 2016). The properties of this plant are caused by its active constituents, among which the essential oil (EO) has high biological potential (Hennebelle et al., 2008). The presence of citral associated with *d*-limonene, one of its main components, is responsible for its pleasant aroma (Hennebelle et al., 2008; Yamamoto et al., 2008; Reis et al., 2010; Glamočlija et al., 2011; Blank et al., 2015).

The aroma may be attributed to the predominate constituents in the essence. These constituents can vary qualitatively or quantitatively depending on several factors, such as seasons of the year, flowering stage, plant age, amount of water in the soil, geographical and climatic factors, soil fertility, and interaction with the soil microbiota (Yamamoto et al., 2008; Reis et al., 2010; Machado et al., 2014; Lermen et al., 2015; Urcoviche et al., 2015; Ospina et al., 2016), including the association with soil fungi.

Soil fungi that form mutualistic symbiosis with plants are known as arbuscular mycorrhizal fungi (AMF). This symbiosis is characterized by the formation of symbiotic structures in the roots with intracellular colonization of the cortex, formation of tangled hyphae and other profusely branched structures (arbuscules), and extraradical mycelium that grow into the soil in the rhizosphere. The AMF are cosmopolitan, being abundant even in highly degraded areas and are found in almost all families of herbaceous and tree species (Smith and Read, 2008).

* Corresponding author.
E-mail addresses: odair@unipar.br, oalberton@yahoo.com.br (O. Alberton).

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Because of its effect on the mineral nutrition of the plant, AMF have positive effects on the condition of the plants, stimulating plant growth (Smith and Read, 2008). Mycorrhizal symbiosis between the fungus and plant occurs as a heterogeneous three-phase system, where the elements, soil, plant, and fungus are in contact, but without mixing. Each component acts on the mycorrhizae in different ways; however, the most important effect of the symbiosis is the dependence of the mycorrhizal plant, as well as the efficiency of the fungus. The availability of P in the soil is considered the most important edaphic factor to the functioning of the mycorrhizal symbiosis (Smith and Read, 2008; Urcoviche et al., 2015).

Phosphorus is considered an essential macroelement for the plant species (Urcoviche et al., 2015). The uptake of P from the soil is carried out by the roots, but it can also be done by external mycelium of the AMF, as shown in studies with *Glomus etunicatum* (Becker W.N. & Gerd C.) in roots of *Lolium perenne* L. (*Poaceae*) (Smith and Read, 2008).

With respect to the substrate, the addition of organic matter favors the growth of the shoot and root system (Lima et al., 2015). Organic matter is made up of products of decomposition of organic residues and microbial metabolism and by humic substances (HSs). These comprise the largest part of the organic matter in soils and are composed of humin, fulvic acid (FA), and humic acid (HA). HA, formed by heterogeneous molecular aggregates and stabilized by hydrogen bridges and hydrophobic interactions, encourage the development of the root system, the accumulation of nutrients, and the biosynthesis of chlorophyll. According to Façanha et al. (2002), HA in plants can promote a partial oxidative phosphorylation in mitochondria and act as growth regulators, increasing the synthesis of biomass and consequently the content of secondary metabolites, such as EO.

There are many benefits of HSs to the metabolism of plants, owing to the positive effect on ion transport, facilitating uptake. The benefits include increased respiration and the speed of reactions of the Krebs cycle, resulting in greater ATP production; increased chlorophyll and synthesis of nucleic acids; selective effects on protein synthesis and increased or inhibited enzyme activity (Façanha et al., 2002).

The objective of this study was to evaluate the effects of AMF *Rhizophagus clarus* (Nicolson T.H. & Schenck N.C.), with or without the addition of HSs (commercial product with 75% of HA plus 25% FA) and with low or high (20 or 200 mg dm^{-3} of soil) P addition to the soil, on the growth and development of *L. alba* and its effect on soil quality, as measured by the microbial biomass C (MB-C), basal soil respiration (BSR), and metabolic quotient (qCO_2) of the soil.

2. Material and methods

2.1. Installation, experimental design, and sampling

The soil samples were collected on an experimental farm at União de Ensino do Sudoeste do Paraná (UNISEP), in Dois Vizinhos County (Paraná State), at 25°46′31.04′S and 53°02′56.78″W, and at 0–20 cm depth (Table 1).

The experimental unit was a pot of polyethylene with 6-kg capacity for soil, which was sieved through a 4-mm mesh, and sterilized by autoclaving at 121 °C for 1 h. The experimental design was a completely randomized design with six replications per treatment in a factorial 2 × 2 × 2 design (with or without AMF, two levels of P, and with or without HSs added to the soil), totaling 48 experimental units. The control was considered the application of 20 mg P dm^{-3} soil without inoculation of AMF and without the addition of HSs. The experiment was conducted in a greenhouse with a mean temperature of 32 °C ± 7 (standard deviation), during a period of 6 months.

The AMF treatments were inoculated with 100 g of soil inoculum (250 spores and roots) in each pot. Soil was inoculated with the AMF *R. clarus* from the bank of glomales of UNIPAR, accession number 10 (Lermen et al., 2015; Urcoviche et al., 2015). In the uninoculated treatments (AMF control), 100 mL of filtrate (filtered using 14-μm paper filter) of the soil inoculum (100 g of soil inoculum L^{-1} of sterile deionized water) was added. In this way, only the effect of the inoculated AMF was obtained. Twenty or 200 mg P dm^{-3} of P was added to the soil according to Urcoviche et al. (2015), and 0 or 0.6 mL HSs (75% of HA plus 25% of FA, extracted from vermicompost; amount suggested by the producer, Tecseed®, 2 L ha^{-1}) was added per pot. The HSs used in the present study were recommended for several annual crops and horticultural plants as a growth promoter, however, they had not yet been tested for *L. alba*.

Young seedlings of *L. alba*, approximately 20 cm tall, were collected from the medical vegetable garden of the Universidade Paranaense and rinsed in tap water. Two seedlings were placed in 70% alcohol for 1 min in a single, previously disinfected vase.

All treatments were fert-irrigated every two days as required, with half concentration of the solution Hoagland and Arnon (1950).

2.2. Spore density and root colonization by AMF

The spores were extracted from subsamples of 10 g of soil with wet sieving meshes (0.710–0.053 mm) (Gerdemann and Nicolson, 1963) and then transferred to Petri dishes for counting and identification using a stereoscopic microscope (40x).

Fine roots were collected, bleached, acidified, and colored with trypan blue as suggested by Phillips and Hayman (1970). The count of colonized root segments was conducted on slides overlaid with coverslips (Giovannetti and Mosse, 1980). In total, 100 segments were analyzed and counted using a stereoscopic microscope (40–100x).

The total root colonization by AMF was transformed by equation 1 to normalize the data.

$$Col._t = (ArcSen\sqrt{Col.\ (\%)/100})\cdot(180/\pi) \quad (1)$$

where $Col._t$ is the total root colonization, ArcSen is the invers of sen, Pi (π).

2.3. Determination of microbial biomass carbon, basal respiration, and metabolic quotient of the soil

The determination of microbial biomass C (MB-C) in the soil was modified according to the fumigation-extraction method proposed by Vance et al. (1987) and Tate et al. (1988) using 10 g of soil and adding 1 mL of ethanol-free chloroform to flasks to be fumigated. Then, flasks

Table 1
Chemical properties of the soil used in the experiment.

	pH ($CaCl_2$)	P	C	Al^{3+}	$H^+ + Al^{3+}$	Ca^{2+}	Mg^{2+}	K^+	SB	CTC	V
		mg dm^{-3}	g dm^{-3}	$Cmol_c$ dm^{-3}							%
Soil	4.30	0.80	1.36	0.00	7.20	1.00	0.75	0.08	1.83	9.03	20.86
Reference[a]	3.8–6.6	16–24	0.8–15.9	–	0.6–5.0	0.3–7.2	0.3–3.3	0.1–0.7	–	2.2–12.5	–

*Methods: P and K extracted by Mehlich-I; Ca. Mg and Al – extracted by KCl 1 mol L^{-1}; C – dichromate/colorimetric. CEC = cation exchange capacity; SB = Sum of bases; V = Base saturation.

[a] Source: (Sambatti et al., 2003).