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RESEARCH ARTICLE

Interactions of the lichen *Cladonia salzmannii* Nyl. with soil, microbiota, mycorrhizae and *Genipa Americana*

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Abstract

The lichen *Cladonia salzmannii* can influence the arbuscular mycorrhizal formation (AMF) of sandy soils in the Brazilian Northeast, thereby contributing to improved vascular plant growth. The objective of this work was to evaluate how *C. salzmannii* changes the biological processes of soil in a tropical savannah ecosystem where soils have low nutrient availability, so any lichen-induced effects would be important. The microbial activity, AMF, growth of *Genipa americana* and chemical-physical characteristics of the soil were analyzed. Soil samples were collected under pillows of lichen thalli or in open spaces without them. Chemical and biological soil attributes were evaluated by analysis of soluble and exchangeable cations, microbial activity and AMF colonization. The presence of barbatic acid (BAR), a major compound of *C. salzmannii*, was detected in the soil, which positively influenced soil properties. AMF colonization was higher in lichen-covered soil. *G. americana* seedlings were grown under greenhouse conditions, the experiment was done in a randomized design of 4 inoculation treatments. The inoculated plants with lichens+AMF presented greater growth parameters. The results could indicate that BAR is capable of stimulating the association between plants and AMF, linking the root and the soil system. This arrangement improves a positive feedback loop established between lichen-AMF-vascular plant.

Keywords: lichen substance, barbatic acid, *cerrado* biome, Glomeromycotina growth, Rubiaceae

1. Introduction

About 180 million hectares of Brazil are occupied by savannah-like vegetation called the *cerrado* biome, *senso stricto*, which extends over the Central-west, part of the Southeast and the Northern and Northeast regions of Brazil. Part of this vegetation originates from climatic characteristics, while other regions of *cerrado senso lato* correspond to an edaphic formation where plant cover is similar, but the climate is different. As an example, in the Brazilian Northeast one may find areas called *Tabuleiros*, which are considered edaphic *cerrados*. *Tabuleiro* soils are naturally acidic and have low fertility and low levels of organic matter. The soils and plant cover of Alhandra (State of Paraiba, Brazil) are characteristic of areas of *cerrado* and Atlantic Forest (Pereira and da Nóbrega Alves, 2006), where several species of the *Cladoniaceae* family (lichen) commonly occur. Interactions of *Cladonia verticillaris* with the physical and chemical properties of these soils have been mentioned (Vasconcelos *et al.*, 2015), as well as the allelophatic effect of compounds produced by this species (Tigre *et al.*, 2012). In addition to *C. verticillaris*, in these areas one may find *C. salzmannii*, whose main phenolic compound is barbatic acid (BAR), a depside composed of two phenolic units with an ester bound. This substance can be leached by rainfall and accumulate in soil. In this way symbiotic organisms play an important ecological role; lichens affect the chemistry of rocks during soil formation (pedogenesis). The weathering action of lichens on rocks involves both biogeophysical and biogeochemical processes. Rhizine penetration and thallus expansion-contraction are import mechanisms involved in biogeophysical weathering, whereas lichen substances are important in biogeochemical weathering due to their ability to release complex metal cations from the mineral substratum (Vasconcelos *et al.*, 2015). The presence and stability of lichen substances may exert a direct or indirect effect on other organisms. The negative influence of these substances against fungi and bacteria and their allelophatic effects have been shown in several *in vitro* growth experiments (Martins *et al.*, 2010; Tigre *et al.*, 2012). Brown and Mikola (1974) observed limited growth of *Pinus silvestris* and *Picea abies* caused by the action of a water extract from *Cladonia alpestris*, where usnic acid is the major substance in this species. The authors associated that growth limitation to inhibition of ectomycorrhizal fungi. The opposite effect has also been observed; after the removal of lichen cover an increase of ectomycorrhizal community diversity was observed which caused a decrease in the mortality of vegetation dependent to this symbiosis (Markkola *et al.*, 2002). After assessing microorganism activity in the soil under *Cladina stellaris,* Stark and Hyvärinen (2003) suggest that lichen substances, such as usnic and perlatolic acids, are carbon sources for the microbial community in the soil under lichen cover. Similarly, Ohtonen and Väre (1998) observed a high incidence of microbial activity in the soil under different species of *Cladoniaceae*. Lichens are drivers of ecosystem patterns where there are few other producers; lichens in desert soil crusts and in the Antarctica are crucial for plant and animal community development in those environments.

In addition to this, arbuscular mycorrhizal fungi (AMF) interact directly with plants, participating in the transfer of mineral nutrients from soil micro-compartments that are not accessible to roots, benefiting plant productivity. AMF belong to the sub-phylum Glomeromycotina (Spatafora *et al.*, 2016) and play a crucial role in soil carbon storage and are widespread in most ecosystems, where they colonize more than

85% of land plants (Pérez *et al.*, 2011). Plants are important in soil aggregate formation and the role of AMF is as vital because AMF symbioses influence the root system, enhancing the consolidation of soil particles (Hallett *et al.*, 2009). Armada *et al.* (2016) suggested that mycorrhizal inoculants may be more important than chemical fertilization orchestrating antioxidant activities along the process of *Retama sphaerocarpa* drought tolerance. It has also been demostrated that arbuscular mycorrhizal symbiosis play a principal role in the aluminum tolerance capacity of wheat developed in those soils with high phytotoxic aluminum levels and fungal native populations adapted to these conditions (Seguel *et al.*, 2016).

Soil and vegetation composition are the main factors that determine the presence and development of AMF. Thus, the presence of allelochemicals in the soil can affect mycorrhyzal associations, acting as inhibitors or stimulators of these symbioses. Some roles of AMF have been related to the production of a fungal substance, termed glomalin. Glomalin-related soil protein (GRSP) pools have influences at the ecosystem scale; the latest studies have demonstrated different factors controlling GRSP production such as fungal community composition, fungal physiology, and cell biology aspects, as well as other soil biota, soil physical and chemical characteristics, and fungus–host plant species combinations (Singh *et al.*, 2013).

Besides the interations of lichens and AMF with *Tabuleiro* soils, plant cover can also be influenced by the actions of both organisms. One of the most representative families of flowering plants in the *cerrado* is that of the Rubiaceae, with *G. americana* (a tree species) being the most representative in this area. In this way, a study assembling lichen, AMF and a representative species of this ecosystem would contribute to the knowledge of its dynamics.

The review of information on lichen-plant interactions supports the idea that there are controversial data of effects between lichens and plants. Lichen allelopathic interferences have mainly been observed *in vitro*, while few examples have been reported in natural settings (Favero-Longo and Piervittori, 2010).

Our study asked whether the lichen metabolites altered the chemical and physical characteristics of the soil, whether the lichen influenced soil microbial communities, mycorrhizal symbiosis and vascular plant growth. This is a relevant ecological study that addresses the less well understood role of soil lichens in the *cerrado*, where they are in direct contact with the soils but represent a small portion of total productivity.

Thus, the objective of this paper was to ascertain the influence of *C. salzmannii*, which contains BAR as a major phenolic, on soil biological processes in a tropical savannah ecosystem, for which soil analysis, measurements of AMF and growth of *Genipa americana* were analyzed. Furthermore, in the light of the little research about this subject, this study represents the first involving BAR-AMF-*G. americana*.

2. Materials and Methods

2.1. Lichen material

C. salzmannii samples were collected during the months of November and December from sandy soils of *cerrado*, in Alhandra-PB (Paraiba, NE of Brazil, Latitude 7º24´S, Longitude 34º57´W, Altitude~107m). In the laboratory the lichen material was separated from its substrate and stored in paper bags. Determination of the lichen was accomplished using standard methods, using chemical and morphological thallus characters. Dried specimens were deposited in the

Herbarium UFP, Dept. of Botany, Universidade Federal de Pernambuco (Brazil), register Nº: 44.143.

2.2. Soil material

Samples of Typic quartzpsamments soil from Alhandra-PB (*cerrado*) were studied during the months of November and December. Samples of this soil were collected under small pillows of *C. salzmannii* thalli (10 samples) or in open spaces without vegetation (10 samples) using a hollow metallic cylinder of 1 cm diameter and 20 cm length (depth). Each sample consisted of three subsamples taken from depths of 0 to 20 cm and mixed. Soil samples were immediately placed in coolers until arrival in the laboratory. Then, the soil was separated from the roots, and stored at 4°C, until the moment of analysis.

2.3. Soil analysis

Analysis of soil chemical and physical characteristics took place at the fertility laboratory of the Empresa Pernambucana Agropecuária (IPA).

Soil CO_2 respiration test method was modified (Haney et al; 2008). CO_2 evolution was determined using 100 g soil, moistened to 10% of its water-holding capacity, placed in hermetically sealed flasks and incubated for 15, 45 and 60 days at 28°C. The CO_2 emitted was collected in 10 mL 0.1 M KOH, unreacted alkali in the KOH traps was back-titrated with 1 N HCl solution to a phenolphthalein endpoint which is relative to the amount of CO_2-C released by soil microorganisms. Basal soil respiration was calculated by subtracting the cumulative 15-day CO_2-C from the cumulative 60-day CO_2-C.

Microbial biomass was determined by the fumigation-extraction method, the most commonly used classical technique. Extractable organic C from soil samples, either fumigated or non-fumigated with chloroform, was extracted with 0.5 M K_2SO_4 and after oxidation with potassium dichromate ($K_2Cr_2O_7$), the C content was measured by titration with 0.033 N $(NH_4)_2Fe(SO_4)_2$ (Vance *et al.*, 1987). Fumigation breaks microbial cells, a variation of the fumigation method, is to do an extraction of the soil after chloroform fumigation. The C content in the extractant is then compared to the amount of C in a similar soil that has not been fumigated. The difference is due to the C released from the microbial biomass.

FDA (Fluorescein Diacetate) Hydrolysis was estimated as described by Swisher and Carrol (1980) with modifications. Five g of soil were incubated in 20 mL of 60 mM K-phosphate buffer pH 7.6. The reaction was started by addition of 0.2 mL of a FDA solution (2 mg mL^{-1} in acetone). After 20 min incubation at 25ºC, 20 mL of acetone were immediately added to stop the reaction. Then, the soil suspensions were filtered and the fluorescein (the product of enzymatic reaction) released in the soil was measured by spectrophotometry at 490 nm. The absorbance was measured with a spectrophotometer (BIOCHROM, Libra S 22). The concentration of fluorescein released was calculated with reference to a standard curve from the results obtained with standards containing 0-400 $\mu g \cdot mL^{-1}$ of fluorescein.

Quantification of glomalin-related soil protein (GRSP) was done according to the Wright and Upadhyaya (1998) method. Soil (0.25 g) was treated with 2 mL of 20 mM sodium citrate (pH 7.0) and autoclaved for 30 minutes at 121ºC. Immediately after autoclaving, samples were centrifugated at 10.000x*g* for 5 min and decanted. The absorbance of the supernatants was measured at 595 nm with a spectrophotometer (BIOCHROM, Libra S 22). The concentration of GRSP was measured using the colorimetric Bradford total protein assay (Bradford, 1976).

Measurements AMF colonization were expressed as Percentage of root colonization and AMF spore num-

ber. Roots present in the soil collected were cleared with 10% KOH (p/v) and stained with Trypan blue in lactophenol (0.05%) as described by Phillips and Hayman (1970). Naturally dark pigmented roots were cleared with 10% H_2O_2 for 1 h before staining with Trypan blue. The percentage of root colonization was calculated by the gridline intersect method (Giovannetti and Mosse, 1980). The number of AMF spores was determined by wet sieving, followed by centrifugation in 40% sucrose (Jenkins, 1964) and counted under a stereoscopic microscope (40X).

Detection and quantification of lichen substances in the soil samples. Soil samples (8 g) and lichen thalli (4 g) were submitted to successive extractions with two solvent systems: 10 mL of diethylether/ethyl acetate (60:40, v/v) for the first extraction and 10 mL chloroform/acetonitrile (65:35, v/v) for the second extraction. Then, the phenolic extracts were analyzed by spectrophotometry at 268 nm and dried for Thin Layer Chromatography (TLC) assays. The content of phenolic organic extract was estimated by using a quantitative UV spectrophotometric method. The absorbances were measured with a spectrophotometer (BIOCHROM, Libra S 22) at 268 nm with a quartz cell (1 cm path length). A calibration curve was plotted using BAR as a standard phenolic compound. The average was expressed as µg of BAR equivalents mL^{-1}.

2.4. Thin Layer Chromatography

The soil samples, lichen extracts and standard substance (BAR) were submitted to TLC, carried out on silica gel plates $F_{254+366}$ MERCK, developed on A solvent system (toluene/dioxan/acetic acid, 180:45:5, v/v/v). The spots were visualized under UV short and long wavelengths (254 and 366 nm). After this procedure, the plates were sprayed with H_2SO_4 solution (10%) and heated to 50ºC for 30 min, for spot color reaction. The BAR used as standard was extracted from *C. salzmannii* and the purification was done according to Martins *et al.* (2010).

2.5. Greenhouse experiments

The experiment was done in a randomized design of 4 treatments: control (C), lichen (L), L+AMF and AMF in 5 replicates, and was carried out under greenhouse conditions at room temperature (28ºC ± 3ºC). *G. americana* seeds collected in the study area were disinfected with 20% sodium hypochlorite, and sown in trays containing sterilized vermiculite. Daily watering took place after the sowing. After 3–4 leaves emerged, seedlings were transplanted into plastic bags containing autoclaved soil from Alhandra-PB, and inoculated with two AMF species (*Gigaspora albida* Schenck & Smith and *Acaulospora longula* Spain & Schenck). The inoculum was a mixture of spores, external hyphae and colonized root fragments in soil. After 20 days, the bags were mulched with 33 g of *C. salzmannii* thallus. The plants were irrigated (50 ml) three times weekly.

The experiment was concluded after 4 months of growth under different treatments (C, L, AMF, L+AMF). Plants were harvested and all growth parameters were measured. Stem diameter (at the collar level) and Height (distance between the collar and the apical region) were measured. Dry weight (DW) of shoot (leaves and stems) and roots were obtained by oven-drying at 50ºC until constant weight (~96 h). AMF colonization was measured as described above.

2.6. Satistical analysis

The results were submitted to analysis of variance (ANOVA) and the means were compared by the Tukey test (P<0.05). The mycorrhizal colonization values and the number of spores were transformed into arcsine (x/100) and log (x+1), respectively.

3. Results

The first part of our study assessed how lichen mats influence the chemical and physical characteristics of the soil. To compare the influence of lichens with areas of bare soil, samples of soil were collected under small pillows of *C. salzmannii* thalli or in open spaces without lichen. Analyses of trends in soil chemistry and physical characteristics from these sites are represented in Table 1. In both cases, sandy soil texture was found, but there were differences in the chemical characteristics of the soils from the different areas assessed. Soils under the lichen *C. salzmannii* presented a higher pH value, sum of bases (S), cationic exchange capacity (CEC), base saturation (V) and calcium concentration (Ca); and lower value of aluminum (Al).

There is evidence that *C. salzmannii* affects some sandy soil parameters. As a result, the second part of this study investigated the biological influence of this lichen. Effects that lichens have on the interaction between soil microbial activity, mycorrhizal colonization and growth of *G. americana* seedlings were evaluated.

Table 1. Chemical and physical characteristics of soil samples from below *C. salzmannii* cover and from areas without lichen. Values are the mean of ten samples consisting of three subsamples taken from depths of 0 to 20 cm and mixed. * H_2O; CEC: cation exchange capacity; S: sum of bases; V: base saturation; m: Al saturation.

Treatments	P	pH*	Ca	Mg	Na	K	Al	H	CEC	S	Classes	V	m
	mg/dm^3		$cmol_c/dm^3$									%	
lichen cover	2±0.10	5.11±0.21	0.85±0.025	0.45±0.018	0.04±0.001	0.03±0.0018	0.55±0.03	5.22±0.35	7.1±0.28	1.4±0.05	Sandy	19±0.7	29±1.2
bare soil	2±0.09	4.85±0.19	0.25±0.010	0.50±0.020	0.04±0.002	0.02±0.0020	0.90±0.02	5.12±0.41	6.8±0.39	0.8±0.03	Sandy	12±1.1	53±2.9

The activity of the microbial community did not respond dramatically to lichens. In bare sandy soils high values of soil carbon dioxide respiration were observed, but these were not statistically different from those found in sandy soils with *C. salzmannii* cover (Table 2). Two assumptions are implicit in the calculation of biomass C from the carbon dioxide flux associated with chloroform fumigation: C from killed microorganisms is mineralized to carbon dioxide more rapidly than that in living microorganisms and death caused by fumigation is essentially complete.

For these reasons, in order to know the effects of *C. salzmannii* cover on microbial activity in soils, the mass of microorganisms was assayed. The lichen-covered sandy soil had a higher microbial biomass than the bare sandy soils, but none that would lead to any general conclusions (Table 2).

Table 2. Microbial activity of soil samples from below *C. salzmannii* cover and from areas without lichen. Values are the mean of ten samples consisting of three subsamples taken from depths of 0 to 20 cm and mixed. Means followed by different letters are significantly different according to Tukey´s test ($P \leq 0.05$).

Treatment	Respiration ($C\text{-}CO_2$ g dw of soil d^{-1})	Microbial biomass (μg C g $soil^{-1}$)	FDA (g dw of soil h^{-1})
lichen cover	6.5±0.29a	1462.3±28.48a	354.6±21.0a
bare soil	7.3±0.79a	1268.6±50.72b	337.4±17.0a

The interactions of *C. salzmannii*- mycorrhizal fungi associations were also evaluated in this research. Extracellular glycoproteins produced by AMF, named glomalin, that are operationally quantified from soil as GRSP were evaluated. In this study *C. salzmannii* did not have significant effects on GRSP levels (Table 3), additional mycorrhizal parameters were also assessed, such as percentage of root colonization and AMF spore number. Arbuscular mycorrhizal abundance was assessed using a stereomicroscope, whose results are also shown in Table 3. The highest values of percentage of root AMF colonization and spore number were observed in the areas with lichen cover, suggesting interaction between AMF and *C. salzamanni* cover.

Table 3. Arbuscular mycorrhizal colonization of soil samples from below *C. salzmannii* cover and from areas without lichen. Values are the mean of ten samples consisting of three subsamples taken from depths of 0 to 20 cm and mixed. Means followed by different letters are significantly different according to Tukey´s test ($P \leq 0.05$). The values were transformed into arcsine x/100 (colonization) and log x +1 (spore numbers).

Treatment	Glomalin (mg/g soil)	Colonization %	Spore Number
lichen cover	2.2±0.10a	3.1±0.20a	13.7±0.56a
bare soil	2.1±0.05a	2.5±0.07b	8.5±0.45b

The chemical characterization of phenolics produced by *C. salzmannii* was determined using TLC and HPLC-UV analyses. The major phenolic compound detected was BAR (Figure 1A). Through TLC assays it was possible to observe that soil extracts also contained this substance (Figure 1A), however HPLC-UV analysis revealed low concentration of the substance, an average of 3.9 µg mL^{-1}.

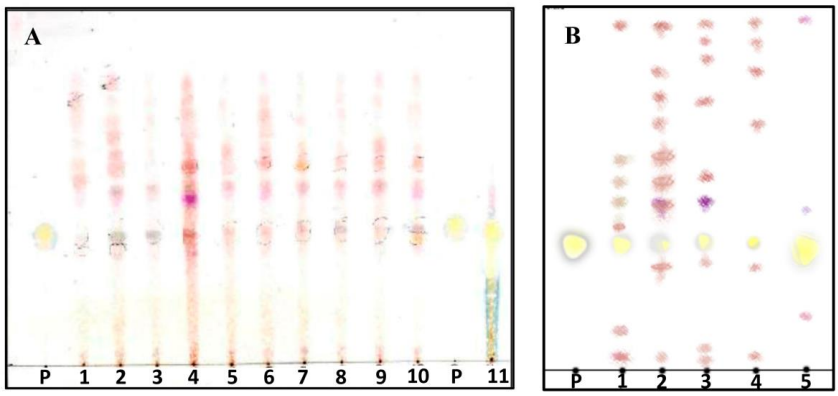

Figure 1. Thin Layer Chromatography. **A)** Natural conditions. 1-10: extracts from soil samples below *C. salzmannii* cover; 11: *C. salzmannii* extract; P: purified barbatic acid. **B)** Greenhouse conditions. P: purified barbatic acid. 1: soil with arbuscular mycorrhiza colonization treatment (AMF); 2: soil with lichen and AMF treatment combined (L+AMF); 3: soil with lichen treatment (L); 4: soil without treatment (C); 5: *C. salzmannii* extract.

Since lichens seem to affect soil parameters (Table 1) and AMF colonization (Table 3), the third part of this study investigated the effects that *C. salzmannii* have on the interaction with a vascular plant. This study was conducted in a greenhouse. Results of lichen and AMF influence on growth parameters (stem diameter, height, shoot and root weight) of *G. americana* seedlings grown under greenhouse conditions are shown in Figure 2**.** To compare the influence of lichens and AMF with that of other ground covers, a transplant grid was used with different aboveground material: lichens (L), mycorrhiza (AMF), L+AMF combined and bare ground (C). The experiment was conducted over the course of 4 months. Analyses of trends in soil chemistry and physical characteristics, mycorrhizal colonization and spore number are shown in Tables 4 and 5 respectively; growth parameters of *G. americana* are shown in Figure 2.

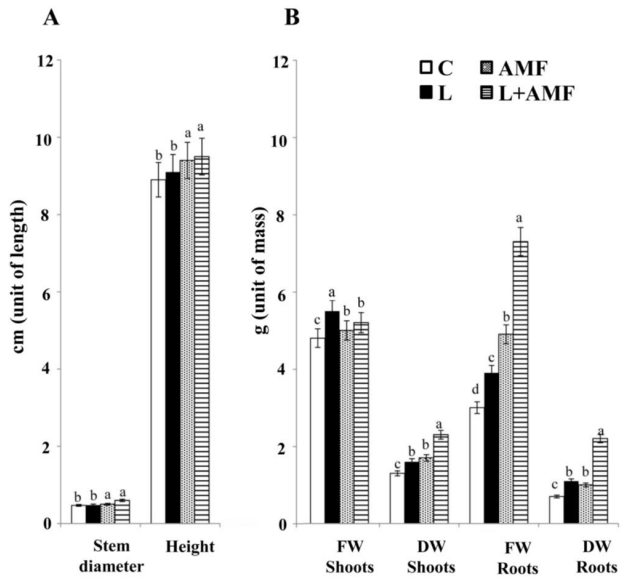

Figure 2. Growth parameters (A, length; B, mass) of *G. americana* seedlings grown in different treated soils, measured after 4 months. Soil with lichen (L), soil with arbuscular mycorrhiza colonization (AMF), soil with L+AMF combined and soil without treatment (C). FW: fresh weight; DW: dry weight. Means followed by different letters are significantly different according to Tukey´s test ($P \leq 0.05$).

In the AMF and L+AMF treatments there were significant differences in stem diameter and height of *G. americana* in relation to C, but there were no significant differences between the two treatments (Figure 2A). In the L+AMF treatment there were meaningfully significant differences showing higher fresh (FW) and dry (DW) weights than the other treatments (Figure 2B), which suggests some synergism between them. The same is observed in the L and AMF treatments, but the plants grown in L or AMF separately presented values lower than those for the plants grown in L+AMF combined. Plants grown with only L presented greater development than the C, and in some cases similar values to the AMF treatment.

As previously described, under natural conditions, *C. salzmannii* promotes AMF colonization (Table 3), this is how the positive effect of L+AMF observed under experimental conditions can be explained. In this case L+AMF treatment was considered higher, and significantly different, compared to the other treatments. AMF colonization (%) and spore number in the rhizosphere of *G. americana* under greenhouse conditions for 4 months are shown in Table 5. Seedlings grown in L+AMF combined had high AMF spore number and mycorrhizal colonization values, which showed significant differences.

Table 5. Soil arbuscular mycorrhizal colonization parameters in different experimental treatments of *G. americana* seedling grown under greenhouse conditions, measured after 4 months. Soil with lichen (L), soil with arbuscular mycorrhiza colonization (AMF), soil with L+AMF combined and soil without treatment. Means followed by different letters are significantly different according to Tukey´s test ($P \leq 0.05$). The values were transformed into arcsine x/100 (colonization) and log x +1 (spore numbers).

Treatments	Colonization (%)	Spore number
C	1.0±0.03b	0.3±0.01b
L	1.3±0.05b	0.4±0.02b
AMF	1.5±0.03b	1.52±0.10b
L+AMF	4.3±0.29a	6.65±0.43a

In the greenhouse soil samples, after 4 months of different treatments, chromatography revealed the presence of BAR (Figure 1B), again at a very low concentration (average 3.9 µg/ml). The chemical and physical properties in different experimental treatments of *G. americana* grown under greenhouse conditions soil are described in Table 4, where higher pH values in the soil were observed than at the start of the experiment. Ca, Mg and Na concentrations also increased in all treatments, as well as the S and V values. Soils after 4 months of AMF treatment presented a higher P value than control and other treatments. Three treatments (L, AMF, L+AMF) influenced the soil Al concentration, lower values were detected than values of control treatment.

Table 4. Soil chemical and physical properties in different experimental treatments of *G. americana* grown under greenhouse conditions. C: control ($t_{0:}$ before experiment, t_4: after 4 months); L, AMF and L+AMF: lichen, arbuscular mycorrizal colonization and combined treatments after 4 months. * H_2O; CEC: cation exchange capacity; S: sum of bases; V: base saturation; m: Al saturation.

Treatments	P	pH*	Ca	Mg	Na	K	Al	H	CEC	S	Classes	V	m
	mg/dm³		cmol$_c$/dm³									%	
Ct_0	3±0.10	4.6±0.21	1.25±0.07	0.25±0.017	0.05±0.001	0.03±0.0011	0.40±0.021	5.45±0.21	7.4±0.42	1.6±0.15	Sandy	21±1.7	20±1.3
Ct_4	3±0.08	5.12±0.19	1.50±0.10	0.25±0.021	0.50±0.024	0.02±0.0013	0.40±0.019	4.57±0.32	6.9±0.37	2.3±0.07	Sandy	33±2.1	2±0.09
L	3±0.10	5.28±0.23	1.75±0.20	0.40±0.019	0.50±0.031	0.02±0.0017	0.10±0.011	3.85±0.27	6.6±0.33	2.7±0.09	Sandy	40±3.1	4±0.21
AMF	5±0.20	5.28±0.17	1.50±0.05	0.50±0.023	0.50±0.012	0.02±0.0020	0.10±0.021	4.10±0.19	6.7±0.44	2.5±0.10	Sandy	38±2.6	4±0.17
L+AMF	2±0.06	5.06±0.13	1.75±0.07	0.30±0.017	0.75±0.027	0.01±0.0009	0.15±0.030	4.13±0.12	7.1±0.49	2.8±0.08	Sandy	40±2.9	5±0.17

4. Discussion

Alhandra (*cerrado* area of the Brazilian Northeast) is a good place to carry out this study because soil lichens are abundant, their communities have been well characterized since about 1960 and the soils have low nutrient availability (Pacheco and Cantalice, 2011; Vasconcelos *et al.*, 2015), so any lichen-induced effects will be important for the ecosystem.

The soils of *cerrado* areas generally present low nutrient availability, due to lower pH values and high Al saturation in soil solutions, which is very toxic to plants. Many soil parameters influence the soil Al exchangeable capacity, especially soil pH. Below pH 5, the proportion of Al as part of exchangeable acidity increases rapidly. The lack of Ca and Mg, frequently related to an excess of Al in the superficial layers of the soil, diminishes root system development, making the plants susceptible to drought (Kliemann, 2003).

According to the results obtained (Table 1), we can state that the values of CEC, S and V vary depending on the presence of the lichen *C. salzmannii*, which can be seen in the low values obtained of Al concentration and Al saturation (m). As detailed before, these parameters are influenced by pH values: soluble Al rises dramatically in nearly all soils below pH 5.0 (bare soil). Therefore, under lichen conditions Al concentration decreases by ~40%, which is according to the high calcium level detected and higher pH value. We can suppose that the presence of *C. salzmannii* modifies *cerrado* soils by making them more fertile.

Typic quartzpsamments soil cover 15% of the *cerrado* area and, as opposed to Litholic Neosols, are quite deep soils and consist almost entirely of sand. The main mineral in the sand fraction of these soils is quartz, which is extremely resistant to weathering and contains no nutrients. Pacheco and Cantalice (2011) reported similar soil characteristics in another *cerrado* area of the Brazilian Northeast: low content in organic matter, low capacity to retain water and nutrients, low cation exchange capacity, low base saturation, sandy texture, predominance of kaolinite in the clay fraction and a fragile, physical structure. According to Vasconcellos *et al.* (2015), and de Armas *et al.* (2016), sandy soil components are chemically modified by exocellular *C. verticillaris* metabolites. Silva *et al.* (2012) reported that phenols from *C. verticillaris* leached out by rainwater promote changes of the chemical composition of the soil. The *de novo* formation of wollastonite implies chemical reorganization of the material in such a way that diverse silicates could capture calcium from salts or chelates and could retain it (Schenkeveld *et al.*, 2007). This biogeochemical transformation of soils could explain the high Ca concentration found under pillows of *C. salzmannii* thalli, 3.4 times higher than that detected in bare soils (Table 1). There is controversial information concerning lichen interactions with mineral substrates, which different authors have associated to biodeterioration or bioprotection effects (Favero-Longo and Piervittori, 2010).

Measurement of soil carbon dioxide respiration is an index of microbial activity and therefore biological soil fertility. However, in semi-arid regions, the high incidence of solar radiation, high temperatures and the great variability of precipitation during the year contribute to loss of CO_2 from the soil. Perhaps this simple and rapid method of chemical titration for soil CO_2 is not an effective means to quantify microbial activity under these analysis conditions. Soils were collected during the months of November and December, days of higher intensity of solar radiation and insolation, and consequently, of higher temperatures in the Brazilian Northeast. This fact could explain the decreasing of respiratory activity detected, probably under these environmental conditions the loss of CO_2 was higher. For these reasons, the mass of microorganisms of soil samples from below *C. salzmannii* cover and from areas without lichen was also assayed

(Table 2). Studies have shown the need to relate soil microbiology to enzymatic activity because measures such as CO_2 emission and C microbial biomass, on their own, do not reflect soil biological activity. In the case of respiration, microorganisms may not be the only source of CO_2 emission, and the microbial biomass does not estimate the isolated microorganism metabolic activity. With the increased interest in integrated soil bioecosystem studies, there was a need to have a method of measuring overall microbial activity. As a measure of total microbial activity, FDA hydrolysis was used in this study, a method commonly used as an indicator of the activity of soil hydrolytic enzymes. FDA hydrolysis has been suggested as a possible measure of total microbial activity because the ubiquitous lipase, protease, and esterase enzymes involved in the hydrolysis of FDA are plentiful in the soil environment. In general, measures of carbon dioxide respiration and FDA were quite similar, but microbial biomass can be distinct from those (Schünurer and Rosswall, 1982). More efficient microbial communities accumulate C in their biomass, and consequently present lower microbial respiration values which result in loss of C to the atmosphere (Dadalto *et al.*, 2015). In this way, the results obtained in our study indicate that soil coverage with *C. salzmannii* can help to increase carbon sequestration and to mitigate, to a lesser extent, global warming.

Lichen presence in the soil resulted in different levels of mass of microorganisms, and although *C. salzmannii* favored FDA hydrolysis, statistical differences have not been found compared to bare soils. A possible consequence is that lichen cover could also affect microbial biomass (Table 2). These results are in agreement with those reported by Sedia and Ehrenfeld (2005). Those authors, in comparing lichen, bryophyte, and grass effects on soil, highlighted that lichens and bryophytes clearly affect soil microbial properties. However, Ohtonen and Väre (1998), in previously assessing the microbial activity in areas with lichens and in areas with bryophytes, did not notice differences in the microbial biomass, but did find that areas with lichen presented higher qCO_2. This could be because soil microbial activity depends on various factors, such as climate, habitat productivity and ecological succession stage. Soil organic matter and microbial biomass can be altered to a lesser or greater extent depending on the vegetation and environmental conditions of the site. Stark and Hyvärinen (2003) observed higher qCO_2 values, which should suggest that lichen substances such as usnic and perlatolic acid are sources of C to the microbial communities existing beneath lichen cover.

The enzymatic conversion of FDA to fluorescein appears to be primarily a hydrolysis reaction followed by a dehydration reaction. The ability to hydrolyze FDA thus seems widespread, especially among the major decomposers: bacteria and fungi. Generally, more than 90% of the energy in a soil system passes through microbial decomposers, therefore an assay which measures microbial decomposer activity will provide a good estimate of total microbial activity. However, the current method for measuring FDA hydrolysis in soils is limited in its application. Adam and Duncan (2000) found that FDA hydrolysis was very low in sandy and/or clayey soils with low microbial activity, which must also be taken into account in this study.

Sandy soils have been defined by their low microbial activity, what makes the measurement of FDA hydrolytic activity more difficult. In addition, Green *et al.* (2006) found that acetone caused a decrease of ~ 37% in the absorbance of fluorescein produced by the soil samples measured, however this solvent was found to be most efficient at stopping the hydrolysis reaction. During this study parameters of the hydrolysis reaction were modified for the measurement of soil samples according different methods previously reported.

The optimization of this method could explain why the increase in FDA hydrolisis activity was not statistically significant while the microbial biomass was. The comparison showed some differences between areas with *C. salzmannii* cover and bare sandy soils in *cerrado* (Brazil), but it is not possible to identify any general conclusions. The only differences were small and seem to favor microbial and biochemical activity in lichen-covered, sandy soils compared to bare areas. Microbial biomass generally comprises approximately 2% of the total organic matter in soil and may easily be dismissed as of minor importance in the soil. However, this is a mistake, microbial biomass is an important agent in controlling the overall biological activity of the soil; the role of microorganisms in soil formation has been proven at different stages of ecosystem development. Higher abundances and activities of microbes have been detected in the rhizosphere of pioneer plants (Schulz *et al.*, 2013). It is unquestionable that plants display a nutrient hotspot in terms of C, as they provide up to 40% of the photosynthetically fixed CO_2 to the microbes. In return, microbes supply the plant with N, phosphate or other nutrients. They are an integral part of all ecosystems, they are often responsible for either fixing or capturing essential nutrients from the air and returning them to the environment.

Lichens are common primary successional species, preparing a previously barren landscape for other plants and organisms to exploit. Lichen secondary products begin the breakdown of sedimentary and igneous rocks, providing a loose soil matrix in which other nonvascular and vascular plants can take root. As other species begin to dominate an ecosystem, decaying lichen material provides additional nutrients.

Based on this, it was also decided to evaluate the interactions of *C. salzmannii*-plant-mycorrhizal fungi associations. This arrangement creates a positive feedback loop established between lichen-microorganisms-vascular plant. Several *in vitro* studies have suggested that competitive relationships between terricolous lichens and plants depend on allelopathic interferences, but other ecological investigations have not supported this finding (Kytöviita and Stark, 2009). Accordingly, these authors have suggested that the antimicrobial and allelopathic interpretation of lichen secondary metabolites in natural systems should be re-evaluated.

Results of arbuscular mycorrhizal colonization of soil samples from below *C. salzmannii* cover and from areas without lichen are shown in Table 3. Lichen did not have significant effects on GRSP levels. GRSP are produced in mycorrhizal fungal cell walls where they remain. Those products are only released into the soil environment during hyphal turnover and after the death of the fungus so production rates of GRSP are not always correlated with AMF abundance in soil. Hence, standing stocks of glomalin in soil have to be determined by its production and decomposition (Singh *et al.*, 2013), environmental conditions could affect the two fluxes independently. In addition, there are also critical assessments of using the Bradford assay to quantify GRSP: cross-reactivity has been shown with non-AMF proteins added before autoclaving and with other non-proteinaceous materials added to the GRSP extract; this can result in an overestimation of GRSP stocks.

It was for those reasons that additional mycorrhizal parameters were also assessed (Percentage of root colonization and AMF spore number). In the areas with lichen cover values of percentage of root AMF colonization and spore number were highest (Table 3). The results could indicate that the lichen substances are capable of providing an association between plants and AMF, which is one of the most important symbioses on earth, linking the root and the soil system. Lichens are well known for the important biological activity of their substances, which accumulate either in the cortex or in the cell walls of medullary

hyphae and thalli, and can be leached out to the soil by rainfall or dew. Thus, such substances can affect the soil in different ways (Asta *et al.*, 2001; Vasconcelos *et al.*, 2015, 2016). As such, the literature data could corroborate the results. Moreover, climatic conditions like water content and radiation are not stable in the *cerrado*, leading to a strongly reduction of phenolic compounds leached into the soil.

The fact that there is no meaningful effect on some parameters analyzed can be a consequence of the low concentration of BAR detected in the soil (~ 3.9 µg mL^{-1}, Figure 1). Alhandra presents a tropical climate As´, according to the classification of Köppen. The maximum rainfall happens in July with approximately 220 mm of average rainfall while the driest period corresponds to October, with an average rainfall of 22 mm. Soil samples were collected in the dry season. The biological modifications of the soil that *C. salzmannii* can provoke depend on the leaching of BAR by rainfall and its percolation in the soil for interactions between fruticose lichens and soil. The interaction between lichen and substrate depends on environmental factors including the distribution of rainfall (de Armas *et al.*, 2016). These authors concluded that proteins, protocetraric and fumarprotocetraric acid from *C. verticillaris* that leached into the soil would be retained, transformed or percolated to deeper soil layers, depending on the amount of rainfall. Further studies, during the rainy season, are still necessary for a better understanding of the processes involved and confirmation of the trends observed here. During the rainy season the effect of lichen substances may be more pronounced than that observed in the dry season. Even so, the results indicate that BAR is favorable to AMF formation. The high physiological cost in energy and carbon for *C. salzmannii* to produce this compound suggests that it could play important ecological roles in soils. Finally, one can summarize that lichens play a crucial role in ecosystem development, but their function can change seasonally.

There are controversial data on the effects of lichens near vascular plants (Favero- Longo and Piervittori, 2010). Most studies of lichens evidence their negative influence on other organisms; such an influence can be related to the action of lichen substances as reported in the introduction. However, the present paper does not evidence such a relationship. This fact indicates that the chemical constitution of the species studied, as well as the concentration of their substances, are responsible for the activity that the lichen may exert on the tested organism. Under experimental conditions, in most cases among the variables measured, higher values were detected for the L+AMF treatment. The fact that seedlings colonized with AMF presented higher values than the control (C), in terms of vegetative growth, is associated with the high capacity of AMF-colonized plants to acquire mineral nutrients from the soil, thereby enhancing plant growth (Pérez *et al.*, 2011). The aforementioned results can indicate a potential lichen effect. *Cladonia* species may contribute to humus formation (Asta *et al.*, 2001), in addition to affecting soil temperature and humidity. These factors may have contributed to the *G. americana* development in the plants with lichen as expected. In other cases, nutrient dissolution from the plant substrate provides mineral nutrition to epiphytic lichen communities. It may, therefore, be a mutual benefit.

While AMF colonization was considered low, colonization and the response to inoculation can vary according to different hosts, depending on the effectiveness of the AMF, P availability in the soil and the fungi-plant combination and the developmental stage of the plants. According to Siqueira (1991), there is a great extent of functional compatibility which results in the establishment of symbiosis between plant and

fungi, controlled by the genetics of both organisms and modulated by the environment.

The phenolics contained in the lichens degraded the soil minerals albite and microcline, a factor that influenced and increased the level of calcium in the soil. It is known that the nutrient availability to the plants is affected by different soil features, such as pH, humidity and microbial activity. Taking into consideration the fact that the presence of Al is one of the main factors that limit plant development in acidic soils, it is important to mention that the Al values decreased after 4 months of different treatments (Table 4). In the L and L+AMF treatments, the V value increased by ~50%, which is related to the high Ca and Mg levels detected and higher pH value. In addition, many reports where different pH conditions were studied have shown root colonization to be reduced at lower pH.

It can be assumed that the presence of *C. salzmannii* and AMF modifies greenhouse soils, making them more fertile as is the case in *cerrado* soils (Table 1). It would seem that treatments can improve the chemical parameters of soil. In addition, a slight presence of AMF spores was observed in the controls (Table 5), perhaps due to some kind of contamination, pointing out that soil is a reservoir of mycobiont partners for plants. The very low vegetative development proves that this probable contamination did not interfere in the results obtained. Given that lichen substances are known for their biological activity, it is possible to suppose that the presence of BAR in the soil may also be influencing the results either directly, by action on the plant, or indirectly by the effects on the AMF. Yano *et al.* (1999), observed that the aqueous extract of *C. verticillaris* stimulates the growth of *Allium cepa*; this fact being related to the presence of fumarprotocetraric acid. Thus, lichens probably exert an ecological influence on the habitat, controlling somehow the surrounding vegetation. The phytotoxic effect of certain lichen metabolites may deter the growth of other lichen populations as well as that of competing bryophytes or vascular plant seedlings for a particular ecological niche. If lichens support the growth of *G. americana*, areas with high lichen cover will be associated with higher cover of *G. americana* and higher AMF colonization in native plants, helping ecological succession. Recent research comparing lichen-rock interactions indicates that a common set of physical and chemical factors can explain the heterogeneous reciprocal effects that exist between lichens and plants, which depend on the species involved, and highlights gaps that need further studies. The contradictory results, reviewed in Favero-Longo and Piervittori (2010), have been consistently supported by both *in vitro* and field data, but differences depend on the lichen metabolites, plant and mycorrhizal fungus species considered, and prevent generalized quantifications of the phenomenon. It must be pointed out that the field portion of this experiment analyzed vegetation communities, thereby reflecting the situation in older established ecosystems, which does not apply to greenhouse conditions. A wider spectrum of environmental conditions should also be taken into account in field studies on the effect of terricolous lichens. Two types of experimental analyses are required to unravel the complex relationships between lichen-AMF-plant vascular-soil.

This is the first time that the influence of BAR on *G. americana* growth and soil microbial activity, specifically concerning AMF, has been reported. If *C. salzmannii* favors AMF associations that could enhance tolerance to conditions of environmental stress, such as high soil salinity, the species could thus prove to be a key component in helping plants to cope with potential adverse environmental conditions. Further investigations of soils are required to find out underlying lichen mechanisms and confirm the tendencies observed.

Conclusions

This work shows that in *cerrado* and under greenhouse conditions; AMF colonization is modified by *C. salzmannii*. Meanwhile, *G. americana* growth was significantly affected by lichen (L) + AMF treatment. The results of the present study contribute to our understanding of how lichens interact with *cerrado* soils and therefore why the maintenance of lichen is important.

It is necessary to examine the overall factors, which act at the lichen interface with any biotic or abiotic substrate. This interdisciplinary and integrated research can serve as a gateway for educating the public about the roles of lichens in ecosystems

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