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Arbuscular Mycorrhizal Fungi in the Phytostabilization of Soil Degraded by Manganese Mining

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Abstract

Mining and processing of manganese (Mn) minerals are activities that may result in the generation of large amounts of wastes and serious environmental impacts. Several strategies have been employed to remediate areas with high Mn concentrations, but many of them imply high investments and high risk of secondary pollution. This study aimed to evaluate the phytostabilization potential of *Mimosa caesalpiniaefolia* Benth. in Mn mining soil influenced by inoculation with arbuscular mycorrhizal fungi (AMF). The experimental design was completely randomized, with four treatments [not inoculated (control), inoculated with *Rhizophagus clarus*; inoculated with *Claroideoglomus etunicatum* and inoculated with *Rhizophagus clarus* + *Claroideoglomus etunicatum* (Mix)], and four replicates. Inoculation with Mix and *C. etunicatum* had higher efficiency in protecting plants against excess Mn, due to the greater retention of this element in the roots and lower translocation to the shoots. Inoculation with *R. clarus* did not influence plant development and reduction of Mn contents in the shoots. The association of the AMF Mix and *C. etunicatum* with the species *Mimosa caesalpiniaefolia* Benth. enhances Mn phytostabilization in mining soils with high concentration of this element. The use of multivariate analyses proved to be an important tool with respect to the behavior of biometric, chemical and microbiological variables in mining soil with high Mn concentration.

Keywords: heavy metals, revegetation, microorganisms

1. Introduction

Mining and processing of manganese (Mn) minerals are activities that may result in the generation of large quantities of wastes and serious environmental impacts. Improper management of this material can result in its dispersion on soil surface by runoff and or it may even reach groundwaters through leaching.

Mn is considered as an essential metal micronutrient but, when in excess, it can be considered as highly toxic to most living organisms (Chen, Yan, Sun, Tian, & Liao, 2016; Mattison et al., 2017). In the case of plants, high Mn concentrations can delay growth and cause symptoms of chlorosis and necrosis, besides interrupting essential metabolic processes such as absorption, translocation and utilization of essential elements (Yang et al., 2015a).

Various strategies have been employed to remediate areas with high Mn concentrations, for instance the use of physical and chemical methods. Nonetheless, such strategies involve high investments, deterioration of soil quality and high risk of secondary pollution (Yang et al., 2013). Phytostabilization is a technique of phytoremediation based on the immobilization of the metal contaminating the soil through its absorption and accumulation inside or outside the root (Brancher & Rogrigues, 2010), thus reducing its mobility and entry into the food chain.

In this context, inoculation with arbuscular mycorrhizal fungi (AMF) is considered as an important strategy in the reestablishment of plants with this purpose, given their capacity to increase the absorption of water and nutrients and to act in the reduction of the availability of metals to the host plant by different mechanisms.

These mechanisms include: dilution in tissues of plants due to their growth (Christie, Li, & Chen, 2004; Schneider, Labory, Rangela, Alves, & Guilherme, 2013), reduction in absorption of metals due to retention and immobilization in certain fungal structures and mycorrhized roots (Gonzalez-Chavez, D'Haen, Vangronsveld, & Dodd, 2002), chelation of metals by compounds secreted by AMF, such as glomalin (Vodnik, Grčman, Maček,

van Elteren, & Kovačevič, 2008; Leung et al., 2013), chelating agents in the cytosol including the use of metallothioneins (plants and fungi), which are metal-binding proteins synthesized in a wide range of organisms exposed to toxic concentrations of metals (Kumar, Dayananda, & Subramanyam, 2005; Cabral, Soares, Giachini, & Siqueira, 2015), accumulation in plant vacuoles and fungal cells (Bertolazi et al., 2010) and temporary immobilization due to the transport by the hyphae (Carneiro, Siqueira, & Moreira, 2001). Given the above, this study aimed to evaluate the phytostabilization potential of *Mimosa caesalpiniaefolia* Benth. in Mn mining soil influenced by inoculation with AMF.

2. Material and Methods

The experiment was carried out in greenhouse at the Department of Soil Science (DCS) of the Federal University of Ceará (UFC), located at the Pici Campus, Fortaleza, Ceará, Brazil (3º45′47″ S; 38º31′23″ W, at mean altitude of 47 m). The climate of the region is classified as hot tropical, with mean annual temperature and rainfall of 27 ºC and 1600 mm, respectively, characterized as Aw' based on Köppen's classification.

The experimental design was completely randomized, with four treatments of inoculation [control – not inoculated, *Rhizophagus clarus*; *Claroideoglomus etunicatum* and *R. clarus* + *C. etunicatum* (Mix)] and four replicates.

The soil used in the experiment was collected in the 0-20 cm layer in an area of Mn mining exploitation located in the municipality of Ocara, Ceará, Brazil. After collection, the soil was sieved through a 4-mm mesh to remove coarser particles, and then autoclaved at 121 ^{o}C at 1 atm pressure for 2 h, to eliminate the native microbiota. Then, soil chemical characteristics were determined by the Laboratory of Soil and Water Routine Analysis of UFC, following the methodological references of EMBRAPA (2009) (Table 1).

Table 1. Chemical characteristics of the soil in the Mn mineral exploration area located in Ocara-CE, Brazil, used to cultivate *M. caesalpiniaefolia* Benth

pH	Al	Ca	Mg	Na	K	S	H+Al	P	N	OM	Mn	Fe	Cu	Zn
(H_2O)	---------- cmolc kg^{-1} ----------							mg kg^{-1}	---- g kg^{-1} ----		---------- mg kg^{-1} ----------			
4.95	0.2	1.4	1.2	0.12	0.15	2.87	4	2.9	0.28	3.93	425.8	82.81	2.39	2.91

The soil was distributed in pots with capacity for 5 kg (4 kg of soil per pot), which received basal fertilization with 6.75 mg of N, 25 mg of K, 10 mg of P and 7.5 mg of Ca per kg of substrate. The sources for the nutrients used were: NH_2CONH_2, KCl, $Ca(H_2PO_4)_2.H_2O$ and $CaSO_4$, respectively.

Mimosa caesalpiniaefolia Benth. seedlings were produced on polystyrene trays, by placing two seeds per cell at 2 cm depth. The substrate used consisted of washed sand, autoclaved at 121 °C and 1 atm pressure for 2 h. At 11 days after sowing, thinning was carried out in each cell, leaving only the most vigorous plant. At transplantation to the plastic pots, the seedlings were inoculated with 40 g of soil-inoculum containing spores and fragments of corn (*Zea mays* L.) roots colonized by the AMF species *Rhizophagus clarus* and *Claroideoglomus etunicatum*, separately and in a mixture (Mix).

The AMF isolates used in this experiment came from the Inoculum Bank of the Soil Microbiology Sector of UFC. The inoculum was placed at approximately 4 cm below the substrate surface. At inoculation, the soil-inoculum used had approximately 258, 437 and 349 spores in 40 g of soil for the treatment with *R. clarus*, *C. etunicatum* and Mix (*R. clarus* + *C. etunicatum*), respectively. In order to reestablish the microbial community in the substrate, the substrate received 8 mL of a "filtrate" from the same soil used in the experiment (not autoclaved), without the presence of AMF propagules.

Plants were kept in the greenhouse and irrigated daily along the entire experimental period, which lasted 60 days. At the end of the experiment, plant shoots were cut close to the substrate and the material was then identified, placed in paper bags and dried in a forced-air oven at temperature of approximately 65 °C, to obtain shoot dry matter. Roots were removed from the substrate, washed with tap water and dried following the same sequence used for the shoots. After obtaining shoot and root dry matter, the material was ground in a Wiley-type mill to determine the contents of phosphorus (P) in the shoots and manganese (Mn) in the shoots and roots, which were determined after nitric-perchloric digestion. P was analyzed by colorimetry and Mn by atomic absorption spectrophotometer.

The density of AMF spores after seedlings' growth was determined through the extraction of 100 g of soil by wet sieving from each sample of the treatments, following the procedures described by Gerdemann and Nicholson

(1963). To evaluate mycorrhizal colonization, roots were washed in running water and placed in a container with 70% alcohol solution. Roots were cleared and stained for analysis of colonization according to the methodology adapted by Koske and Gemma (1989). Colonization percentage was obtained according to McGonigle et al. (1990).

The data were subjected to analysis of variance by F test ($p \leq 0.05$), and means were compared by Scott-Knott test ($p \leq 0.05$), using the statistical program ASSISTAT, Beta version 7.7 (Silva & Azevedo, 2016). Additionally, cluster and principal component analyses were carried out using the program STATISTICA, version 7.0 (STATSOFT, 2004).

3. Results and Discussion

According to the analysis of variance (Table 2), the inoculation of *M. caesalpiniaefolia* Benth. plants with AMF significantly influenced ($p \leq 0.01$) shoot and root dry matter, AMF spore density in soil, arbuscular mycorrhizal colonization, P content in the shoots and Mn contents in shoots and roots.

Table 2. Results of the analysis of variance (ANOVA) for the variables shoot dry matter (SDM), root dry matter (RDM), AMF spore density in soil (SpD), mycorrhizal colonization (MC), phosphorus content in the shoots (P) and manganese contents in the shoots (Mn Shoot) and roots (Mn Root)

Variables	Units	MS[1]	CV (%)[2]	AO[3]	F test
SDM	(g plant^{-1})	54.08	17.43	3.6	136.62**
RDM	(g plant^{-1})	12.82	15.67	1.64	192.26**
SpD	(100 g^{-1} soil)	441894.75	22.63	399.37	50.42**
MC	(%)	1911.89	22.03	21.59	84.52**
P	(g kg^{-1})	0.07	10.45	0.65	15.38**
Mn Shoot	(mg kg^{-1})	7682.41	4.53	1027.59	3.54**
Mn Root	(mg kg^{-1})	82469102.93	7.53	8262.65	212.95**

Note. [1] Medium square; [2] Coefficient of variation; [3] Average Overall; **, *, ns: significant by the F test at the level of 1% and 5% of significance and not significant, respectively.

Mimosa caesalpiniaefolia Benth. plants inoculated with the AMF, Mix and *C. etunicatum*, showed significant increases of 29.1% and 25.2%, respectively, in shoot dry matter (SDM) compared with non-inoculated plants (Figure 1A). Similar response was also observed for root dry matter (RDM), where inoculation with the AMF Mix followed by *C. etunicatum* led to significant increases of 26% and 16.5%, respectively, compared with non-inoculated plants (Figure 1B).

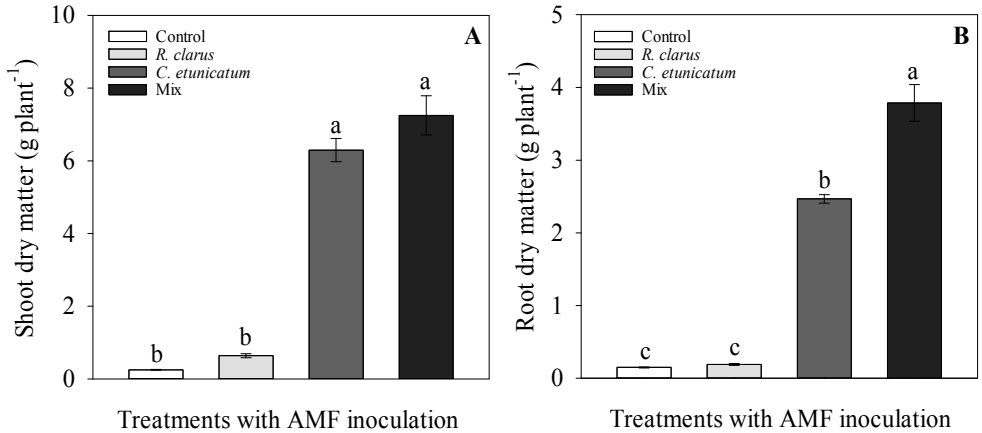

Figure 1. Shoot dry matter (SDM) and root dry matter (RDM) as a function of different treatments with AMF inoculation in *M. caesalpiniaefolia* Benth. plants grown in Mn mining soil. The values represent the mean of four replicates±standard error. Means followed by equal letters do not differ by Scott-Knott test ($p \leq 0.05$)

Enhancement in nutrient absorption from the soil solution and protection of plants against toxicity by heavy metals are benefits caused by the AMF which are related to the greater growth, generally in terms of dry matter, in mycorrhized plants (Carrenho, Alves, & Santos, 2018; Gunathilakae, Yapa, & Hettiarachchi, 2018). Li, Sun, Jiang, Chen, and Zhang (2018), studying the attenuation of toxicity by arsenic in *Medicago sativa* plants colonized by AMF, reported significant increase in shoot and root dry matter of mycorrhized plants at all levels of arsenic. Similar responses of inoculation with AMF have also been observed by Nogueira, Nehls, Hampp, Poralla, and Cardoso (2007) in soybean plants grown in soil with high Mn content and by Garcia et al. (2017) in *Mimosa caesalpiniaefolia* Benth. plants grown in soil degraded by Mn mining.

AMF spore density in soil was significantly higher in the rhizosphere of plants inoculated with *C. etunicatum*, followed by the treatment Mix, compared with the rhizosphere of plants inoculated with *R. clarus* (Figure 2A).

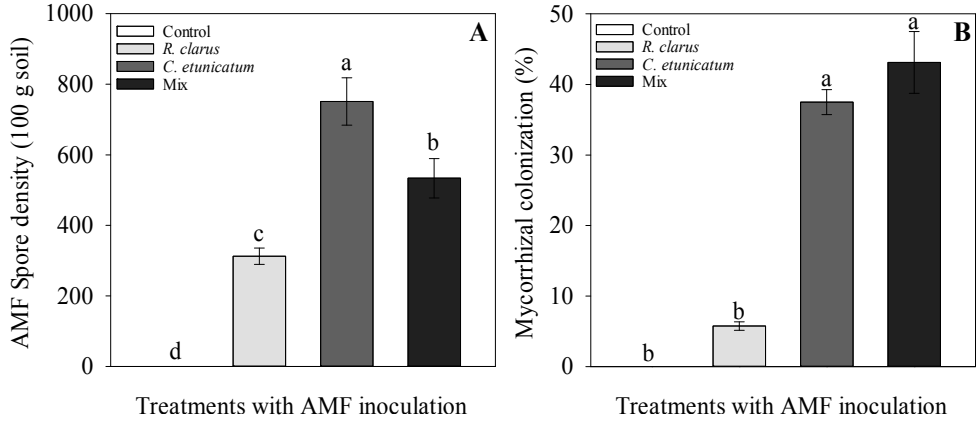

Figure 2. AMF spore density (SpD) and arbuscular mycorrhizal colonization (MC) as a function of different treatments with AMF inoculation in *M. caesalpiniaefolia* Benth. plants grown in Mn mining soil. The values represent the mean of four replicates±standard error. Means followed by equal letters do not differ by Scott-Knott test ($p \leq 0.05$)

The number of AMF spores in soil has been a parameter widely used by researchers to evaluate the effect of heavy metals on AMF (Rivera-Becerril et al., 2013; Schneider, Bundschuh, Rangel, & Guilherme, 2017; Garcia et al., 2017). A series of limitations, such as the reduction in spore germination, extraradical mycelium development and sporulation of some AMF species, has been reported in the presence of excess heavy metals in the soil (Pawlowska & Charvat, 2004; Yang et al., 2015b). Cardoso, Navarro, and Nogueira (2002), working with Mn and in vitro germination of AMF spores, concluded that AMF spore germination was differently damaged between species and Mn^{2+} doses. On the other hand, in soil contaminated by cadmium, Martins, R. Melloni, and E. G. P. Melloni (2017) reported different behavior between AMF species at the different doses of this element, thus demonstrating higher tolerance of some AMF to the excess of this metal. In areas of lead and zinc mining, Yang et al. (2015b) observed variation from 46 to 670 spores in 100 g of soil, in soil with higher and lower levels, respectively, of contamination by these metals.

Regarding arbuscular mycorrhizal colonization, plants inoculated with *C. etunicatum* and Mix had the highest percentages of colonized roots, 37.5% and 43.12%, respectively (Figure 2B). There were no spores or signs of root colonization in plants of the control treatment, thus indicating the absence of contamination. Variations in the responses of mycorrhizal colonization by different AMF species in the same plant grown in soil with excess of heavy metals have already been evidenced in several studies (Schneider et al., 2017; Li et al., 2017; Spagnoletti, Carmona, Gómez, Chiocchio, & Lavado, 2017; Brito, Carvalho, Alho, & Goss, 2014). In the present study, it is also possible to observe that the same treatments of inoculation with AMF (*C. etunicatum* and Mix) which had positive effect on plant development were also responsible for the highest percentages of mycorrhizal colonization. The effects of AMF on plant development depend on fungal isolate, plant species and heavy metals involved in the association (Ferrol, Tamayo, & Vargas, 2016). In addition, the data of the present study also allow to state that there may have been a synergistic effect of the treatment Mix, when both symbionts were inoculated together, resulting in higher percentage of mycorrhizal colonization and other benefits, as also reported by Tian, Drijber, Li, Miller, and Wienhold (2013).

As to the P contents in the shoots (Table 3), inoculation with *C. etunicatum* followed by the treatment Mix caused significant increments of 47.3% and 17.5%, respectively, in this variable, compared with non-inoculated plants. In general, mycorrhized plants had higher P contents than non-mycorrhized plants.

Table 3. Phosphorus contents in the shoots as a function of different treatments with AMF inoculation in *M. caesalpiniaefolia* Benth. plants grown in Mn mining soil

Treatments with AMF inoculation			
Control	*R. clarus*	*C. etunicatum*	Mix
---------- P contente in the shoot (g kg^{-1}) ----------			
0.57±0.03 c	0.54±0.01 c	0.84±0.04 a	0.67±0.03 b

Note. The values represent the mean of four replicates±standard error. Means followed by equal letters do not differ by Scott-Knott test ($p \leq 0.05$).

This fact is related to the larger soil volume explored by the AMF hyphae, which work as an extension of the root system (Folli-Pereira, Meira-Haddad, Bazzolli, & Kasuya, 2012). Under adequate nutrition with P, plants may exhibit higher vigor and capacity to withstand the negative effects caused by the excess Mn (Nogueira et al., 2007). According to Foy (1984), the formation of low-solubility complexes between P and Mn may act in the reduction of toxicity by Mn. Our results also corroborate those found by Zhou et al. (2017), and Sadia, Asma, and Riffat (2016).

Mn contents in the shoots decreased significantly in plants inoculated with AMF, compared with non-inoculated plants, except for the treatment with *R. clarus* (Figure 3). On the other hand, Mn contents in the roots were significantly higher in plants inoculated with AMF, except for *R. clarus* (Figure 3).

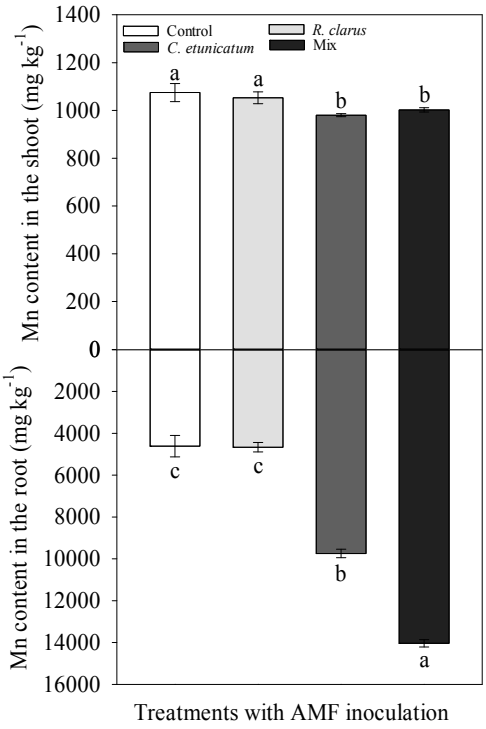

Figure 3. Manganese contents in the shoots and roots as a function of different treatments with AMF inoculation in *M. caesalpiniaefolia* Benth. plants grown in Mn mining soil. The values represent the mean of four replicates±standard error. Means followed by equal letters do not differ by Scott-Knott test ($p \leq 0.05$)

Mimosa caesalpiniaefolia Benth. plants inoculated with AMF had mean Mn content of 1074.86 mg kg^{-1}, which is above the levels considered as toxic for this metal (Mn = 400 mg kg^{-1} in shoot dry matter) (Kabata-Pendias &

Pendias, 2001; Foy, Chaney, & White, 1978). However, although the inoculation with *C. etunicatum* and Mix reduced Mn contents in the shoots, compared with non-inoculated plants, these contents were still very high (980.29 to 1002.21 mg kg^{-1}) and above the range considered as toxic for plant species in general (Kabata-Pendias & Pendias, 2001). In spite of that, colonized plants did not exhibit symptoms of toxicity. It has been demonstrated that mycorrhizal colonization and tolerance of AMF to heavy metals have shown high variability, resulting from both AMF species and host plant (Liu et al., 2015). Frequently, mycorrhization in some plants has led to higher contents of metals in the roots and lower contents in the shoots (Ferrol et al., 2016; Wu et al., 2016; Gunathilakae et al., 2018). In the present study, we also observed that plants colonized by *C. etunicatum* and Mix showed higher Mn contents in the roots than in the shoots. The reason for this response can be related to the capacity of arbuscular mycorrhizae to retain and immobilize metals in certain fungal structures and colonized roots (Gonzalez-Chavez et al., 2002). However, a more detailed investigation using scanning electron microscopy associated with X-ray microanalysis would be necessary to clarify such hypothesis. The obtained results also confirm the potential of the species *M. caesalpiniaefolia* Benth., mainly when associated with the AMF, to act as phytostabilizing plant in areas of Mn mining.

The responses of cluster analysis illustrated by the dendrogram (Figure 4) allow the identification of two main groups which describe the interactions between the and biometric, chemical and microbiological variables of the present study: Group 1, containing only Mn Shoot, and Group 2, including SDM, RDM, MC, SpD, P and Mn Root.

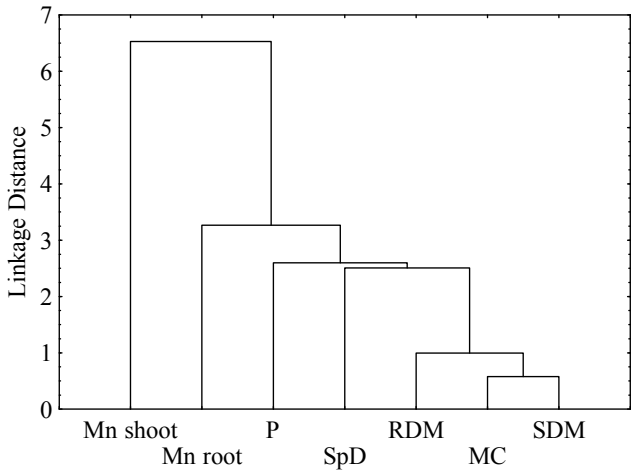

Figure 4. Dendrogram for the 7 variables (Mn Shoot, Mn Root, P, SpD, RDM, MC, SDM) analyzed as a function of different treatments with AMF inoculation in *M. caesalpiniaefolia* Benth. plants grown in Mn mining soil

The method most used to extract factors in factor analysis is the principal component (PCA) method. It is used to reduce the number of variables in order to obtain a lower number of factors necessary to explain as much as possible the variance represented by the original variables (Ho, 2006).

The variables composing the principal components (PC) were selected based on the homogeneity and heterogeneity of the groups identified in the Cluster Analysis. In this context, no variable was disregarded for having short linkage distances and, therefore, high intensities of correlation. Thus, the PCA was conducted in the data matrix composed of 7 variables. According to Vicini (2005), the selection of the number of components can take into account different criteria, and a very important one is the Kaiser's or latent root criterion, which selects only the factors with eigenvalues higher than 1 (one).

Based on this premise, two components with eigenvalues above 1 (one) were extracted and together explained about 83.62% of the total data variability (Table 4).

Table 4. Extraction of the principal components for biometric (SDM, RDM), chemical (P, Mn Shoot and Mn Root) and microbiological (MC and SpD) values and their respective eigenvalues, total variance and cumulative variance

Variables	Factor 1	Factor 2
SDM	0.96066	0.24911
RDM	0.90651	0.38035
MC	0.93986	0.32475
SpD	0.88808	-0.18755
P	0.81311	-0.42117
Mn Shoot	-0.74547	0.14285
Mn Root	0.73133	-0.37442
Eigenvalues	5.16834	1.10000
Total variance (%)	73.8335	9.79058
Cumulative variance (%)	73.8335	83.62400

The rotated matrix of the components containing the factor loadings shows the degree of correlation existing between the variables and the principal component 1 (PC1). The rule generally adopted is that the minimum value for the loadings is 0.30, but loadings with values above 0.5 are the significant ones (Hair, Anderson, Tatham, & Black, 2005).

The correlations between the studied variables and PC1 were: SDM (0.960657), MC (0.939855), RDM (0.906511), SpD (0.888075), P (0.813111) and Mn Root (0.731332); for Mn Shoot (-0.745465) the sign of the loading was negative, indicating that the correlation is inversely proportional, i.e., as the quantitative values of SDM, MC, RDM, SpD, P, Mn Root, in this order of importance, increase, there is a reduction in Mn Shoot values. The total variance explained by PC1 was 73.83%, that is, 73.83% of the behavior of the variables is being retained by the factor (Table 4).

The results obtained in the analysis of all variables support the hypothesis that the reduction in Mn content in the shoots of plants colonized by AMF is related to its higher accumulation in the roots. This again reinforces the role of AMF in enhancing the tolerance of *Mimosa caesalpiniaefolia* Benth. plants and favoring their development in Mn mining soil. Similar results with AMF have also been observed by Nogueira et al. (2007) in *Glycine max* L. Merrill plants grown in soil with excess Mn and by Hristozkova et al. (2016) in *Calendula officinalis* L. plants grown in soil contaminated with cadmium and lead.

Although the second principal component (PC2) showed eigenvalues above 1 and explained 9.27% of the total variability, its correlations were considered as low ($R < 0.5$) (Table 4), and were inexpressive to explain the interaction existing between the analyzed variables. However, some studies found in the literature use correlations from 0.3 to explain the data. The present study evaluated correlations between variables higher than or equal to 0.5. The explanation for the low interaction between variables in PC2 is related to the high variability explained in PC1 (Figure 5).

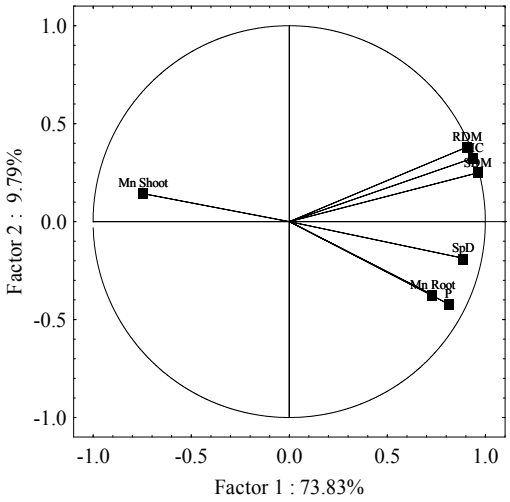

Figure 5. Two-dimensional projection (Biplot) of biometric, chemical and microbiological variables evaluated in the first two principal components

4. Conclusions

The association of the AMF Mix and *Claroideoglomus etunicatum* with the species *Mimosa caesalpiniaefolia* Benth. enhances Mn phytostabilization in mining soils with high concentration of this element.

Further research is necessary to study the capacity of *Mimosa caesalpiniaefolia* Benth. plants associated with AMF to develop under field conditions.

Approaches using multivariate analyses are important tools to provide more in-depth knowledge on the behavior of biometric, chemical and microbiological variables in mining soil with high concentration of manganese.

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