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EFFECT OF ARBUSCULAR MYCORRHIZAL FUNGI *Glomus* spp. INOCULATION ON ALFALFA GROWTH IN SOILS WITH COPPER

Daniela Novoa M.[1], Soledad Palma S.[1], and Hernán Gaete O.[1*]

ABSTRACT

Soils near mining centers usually have high heavy metal (HM) levels. It has been found that some plants associated with arbuscular mycorrhizal fungi (AMF) improve growth and tolerance to HM in soils. This symbiosis is a biological resource for degraded soil recovery. The objective of this study was to determine the effect of inoculating AMF (*Glomus* spp.) on alfalfa (*Medicago sativa* L.) growth in agricultural soils with different copper (Cu) levels for degraded soil recovery. To this effect, alfalfa seeds were grown in soils from the Catemu and Casablanca valleys and inoculated with AMF. Plant height, stem diameter, and number of leaves were measured weekly. Dry matter, mycorrhizal colonization, and Cu concentration in alfalfa plant tissues were measured after 81 days. Inoculation increased plant height by 24%, stem diameter by 11%, and number of leaves by 34%. Inoculation had a significant effect ($p \leq 0.05$) on alfalfa plants that were grown in soil with the highest Cu concentration, but had no effect on Cu accumulation in alfalfa plant tissues. A direct relationship was observed between Cu accumulation in alfalfa and Cu concentration in soils. It was concluded that alfalfa inoculated with *Glomus* spp. is applicable to the soil recovery process whenever soil properties can ensure inoculum effectiveness on alfalfa growth, and avoid toxicity by excessive Cu in alfalfa plant tissues.

Key words: *Medicago sativa*, mycorrhizal colonization, soil recovery.

INTRODUCTION

Copper (Cu) mining is Chile's most important economic activity but also one of its major pollutants. The environmental problem provoked by this activity is related to soil contamination by heavy metals (HM), particularly Cu (De Gregori *et al*., 2003; Ávila *et al*., 2009). Although Cu is considered to be an essential nutrient for plants, it can be toxic in high concentrations (Lasat, 2000; Adriano, 2001; Ávila *et al*., 2009).

A plant community called metallophyte flora has developed specialized physiological mechanisms to survive in HM-rich soils (Ginocchio and Baker, 2004). Some tolerate HM in the soil by restricting absorption and/or translocation to their leaves, or act as indicators reflecting soil metal concentration in their tissues. Other species, however, show specialized mechanisms allowing them to accumulate or hyperaccumulate more than 1000 mg kg^{-1} Cu in their aerial biomass without showing any visible symptoms of toxicity (Lasat, 2000), but are characterized by their slow growth and scarce biomass due to energy used in adaptation mechanisms to high metal concentrations in their tissues (Citterio *et al*., 2005; Wang *et al*., 2007). This plant community is made up of a potentially valuable biological resource for the mining sector, for better closure and soil recovery practices of HM-enriched soils (Ginocchio and Baker, 2004). Lins *et al*. (2006) and Chen *et al*. (2007) point out that the use of vegetation to stabilize and control contamination would be the best way to recover mine-waste impacted soils.

Plant metal absorption can be influenced by soil microorganisms or arbuscular mycorrhizal fungi (AMF) which are closely related to the plant roots (Citterio *et al*., 2005). The importance of this association lies in the fact that the plant transfers carbon products and energy derived from photosynthesis to the fungus, as well as an ecological niche. As regards the fungus, it helps plant growth and its capacity to supply water and nutrients, particularly phosphate and trace elements, obtained by its greater access to resources far from the root system (Chen *et al*., 2007; Jankong and Visoottiviseth, 2008).

[1]Universidad de Valparaíso, Facultad de Ciencias, Av. Gran Bretaña 1111, Playa Ancha, Valparaíso, Chile. *Corresponding author (hernan.gaete@uv.cl).
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Leyval *et al*. (1997) and Lins *et al*. (2006) point out that AMF increase plant tolerance to HM in soils allowing its application in degraded soil recovery. However, the effect of AMF on HM tolerance and accumulation in plants depends on the type of AMF, species of host plant, type of HM, physical and chemical soil properties, and environmental conditions (Chen *et al*., 2007; Wang *et al*., 2007; Jankong and Visoottiviseth, 2008).

Jankong and Visoottiviseth (2008) worked with a commercial inoculum (*Glomus* spp. mixture) and distinct inoculated plants with results showing that the highest growth and metal absorption depends on the type of host plant. Wang *et al*. (2007) used corn (*Zea mays* L.) because it is mycotrophic-dependent with a high biomass, and can extract considerable quantities of Pb, Cd, and Zn from contaminated soils. However, it presented sensitivity to Cu when not translocating to the aerial tissue Cu absorbed from a moderately contaminated soil.

De Gregori *et al*. (2000) worked with alfalfa samples from the Puchuncaví and Catemu Valleys, Valparaíso Region, Chile. Their results show that when the level of Cu in the soils is higher, the capacity of alfalfa to accumulate it is greater. In turn, Peralta *et al*. (2004) demonstrated that alfalfa has the capacity to grow in Cu-contaminated sites, and it is therefore feasible to use it to recover soils with sufficiently high Cd, Cu, or Zn concentrations, but do not impede seed germination.

The objective of this study was to determine the effect of arbuscular mycorrhizal fungi (*Glomus* spp.) inoculation on alfalfa growth in agricultural soils with distinct levels of Cu for degraded soil recovery.

MATERIALS AND METHODS

Site selection and soil sampling

Soil sampling was carried out between October and November 2007 in two agricultural zones of the Valparaíso Region, Chile. The first zone corresponds to the Catemu Valley located in the Aconcagua River valley. According to previous studies (De Gregori *et al*., 2000; 2003), this zone can be affected by particulate emissions containing Cu from the Chagres Foundry (32º48' S 70º57' W), which is why three sectors were sampled with different distances from the Foundry. The second zone corresponds to Casablanca Valley (33º18' S 71º24' W). This zone is made up of an area without direct impact of Cu mining and metallurgical activities, and with edaphic and climatic characteristics similar to those in Catemu Valley (De Gregori *et al*., 2000; 2003).

In each sampled sector, 10 kg of soil was obtained from a depth between 0 and 20 cm. Subsequently, soils were moved to the Environmental Biotechnology Laboratory of the Universidad de Valparaíso where they were passed through a 2-mm mesh sieve. Furthermore, soils were sterilized to avoid the presence of native mycorrhizal fungi and other microorganisms that could interfere with the experiment and alter measurements. Sterilization was carried out in an autoclave for 20 min on two consecutive days (Sadzawka, 1990).

Physical and chemical soil analysis

The physical and chemical soil sample analysis was carried out in the Soils and Foliar Analysis Laboratory of the Pontificia Universidad Católica de Valparaíso (Table 1). Granulometry and texture were determined by the simplified hydrometer method according to Sheldrick and Wang (1993). Percentage organic matter (OM) was obtained by the humid combustion method and colorimetric determination of reduced chromate (Sadzawka *et al*., 2006). The concentration of P (P-Olsen) was extracted with a Na 0.5 mol L^{-1} bicarbonate solution with 8.5 pH. Phosphorus in the extract was determined by colorimetry and molybdenum blue method, and with ascorbic acid as a reducer (Sadzawka *et al*., 2006). Furthermore, pH was measured with a digital pH meter (model Q-400M2, QUIMIS, Diadema, Sao Paulo, Brazil), and electrical conductivity with a digital conductivity meter (model SC-12, Suntex, Taipei, Taiwan), according to the methodology described by Jackson (1964).

Total Cu concentration was determined by atomic-absorption spectrophotometry with direct aspiration to the flame, then total digestion of the soils with nitric acid, hydrochloric acid, and peroxide (Sadzawka *et al*., 2005). Soluble Cu was determined with a KNO_3 0.1 M solution as an extractor. The soluble Cu concentration was determined by atomic-absorption spectrophotometry (Sadzawka *et al*., 2005). Finally, the Cu^{+2} (pCu^{+2}) free ion activity in the saturated paste extract (Sadzawka, 1990) was measured with a Cu ion-selective electrode (Sauvé *et al*., 1995; Rachou *et al*., 2007).

Plants

For this study, alfalfa 'California 55' was the variety recommended for Central Chile by ANASAC (Agrícola Nacional S.A.C.). Alfalfa is a mycotrophic-dependent legume. Its main characteristics are high biomass production, adaptation to different ecological regimes, and resistance to pests, diseases, and toxic elements (Tovar, 2006).

Arbuscular mycorrhizal fungi (AMF) inoculum

The inoculum applied was the commercial MYCOSYM TRI-TON® from MYCOSYM International AG Company which has a production plant in Málaga, Spain and commercial offices in Basel, Switzerland. The inoculum corresponds to a granular formulation product containing porous clay particles and fine roots with infection

Table 1. Physical and chemical properties of soil samples.

Properties	Soil 1	Soil 2	Soil 3	Soil 4
Physical				
Zone	Casablanca Valley	Catemu Valley	Catemu Valley	Catemu Valley
Geographical coordinates	33º18' S 71º24' W	32º47' S 70º51' W	32º47' S 70º57' W	32º46' S 70º59' W
Distance to foundry, km	nd	13.5	1.2	4.3
Texture	Clay loam	Clay loam	Clay loam	Sandy clay loam
Chemical				
Initial pH	5.93	7.38	7.71	7.07
Inoculated soil pH	6.42	8.13	8.01	7.61
Non-inoculated soil pH	5.57	7.28	6.83	6.64
Electrical conductivity, S m^{-1}	0.08	0.11	0.06	0.05
Organic matter, %	3.35	3.36	3.17	4.47
P-Olsen, mg kg^{-1}	31.8	53.6	48.1	18.3
Total Cu, mg kg^{-1}	53.8	96.4	128	620
Soluble Cu, mg kg^{-1}	0.09	0.04	0.37	0.71
pCu^{+2}	13.2	14.6	12.8	13.3

nd: not determined. pCu^{+2}: -log (Cu^{+2} free ion activity).

units (spores and hyphae) of *Glomus etunicatum, G. intraradices,* and *G. fasciculatum* fungi.

Experimental design

A bifactorial 4 x 2 design was carried out for sowing with four soil samples and two treatments for each sample: inoculated with AMF and non-inoculated. Each treatment had four experimental units. Thirty-two 1-kg capacity plastic pots were used. To each experimental unit in the inoculated treatment, 500 g of the respective soil sample and 15 g of inoculum were added, 30 alfalfa seeds were homogeneously scattered on the surface, and then covered with 300 g of soil sample. To each experimental unit in the non-inoculated treatment, 500 g of the respective soil sample was added and 30 alfalfa seeds were covered with 300 g of soil sample. The experiment was carried out between December 2007 and February 2008. Plants grew in greenhouse conditions at environmental temperature (17 ºC mean), 50% air relative humidity, and photoperiod 14:10 h. Plants were irrigated every 2 d with 22 mL of potable water (Ginocchio and Narváez, 2002). Plant height, stem diameter, and number of leaves were measured once a week throughout the experiment.

Plant analysis

Alfalfa plants were harvested after 81 d of growth. Plants were washed in hydrochloric acid 0.01 N, distilled water, EDTA 0.05 M, and once again with distilled water (Ginocchio *et al*., 2002). Subsequently, biomass (dry weight) was determined by separating aerial (leaves and stem) and root tissues of the harvested plants. These were placed in a drying oven (model LDO-150N, Labtech Hebro, Santiago, Chile) at 60 ºC for 48 h, and then weighed (Ginocchio and Narváez, 2002). To determine mycorrhizal colonization, a 1 cm fragment of undried alfalfa root was separated, classified in KOH 2.5% p/v for 3 d, then left in HCl 1% for 1 d to eliminate excess KOH, and finally stained with trypane blue 0.05% p/v (Phillips and Hayman, 1970). The stained roots were randomly distributed in a squared Petri dish and the percentage of mycorrhizal colonization was counted with a stereoscopic microscope (model Stemi DV4, Zeiss, New York, USA) in accordance with the line intercept method (Giovanetti and Mosse, 1980).

The concentration of Cu in alfalfa aerial and root tissues in the presence and absence of mycorrhizae was obtained by atomic-absorption spectrophotometry with an air-acetylene flame for direct aspiration (model 902, GBC, Melbourne, Australia), and dried and ground samples of plant tissue were then transferred to Teflon containers for digestion with nitric acid, peroxide, and hydrofluoric acid according to the description in Sadzawka *et al*. (2007).

The bioconcentration factor (BF) corresponds to the plant's ability to capture and transport metals from the soil to its tissues. This factor was obtained by dividing total plant Cu concentration (aerial and root) by soil total Cu concentration (McGrath and Zhao, 2003). The translocation factor (TF) corresponds to the plant's ability to transport the metal from the root to the aerial tissue, and was obtained by

dividing Cu concentration in the plant aerial tissue by Cu concentration in the root tissue (Wang *et al*., 2007).

Statistical analysis

One factor was analyzed by ANOVA and followed by multiple comparisons Tukey test with 5% probability to determine the statistically significant differences between inoculated and non-inoculated treatments. Pearson linear correlations were applied to the variables analyzed in the plants and soil Cu concentrations. These analyses were done by the statistical program Minitab 15 (Minitab, State College, Pennsylvania, USA). Results having more than one replicate are shown as mean ± standard deviation.

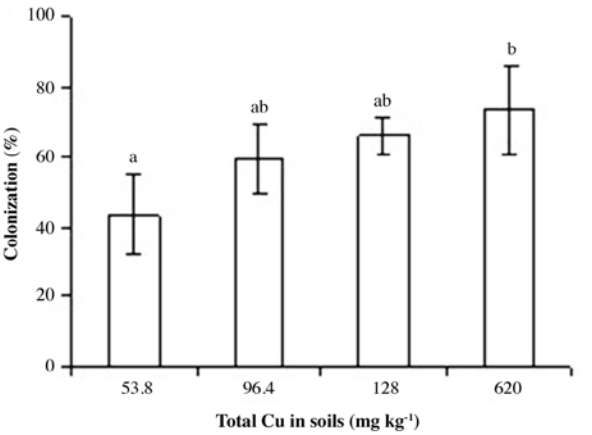
RESULTS AND DISCUSSION

The soil sample from the Casablanca Valley showed the lowest total Cu concentration while soil samples from the Catemu Valley showed the highest concentrations (Table 1). High Cu concentrations in Catemu Valley are explained mostly by mining activity, while the variation in the concentration is explained by the distance to the copper foundry. Soil Cu levels in this study are higher than those reported by De Gregori *et al*. (2000) in the same sectors. Soluble Cu concentrations were in the range of 0.09 and 0.71 mg kg^{-1} and directly related to total Cu concentrations ($r = 0.92$; $p > 0.05$), for this reason soils were identified according to total Cu concentration.

Mycorrhizal colonization

Soil pH in inoculated treatments increased significantly ($p \leq 0.05$), and it was not significant ($r = 0.74$; $p > 0.05$) even when there was a relationship with mycorrhizal colonization (Table 1). On this subject, Lins *et al*. (2007) point out that pH can influence mycorrhizal colonization since fungi of the *Glomus* genus are mostly found in soils with pH equal or greater than 6.1.

It can be observed in Figure 1 that AMF inoculum colonized alfalfa roots in four soils. The highest



Different letters indicate significant differences among treatment according to Tukey test ($p \leq 0.05$).

Figure 1. Percentage of mycorrhizal colonization (mean ± SD) in inoculated alfalfa roots for different soil Cu concentrations.

Table 2. Comparison of arbuscular mycorrhizal fungi inoculated and non-inoculated treatments for growth parameters of alfalfa plants: plant height, stem diameter, number of leaves, shoot and root dry matter after 81 d of growth in soils with increasing Cu concentrations.

	Total Cu in soils			
	53.8	**96.4**	**128**	**620**
	mg kg^{-1}			
Plant height, cm				
Inoculated	34 ± 3.5	31.8 ± 2.5*	35.1 ± 8.5	25.9 ± 8*
Non-inoculated	29 ± 9.1	24.5 ± 5.1	27.4 ± 2	15.4 ± 2.2
Stem diameter, mm				
Inoculated	2 ± 0.3	2 ± 0.1*	2 ± 0.1	1 ± 0.3*
Non-inoculated	1.9 ± 0.3	1.6 ± 0.3	1.9 ± 0.3	1 ± 0.1
Number of leaves				
Inoculated	109 ± 35*	59 ± 6	71 ± 5	44 ± 5**
Non-inoculated	61 ± 20	48 ± 13	63 ± 9	16 ± 2
Aerial biomass, g				
Inoculated	2.9 ± 0.8	2.2 ± 0.3	2.3 ± 0.3	1.1± 0.3
Non-inoculated	2.2 ± 1.4	1.6 ± 0.9	1.9 ± 0.3	0.9 ± 0.7
Root biomass, g				
Inoculated	1.1 ± 0.6	0.3 ± 0.2	0.7 ± 0.3	0.6 ± 0.5
Non-inoculated	1.1 ± 0.8	0.4 ± 0.3	0.4 ± 0.2	0.3 ± 0.2

*$p \leq 0.05$; ** $p \leq 0.01$. Difference between inoculated and non-inoculated treatment according to ANOVA. Mean ± SD is indicated.

colonization percentage (73.6%) was in the soil with the highest Cu concentration (620 mg kg^{-1}). This demonstrates *Glomus* spp. inoculum tolerance to Cu concentrations present in soils.

Alfalfa growth

Whether the treatment was inoculated or non-inoculated, growth of plants cultivated in the soil with the highest Cu concentration (620 mg kg^{-1}) was significantly lower ($p \leq 0.05$) than growth of plants cultivated in soils with lower Cu concentrations (Table 2).

Comparing treatments, plant height was on the average 24% higher, stem diameter 11% higher, and number of leaves 34% higher in the inoculated treatments. This difference between treatments was significant when alfalfa plants were cultivated in soil with a higher Cu concentration (Table 2). Results were similar to those reported by Lins *et al*. (2006) in *Leucaena leucocephala* (Lam.) plants. In their study, they inoculated plants with AMF *Glomus etunicatum*, and those cultivated in soil with higher Cu concentration showed a higher height and number of leaves than non-inoculated plants.

As regards biomass (dry weight), independently of the treatment, alfalfa plants cultivated in soil with the lowest Cu concentration (53.8 mg kg^{-1}) had a significantly higher ($p \leq 0.05$) aerial and root biomass than plants cultivated in soils with the highest Cu concentration (620 mg kg^{-1}) (Table 2). Alfalfa plant aerial and root biomass was 23 and 19% higher in the inoculated treatments, respectively, though this difference was not significant ($p > 0.05$). This is similar to what Citterio *et al*. (2005) found in *Cannabis sativa* L. plants inoculated with AMF *Glomus mosseae* cultivated in soils contaminated with HM and where there was no significant difference between the biomass of plants inoculated with AMF and those non-inoculated.

Accumulation of Cu in alfalfa

Copper concentrations in alfalfa aerial tissues are in the 20 to 100 mg kg^{-1} range (Table 3), corresponding to excessive or toxic concentrations for agricultural crops (Adriano, 2001). Chlorosis in the leaves of alfalfa plants cultivated in soil with a higher Cu concentration appeared after 25 d of growth. Chlorosis continued until the plants were harvested without killing them. Ginocchio and Narváez (2002) point out that when the tolerance to excess Cu accumulated in the roots is surpassed, translocation of this element to the shoot takes place affecting photosynthesis and other cell functions. Peralta *et al*. (2004) point out that HM reduce plant aerial tissue growth which decreases chlorophyll content and photosystem I activity. This would have originated chlorosis and lower aerial tissue growth of plants sown in the soil with the highest Cu concentration.

There was generally no significant difference between Cu accumulation in alfalfa plants inoculated with *Glomus* spp. and those non-inoculated. Except for the plants sown in the soil with the lowest Cu concentration, alfalfa plants accumulated a higher Cu concentration in root tissue than in aerial tissue (Table 3). These results are similar to those reported by Lins *et al*. (2006) working with *Leucaena leucocephala* (Lam.) plants inoculated with AMF *Glomus etunicatum*.

Copper accumulation in alfalfa aerial and root tissue in the inoculated, as well as the non-inoculated treatment, had a direct relationship with soil total Cu concentration (Table 3). There is a tendency toward higher Cu accumulation in alfalfa tissues when soil Cu concentration increases. The results of this study coincide with results obtained by De Gregori *et al*. (2000) where

Table 3. Comparison of arbuscular mycorrhizal fungi inoculated and non-inoculated treatments for Cu concentration in alfalfa plant shoot and root tissues expressed as dry weight. Pearson correlations between Cu concentration in alfalfa and Cu concentration in soils and their significance levels are shown.

Treatment		Total Cu in soils				R	P
		53.8	96.4	128	620		
		mg kg^{-1}					
Cu in alfalfa							
Aerial	Inoculated	39.5 ± 2.5*	25.3 ± 0.3	34.1 ± 7.5	79.7 ± 24.2	0.95	0.04
	Non-inoculated	48.2 ± 1.2	25.7 ± 3.5	31.7 ± 5.3	75.9 ± 6.4	0.86	0.14
Root	Inoculated	27.7 ± 3	33.6 ± 0.8**	51.8 ± 1	170[1]	0.99	0.002
	Non-inoculated	19.5 ± 3.6	38.9 ± 0.1	47.4 ± 13.4	226[1]	0.99	0.001
BF	Inoculated	1.2 ± 0.06	0.6 ± 0.01**	0.7 ± 0.05	0.4 ± 0.04	-0.71	0.30
	Non-inoculated	1.3 ± 0.06	0.7 ± 0.04	0.6 ± 0.1	0.5 ± 0.01	-0.6	0.34
TF	Inoculated	1.4 ± 0.07	0.8 ± 0.03	0.7 ± 0.13	0.5 ± 0.14	-0.69	0.31
	Non-inoculated	2.5 ± 0.53	0.7 ± 0.09	0.7 ± 0.31	0.3 ± 0.03	-0.6	0.41

*$p \leq 0.05$; ** $p \leq 0.01$. Difference among treatments according to ANOVA. Mean ± SD is indicated.
[1]Only one value; BF: bioconcentration factor. TF: translocation factor.

alfalfa plants from the Puchuncaví and Catemu valleys tended to accumulate a greater quantity of Cu in their tissues when soil Cu levels increased. Likewise, Wang *et al*. (2007) reported that in corn plants inoculated with AMF *Acaulospora mellea*, Cu concentration in the roots tended to increase when soil Cu levels increased, whether the treatment was inoculated or non-inoculated.

Bioconcentration (BF) and translocation (TF) factors (Table 3) tend to decrease when soil Cu concentration increases. Alfalfa plants cultivated in the soil with the lowest Cu concentration showed higher BF and TF values, whereas alfalfa plants cultivated in the soil with the highest Cu concentration had lower BF and TF values. This behavior indicates the beneficial effect of mycorrhizal colonization under excessive HM conditions where AMF acts as a protective barrier restricting soil metal transfer to the plant and the subsequent metal translocation from the root to aerial tissue (Wang *et al*., 2007; Jankong and Visoottiviseth, 2008).

CONCLUSIONS

Arbuscular mycorrhizal inoculum (*Glomus* spp.) tolerated Cu concentrations in the soil samples from the Catemu and Casablanca valleys. Inoculation had a beneficial effect on alfalfa growth in soils with Cu, but not on tissue Cu accumulation. There was a tendency for alfalfa to accumulate a greater quantity of Cu in its root and aerial tissues when soil Cu concentration increased. These results suggest the potential use of alfalfa inoculated with mycorrhizal fungi (*Glomus* spp.) in Cu-degraded soil recovery processes. However, to apply this, associated costs and soil properties must be considered, such as the quantity of Cu, in order to ensure inoculum effectiveness on alfalfa growth and avoid toxicity symptoms in the plants as a result of excessive Cu accumulation in their tissues

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RESUMEN

Efecto de la inoculación con hongos micorrízicos arbusculares *Glomus* spp. sobre el crecimiento de alfalfa en suelos con cobre. Los suelos cercanos a centros de actividad minera suelen presentar altos niveles de metales pesados (HM). Se ha encontrado que algunas plantas asociadas a hongos micorrízicos arbusculares (AMF) mejoran su crecimiento y tolerancia a los HM presentes en los suelos. Esta simbiosis constituye un recurso biológico para la recuperación de suelos degradados. El objetivo de este estudio fue determinar el efecto de la inoculación con AMF (*Glomus* spp.) sobre el crecimiento de alfalfa (*Medicago sativa* L.) en suelos agrícolas con distintos niveles de cobre (Cu) para la recuperación de suelos degradados. Para ello se sembraron semillas de alfalfa en suelos del Valle de Catemu y Casablanca y se inocularon con AMF. Semanalmente se midió la altura de las plantas, diámetro de tallo y número de hojas. Transcurridos 81 días se determinó biomasa, colonización micorrízica y concentración de Cu. La inoculación incrementó un 24% la altura de la planta, 11% el diámetro del tallo y 34% el número de hojas. La inoculación tuvo un efecto significativo ($p \leq 0.05$) sobre el crecimiento de alfalfa en el suelo con mayor concentración de Cu, pero no sobre la acumulación de Cu en sus tejidos. La acumulación de Cu en alfalfa se relacionó directamente con la concentración de Cu en los suelos. Se concluye que alfalfa inoculada con *Glomus* spp. es aplicable a procesos de recuperación de suelos, siempre que se consideren las propiedades del suelo para asegurar la efectividad del inóculo sobre el crecimiento de alfalfa y evitar la toxicidad por exceso de Cu en sus tejidos.

Palabras clave: *Medicago sativa* L., colonización micorrízica, recuperación de suelos.

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