Glomalin-related soil protein in a Mediterranean ecosystem affected by a copper smelter and its contribution to Cu and Zn sequestration

Pablo Cornejo[a,*], *Sebastián Meier*[a], *Gilda Borie*[b], *Matthias C. Rillig*[c], *Fernando Borie*[a]

[a] *Departamento de Ciencias Químicas, Universidad de La Frontera, Casilla 54-D, Temuco, Chile*
[b] *Departamento de Química Inorgánica y Analítica, Universidad de Chile, Casilla 203, Santiago, Chile*
[c] *Institut für Biologie, Freie Universität Berlin, Altensteinstr. 6, D-14195 Berlin, Germany*

A B S T R A C T

The amount of glomalin-related soil protein (GRSP), a glycoprotein produced by arbuscular mycorrhizal fungi (AMF), its contribution to the sequestering of Cu and Zn in the soil, and the microsite variation of other soil traits (pH, water-stable aggregates—[WSA], soil organic carbon—[SOC]) was studied in a semi-arid Mediterranean ecosystem near a copper smelter and affected by deposit of metal-rich particles since 1964. Rhizospheric (R) and non-rhizospheric (NR) soil of four representative plants (*Argemone subfusiformis*, *Baccharis linearis*, *Oenothera affinis* and *Polypogon viridis*) was analyzed. The results showed a strong variability in GRSP (6.6–36.8 mg g^{-1}), Cu content (62–831 mg kg^{-1} for the total Cu and 5.8–326 mg kg^{-1} for the available Cu) and pH (4.2–5.5) in the different plant and rhizospheric zones analyzed. A strong relationship between the GRSP with the soil Cu and Zn contents was found ($r = 0.89$ and 0.76 for Cu and Zn respectively, $p < 0.001$). The GRSP-bound Cu ranged from 3.76 to 89.0 mg g^{-1} soil and represents 1.44–27.5% of the total Cu content in soil. Moreover, the WSA reached 89% in *P. viridis* R. For this plant, the C contained in GRSP represented up to 89% of SOC, and this coincided with the most extreme conditions of soil degradation within the ecosystem (the highest content of heavy metals and low pH values). This study provides evidence on the role of the GRSP in Cu and Zn sequestration and suggests a highly efficient mechanism of AMF to mitigate stress leading to stabilization of soils highly polluted by mining activities.

Keywords:
Glomalin
Copper
Zinc
Heavy metal pollution
Soil stabilization
Soil organic carbon

1. Introduction

Copper (Cu) mining is the main economic activity in Chile, which represents about a third of global Cu production. It is well known that the negative effects of mining on natural ecosystems are mainly due to the discharge of heavy metals (HM) (González and Ite, 1992), which are toxic to higher organisms and microbes (Adriano, 2001). This toxicity can cause losses in diversity and functionality of native plant species, leading to extensive damage to local vegetation, a strong change of soil characteristics and limiting vegetation establishment (Giller et al., 1998; Ginocchio, 2000).

However, the exposure to HM may cause the selection and development of tolerant populations, both plants and soil microorganisms (Ellis et al., 2003), which could be useful in the restoration/remediation of ecosystems affected by this kind of pollution. Among soil microorganisms arbuscular mycorrhizal fungi (AMF) are particularly important; they establish mutual symbioses with the majority of higher plants, forming a direct link between soil and plant roots (Allen, 1996; Jeffries et al.,

E-mail addresses: pcornejo@ufro.cl, pablo.cornejorivas@gmail.com (P. Cornejo).

2003; Reinhardt, 2007). AMF occur in practically all the ecosystems around the world (Barea et al., 1997), including in degraded soils with high contents of HM (Del Val et al., 1999) like those affected by mine activities. Therefore, the use of AMF and plants that could form arbuscular mycorrhizae (AM) can be a valuable tool to enhance site-remediation processes (Khan 2005; Leung et al., 2007).

Glomalin is a glycoprotein produced by AMF (Gadkar and Rillig, 2006). Operationally defined by extraction and detection conditions (Wright and Upadhyaya, 1996, 1998; Nichols, 2003; Nichols and Wright, 2006; Purin and Rillig, 2007), it is detected in large amounts in diverse soils as glomalin-related soil protein (GRSP; Rillig 2004). As such, is widely studied for its implications in the carbon storage, sequestration of potentially toxic element as HM, and its role in soil stability. GRSP could represent a significant fraction of the pool of soil proteins due to its persistence (Rillig et al., 2001, 2007). While the identity of the protein proper has been revealed to be a putative hsp60 homolog (Gadkar and Rillig, 2006), the biochemical nature of the substance extracted from soil is still not fully revealed. GRSP appears to be a component of the hyphae and spore wall of AMF, likely released into the soil by mycelium turnover (Driver et al., 2005), where it subsequently contributes to linking soil particles and stabilizing aggregates (Rillig and Mummey, 2006).

Moreover, recent studies indicate that GRSP can bind and sequester some heavy metals such as Cu, Cd, Pb and Zn (González-Chávez et al., 2004; Chern et al., 2007; Vodnik et al., 2008). Based on these data we hypothesized that the release and accumulation of GRSP in soils can be a very important mechanism for the stabilization of soils degraded by mining activities, and that this substance may also contribute to sequestration of significant quantities of HM characteristic of this kind of environmental pollution.

Therefore, the aim of this study was to evaluate the role played by GRSP in the sequestration of Cu and Zn in soils of an ecosystem that shows high levels of pollution by mining activities. Additionally, we evaluated the contribution of GRSP in the storage of organic C in soil and its role in stabilizing soil aggregates in order to characterize its ability to promote site-remediation processes. The study used rhizospheric and non-rhizospheric soil from four plant species representative of a Mediterranean ecosystem of central Chile strongly affected by the deposit of metal-rich particles from a smelter.

2. Materials and methods

2.1. Study area

The study area was located in a Mediterranean environment (32° 46′ 30″ S 71° 28′ 17″ O), located approximately 1.5 km southeast from the Ventanas smelter (ENAMI), in the Puchuncaví valley, central Chile. This area is characterized by marked Cu pollution due to the continuous discharge of gaseous emissions and deposition of metal-rich particles from the smelter since 1964. Concentrations of soil Cu as high as 680 mg kg^{-1} were previously reported (De Gregori et al., 2003; Ginocchio et al., 2004). As a result of this pollution the local ecosystems have been heavily affected, decreasing drastically their plant diversity and density, which currently consists of a few species tolerant to high levels of Cu (Ginocchio, 2000; Ginocchio et al., 2004). The soils correspond to the Chilicauquén series, fine sandy loam in texture, moderately deep, formed on a substrate of sandstone cemented with clay from the upper horizons.

2.2. Collection of samples

Zones colonized by four plant species representative of the semi-arid Mediterranean ecosystem were selected. The plant species considered were *Argemone subfusiformis* Ownbey (Papaveraceae), *Baccharis linearis* (R. et P.) Pers. (Asteraceae), *Polypogon viridis* (Gouan) Breistr. (Poaceae) and *Oenethera affinis* Cambess. (Onagraceae). *P. viridis* was found forming a homogeneous cover in one limited area, while the other species were heterogeneously distributed throughout the ecosystem. Rhizospheric (under plant canopy, R) and non-rhizospheric (at a distance of 1 m from the plant, NR) soils were collected, at 0–25 cm depth, and were subsequently sieved (2 mm) and air-dried.

2.3. Measurements

GRSP, operationally defined as Bradford-reactive soil protein following extraction (Rillig, 2004), was determined according to the method described by Wright and Upadhyaya (1998) with minor modifications. For the easily extractable fraction of GRSP (EE-GRSP) samples of 1 g soil were subjected to extraction with 8 mL of 20 mM citrate, pH 7.0, and autoclaving for 30 min at 121 °C. The total GRSP (T-GRSP) was extracted from 1 g of soil with 8 ml of 50 mM citrate, pH 8.0, and autoclaving for 1 h at 121 °C. In both cases, the supernatant was separated by centrifugation at 8000 *g* for 20 min and filtrated through paper Watman No 1. For T-GRSP, the procedure described was repeated several times on the same sample until the reddish-brown color typical of GRSP disappeared from the supernatant, combining all extracts from a soil sample. The protein content in the crude extract was determined by Bradford assay (Bio Rad Protein Assay; Bio Rad Laboratories) with bovine serum albumin as the standard.

To determine GRSP-bound Cu (GRSP-Cu) and GRSP-bound Zn (GRSP-Zn) and their content of C (C-GRSP) and N (N-GRSP) we used the follow protocol. GRSP was precipitated by slow addition of 2 M HCl up to pH 2.5, centrifuged at 8000 *g* for 20 min, re-dissolved in 0.5 M NaOH, dialyzed against deionized H_2O and freeze-dried. Dried GRSP was acid-digested (H_2O/HCl/HNO_3; 8/1/1 v/v/v) and GRSP-Cu and Zn were determined by atomic Absorption Spectroscopy (–AAS–, Perkin-Elmer 3110). C-GRSP and N-GRSP were determined by dry combustion (VARIO/EL).

Available Cu and Zn in the soil were extracted with DTPA solution (5 mM diethylene triamine pentaacetic acid, 0.108 M triethanolamine, 10 mM $CaCl_2$, pH 7.3), while total Cu and Zn were obtained by microwave-assisted (Milestone MLS-1200 MEGA) digestion with an acid mixture (HNO_3/H_2O_2/HF; 3/2/1 v/v/v) that consisted of the following steps: 6 min at 250 W, 6 min at 400 W, 6 min at 650 W and 6 min at 250. Cu and Zn total and available contents were determined by AAS (Perkin-Elmer 3110). The soil organic C (SOC) was determined by means of the Walkley–Black wet oxidation method (Nelson and Sommers, 1982) and N in soil was determined by dry combustion (VARIO/EL). The water-stable aggregates of soil (–WSA–, 1–

Table 1 – pH values, Cu content (total, DTPA extractable and GRSP-Cu) and Zn content (total, available and GRSP-Zn) determined in soil from four plant species of a Cu polluted Mediterranean ecosystem

Plant	Zone	pH_w	Cu (mg kg^{-1})			Zn (mg kg^{-1})		
			Total	DTPA	GRSP-Cu	Total	DTPA	GRSP-Zn
Argemone subfusiformis	R	4.71 (0.03) d	269 (13.6) c	50.2 (4.97) bc	4.63 (0.44) e	131 (1.78) g	7.83 (0.30) b	2.42 (0.31) c
	NR	5.23 (0.02) b	106 (3.52) c	16.3 (1.89) bc	3.76 (0.37) e	157 (1.41) d	2.57 (0.47) d	2.24 (0.31) c
Baccharis linearis	R	4.47 (0.06) e	97.3 (10.4) b	93.2 (12.6) b	8.77 (0.11) e	148 (0.82) e	13.0 (0.16) a	4.58 (1.41) c
	NR	5.47 (0.01) a	62.2 (7.20) c	5.80 (0.37) c	30.2 (2.71) cd	164 (0.82) c	7.24 (0.55) bc	5.63 (0.50) bc
Oenothera affinis	R	5.03 (0.04) c	154 (4.64) c	18.8 (6.49) bc	42.4 (2.01) bc	137 (1.55) fg	5.36 (0.88) c	4.95 (0.56) c
	NR	4.89 (0.00) c	742 (213) a	48.0 (8.26) bc	14.4 (0.36) de	138 (1.65) f	8.76 (0.37) b	4.70 (0.16) c
Polypogon viridis	R	4.52 (0.01) e	831 (4.71) a	326 (16.3) a	89.0 (5.44) a	198 (1.55) a	13.7 (0.03) a	11.3 (1.64) a
	NR	4.19 (0.02) f	827 (14.5) a	318 (45.3) a	58.1 (8.97) b	180 (0.24) b	14.3 (0.23) a	9.50 (0.32) ab
ANOVA								
F-values		178^{***}	18.9^{***}	54.7^{***}	60.3^{***}	306^{***}	89.6^{***}	14.7^{***}
Error		0.00	23066	1306	60.9	7.03	0.80	2.77

Assay values are means of four replicates. Standard error is presented in parentheses. Significance conventions: ns = not significant. $^{*}p<0.05$. $^{**}p<0.01$. $^{***}p<0.001$. Different letters indicate significant differences according to orthogonal contrasts test ($p<0.05$). Abbreviations: R = rhizospheric soil; NR = non-rhizospheric soil.

2 mm size class aggregates) was determined based on the method described by Kemper and Rosenau (1982). Soil pH was measured in a soil/water mix (2/5; w/v).

2.4. *Statistical analysis*

The design was fully factorial, with four plant species, two rhizospheric zones and four replicates in each combination. For all the variables studied, ANOVA was performed to test for the effect of the plant or the zone sampled for each variable studied. Data sets not meeting assumptions for ANOVA were transformed as required, but the results are presented in their original scale of measurement. Where there were significant differences ($p<0.05$), the means were compared by the orthogonal contrast test. All the data sets obtained were subjected to principal component analysis (PCA) and the correlation among the different variables and the principal components (PC) obtained were analyzed using the Pearson correlation coefficient. A non-hierarchical cluster analysis using the farthest-neighbor as agglomerative method was used for grouping the different experimental units. Statistical analyses were performed with the SPSS software v. 10.0 (SPSS, Inc., Chicago, Il.).

3. Results

All the soils analyzed for different plant species had pH values between very strong and extremely acidic (Soil Survey Division Staff, 1993) (Table 1), with the lowest values in *P. viridis* NR. Conversely, *B. linearis* NR had the highest pH values (1.3 units of difference). Cu total contents ranged between 62 and 830 mg kg^{-1}, with the lowest levels corresponding to *B. linearis* NR, and the highest by far occurring in *P. viridis*. A similar pattern was observed for available Cu, showing that *P. viridis* (R and NR) had 54 fold more available Cu than *B. linearis* NR. Except in *P. viridis*, there were strong differences in Cu contents (total and available) among the soil zones analyzed for each plant. In *A. subfusiformis* and *B. linearis* there were larger contents of total and available Cu in the R zone (2.5 and 3.1 fold in *A. subfusiformis*; 1.6 and 16.1 folds in *B. linearis*) while

Table 2 – Glomalin-related soil protein (GRSP) bound Cu (GRSP-Cu) and Zn (GRSP-Zn) (%) and contribution of GRSP-Cu and GRSP-Zn relative to the total content of each metal (%) in soil from four plant species of a Cu polluted Mediterranean ecosystem

Plant	Zone	GRSP-Cu (%)	GRSP-Cu in soil (%)	GRSP-Zn (%)	GRSP-Zn in soil (%)
Argemone subfusiformis	R	0.66 (0.04) d	4.33 (0.32) de	0.35 (0.03)	1.84 (0.23) cd
	NR	0.56 (0.04) d	1.44 (0.20) e	0.33 (0.04)	1.44 (0.21) d
Baccharis linearis	R	1.16 (0.03) cd	14.8 (1.41) b	0.34 (0.07)	1.77 (0.42) cd
	NR	2.17 (0.00) b	6.05 (0.43) d	0.41 (0.00)	3.44 (0.31) bc
Oenothera affinis	R	2.85 (0.15) a	27.5 (0.82) a	0.33 (0.04)	3.62 (0.41) bc
	NR	1.47 (0.01) c	14.9 (0.45) b	0.48 (0.01)	3.40 (0.12) bc
Polypogon viridis	R	2.41 (0.08) ab	10.8 (0.73) c	0.30 (0.04)	5.75 (0.86) a
	NR	2.30 (0.33) ab	7.01 (1.12) d	0.38 (0.02)	5.26 (0.17) ab
ANOVA					
F-values		40.6^{***}	107^{***}	1.96ns	15.3^{***}
Error		0.07	2.53	0.03	0.67

Assay values are means of four replicates. Standard error is presented in parentheses. Significance conventions: ns = not significant; $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$. Different letters indicate Significant differences according to orthogonal contrasts test ($p<0.05$). Abbreviations: R = rhizospheric soil; NR = non-rhizospheric soil.

Table 3 – Total glomalin-related soil protein (T-GRSP), easily extractable GRSP (EE-GRSP), proportion of C in GRSP (C-GRSP1), C-GRSP relative to the total C in soil (C-GRSP2), proportion of N in GRSP (N-GRSP1), N-GRSP relative to the Total N in soil (N-GRSP2) and water-stable aggregates (WSA) in soil from four plant species of a Cu polluted Mediterranean ecosystem

Plant	Zone	T-GRSP (mg g^{-1})	EE-GRSP (mg g^{-1})	C-GRSP1 (%)	C-GRSP2 (%)	N-GRSP1 (%)	N-GRSP2 (%)	WSA (%)
Argemone subfusiformis	R	6.60 (0.29) e	2.17 (0.07) e	17.7 (0.08) f	17.1 (0.65) de	0.53 (0.05) d	3.40 (0.36) d	74.3 (1.04) b
	NR	6.89 (0.30) de	2.23 (0.03) e	18.6 (0.11) f	16.1 (0.67) e	0.68 (0.04) d	2.61 (0.16) d	62.2 (1.63) cd
Baccharis linearis	R	13.9 (1.25) c	3.36 (0.17) d	26.9 (0.10) d	25.3 (0.29) cd	1.29 (0.12) cd	4.65 (0.43) d	57.1 (0.50) de
	NR	7.60 (0.11) de	2.61 (0.04) e	31.2 (0.29) c	47.0 (4.12) b	1.85 (0.32) bc	11.3 (3.21) cd	51.4 (0.54) e
Oenothera affinis	R	9.83 (0.22) d	3.32 (0.11) d	33.6 (0.19) b	52.6 (0.88) b	2.22 (0.07) b	15.3 (0.70) c	66.2 (2.69) c
	NR	14.9 (0.57) c	4.07 (0.10) c	25.3 (0.15) e	28.9 (1.05) c	0.61 (0.18) d	3.06 (0.73) d	74.5 (1.06) b
Polypogon viridis	R	36.8 (1.15) a	8.03 (0.25) a	39.2 (0.45) a	88.6 (2.77) a	2.43 (0.15) b	28.6 (3.67) b	89.4 (0.16) a
	NR	25.0 (0.50) b	5.89 (0.09) b	38.6 (0.27) a	82.0 (0.63) a	3.25 (0.20) a	52.4 (3.99) a	66.3 (0.01) c
ANOVA								
F-value		265.0***	260.2***	1210***	229***	35.4***	59.2***	87.7***
Error		1.72	0.06	0.22	335	2.69	494	6.34

Assay values are means of four replicates. Standard error is presented in parentheses. Significance conventions: ns = not significant; *$p<0.05$; **$p<0.01$; ***$p<0.001$. Different letters indicate significant differences according to orthogonal contrasts test ($p<0.05$). Abbreviations: R = rhizospheric soil; NR = non-rhizospheric soil.

O. affinis NR soil had 4.8 and 2.6 fold higher total and available Cu, respectively, compared to the R zone.

The total and available Zn contents exhibited lower variability, with values between 131 and 198 mg kg^{-1} for total Zn, and 2.6–14.3 mg kg^{-1} for available Zn. *P. viridis* in both zones presented the highest values of total and available Zn, significantly different from the other plants studied. Moreover, the GRSP-Cu was lower in *A. subfusiformis* NR, reaching 0.56% of GRSP mass, while GRSP-Cu from *O. affinis* R reached 2.85% (Table 2). In fact, the GRSP-Cu represented up to 27.5% of total Cu in soil in *O. affinis* R. The GRSP-Zn showed more homogeneous levels (between 0.30 and 0.48% in GRSP mass) and contributed to Zn sequestration representing up to 5.8% of total Zn in *P. viridis* R.

P. viridis (R and NR) showed GRSP values between 2.5 and 5.6 fold the ones measured in the other plants (Table 3). The EE-GRSP ranged from 21.8% in *P. viridis* R to 34.3% in *B. linearis* NR. C-GRSP showed differences depending on the plant, with values of 17–19% in *A. subfusiformis*, while in *P. viridis* the levels were close to 39%. A similar pattern was observed for the N-GRSP, with the lower contents found in *A. subfusiformis* R (0.53%) and higher contents in *P. viridis* NR (3.25%).

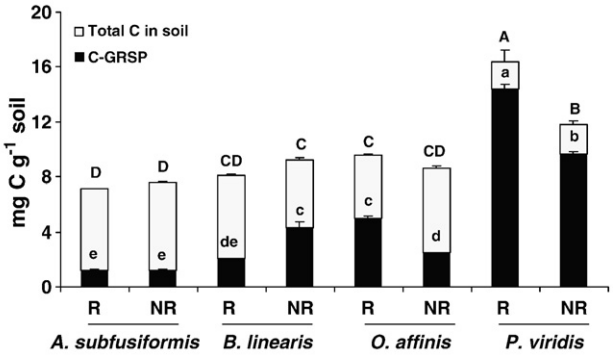

Fig. 1 – Soil organic carbon (total and C-GRSP) in soil from four plant species from a Mediterranean ecosystem in central Chile. Abbreviations: R = rhizospheric soil; NR = non-rhizospheric soil. Different letters indicate significant difference according to the orthogonal contrasts test ($p<0.05$). Bars denote mean ± S.E. ($n=4$).

The SOC presented the same pattern observed in GRSP, with contents between 7.1 and 16.4 mg g^{-1} in *A. subfusiformis* R and *P. viridis* R, respectively. C-GRSP represented a significant fraction of the SOC, ranging from 16% in *A. subfusiformis* NR to 89% in *P. viridis* R. However, the C remaining in soil (non-C-GRSP) was lower with increasing contribution of C-GRSP to total SOC, reaching in all cases from 4.5 to 6.4 mg C g^{-1} soil, except in *P. viridis*, which showed levels close to 2.0 mg C g^{-1} soil (Fig. 1). This latter case was also where the highest levels of WSA were found; *P. viridis* R had values of WSA higher than 89%, significantly different from the other plants and locations studied.

Several strong relationships among different parameters were found (Table 4). Notable are the direct correlations found between Cu and Zn contents (total and available), GRSP and SOC; and the inverse correlation between pH and the other variables studied. On the other hand, the principal components 1 and 2 accounted for 75.8% of the total sample variance (66.6 and 9.2% for PC1 and PC2, respectively) (Fig. 2). PC1 showed a high correlation with all the variables studied (Table 4). Five different groups were obtained by non-hierarchical cluster analysis. *P. viridis*, *A. subfusiformis* and *O. affinis* R form homogeneous groups and with great distances between them, while *O. affinis* NR and *B. linearis* (R and NR) are at intermediate distance between the groups previously mentioned (Fig. 2).

4. Discussion

4.1. *Glomalin content and HM sequestration*

The ability of GRSP to sequester several HM has been previously demonstrated (González-Chávez et al., 2004; Chern et al., 2007; Vodnik et al., 2008). In this study we found higher contents of GRSP-Cu and GRSP-Zn (5.6–28.5 and 3.3–

Table 4 – Correlation matrix of some selected variables studied and the principal components (PC) obtained

Variables	pH_w	Cu	DTPA Cu	GRSP-Cu	Zn	DTPA Zn	GRSP-Zn	GRSP	SOC	C-GRSP	N-GRSP	WSA
Cu[a]	−0.65***											
DTPA Cu[b]	−0.76***	0.67***										
GRSP-Cu[c]	−0.75***	0.59***	0.80***									
Zn[d]	−0.82***	0.65***	0.89***	0.77***								
DTPA Zn[e]	−0.81***	0.52**	0.79***	0.75***	0.85***							
GRSP-Zn[f]	−0.64***	0.63***	0.80***	0.87***	0.76***	0.72***						
GRSP[g]	−0.77***	0.66***	0.89***	0.97***	0.85***	0.76***	0.89***					
SOC[h]	−0.74***	0.64***	0.85***	0.95***	0.83***	0.71***	0.88***	0.98***				
C-GRSP[i]	−0.62***	0.64***	0.88***	0.94***	0.80***	0.83***	0.86***	0.95***	0.88***			
N-GRSP[j]	−0.59***	0.56***	0.83***	0.75***	0.69***	0.74***	0.73***	0.76***	0.65***	0.87***		
WSA (%)[k]	−0.27ns	0.38ns	0.43*	0.50**	0.28ns	−0.02ns	0.40*	0.57**	0.60***	0.39*	0.22ns	
PC1	−0.80***	0.69***	0.90***	0.97***	0.86***	0.85***	0.91***	0.98***	0.95***	0.98***	0.82***	0.43*

Pearson correlation coefficients (r) were calculated from four replicates of each sampling situation ($n=32$).
Significance conventions: ns = not significant; *$p<0.05$; **$p<0.01$; ***$p<0.001$.
[a] Total Cu in soil (μg g^{-1}).
[b] DTPA extractable Cu (μg g^{-1}).
[c] GRSP-bound Cu per gram of soil (μg g^{-1}).
[d] Total Zn in soil (μg g^{-1}).
[e] DTPA extractable Zn (μg g^{-1}).
[f] GRSP-bound Zn per gram of soil (μg g^{-1}).
[g] Glomalin-related soil protein (mg g^{-1}).
[h] Soil organic carbon (mg g^{-1}).
[i] C associated to GRSP in soil (mg g^{-1}).
[j] N associated to GRSP in soil (mg g^{-1}).
[k] Water-stable aggregates (%).

4.8 mg g^{-1} for Cu and Zn, respectively; Tables 1 and 2) compared to those found by González-Chávez et al. (2004). This may be due to higher pollutant levels (especially in the available fraction) in the soils studied here. Vodnik et al. (2008) found high GRSP-bound Pb contents, suggesting that GRSP was preferentially binding Pb, which would replace the Zn, supported by the negative correlation between soil Pb content and GRSP-Zn. In our study a positive and highly significant correlation was obtained between GRSP-Cu and GRSP-Zn ($r=0.87$, $p<0.001$; Table 4). In this case, the higher amount of GRSP-Cu compared to the amount of GRSP-Zn was likely due to the greater abundance of Cu in the soil and not a Zn-replacement effect.

Moreover, strong positive correlation between GRSP content and Cu and Zn contents (total and available fractions) were found (Table 4). Previous studies have shown that under suboptimal conditions for AMF hyphal growth, GRSP content increased. Presumably this could be a mechanism to improve the fungal habitat (Purin and Rillig, 2007), perhaps in regard to high levels of potentially phytotoxic elements (such as Fe and Al, Lovelock et al., 2004), or to alleviate physical/spatial constraints for hyphal development (Rillig and Steinberg, 2002). Our results agree with such previous observations, and suggest that the HM sequestration in GRSP may be another mechanism by which AMF could improve environmental conditions for their development, representing in our case up to 27% and 5.8% of total Cu and Zn in the soil, respectively (Table 2).

High contents of GRSP along with high contents of HM could be explained by the presumably low activity of microorganisms able to degrade GRSP under these extreme pollution conditions (Dai et al., 2004), and its recalcitrance in the soil (Steinberg and Rillig, 2003). Another "protection" effect of GRSP could be its iron binding (Rillig et al., 2001), agreeing with previous studies in these soils that shown high content of this elements (Ginocchio et al., 2004). Given the recent finding of high homology between glomalin and hsp 60 (Gadkar and Rillig, 2006), which are stress-related proteins, it should not be neglected that a strong stress presented by high levels of HM may cause overexpression of this protein. This hypothesis should be tested under controlled-sterile culture conditions.

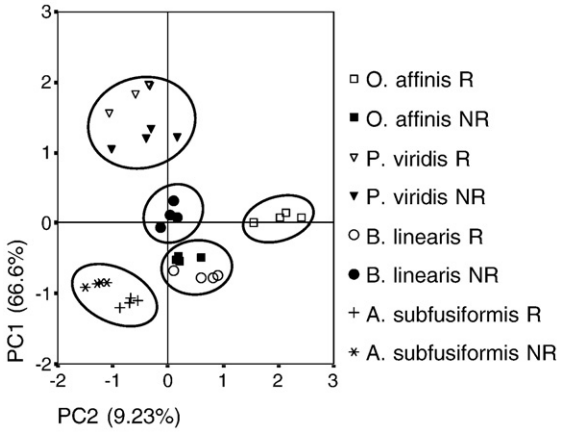

Fig. 2 – PCA scores in the respective plant species and zones analyzed in a Mediterranean ecosystem Cu polluted in central Chile. Four replicates of each sampling situation. Percentage values in parenthesis indicate the variation explained by each PC. Abbreviations: R = rhizospheric soil; NR = non-rhizospheric soil. The circles comprise individuals of similar characteristics according to the cluster analysis, and should be understood as a visual aid for the discrimination of groups.

4.2. *Soil organic carbon and aggregate stability*

Most of the soils had GRSP contents within the range previously described for non-organic soils (Wright and Upadhyaya, 1998) (Table 3). However, soils influenced by *P. viridis* presented GRSP contents similar to those reported in organic soils by Vodnik et al. (2008). The high GRSP contents found at *P. viridis* were associated with a higher hyphal density (Cornejo et al., unpublished data), which may explain a greater amount of GRSP because its feature as hyphal wall component (Driver et al., 2005).

A strong correlation between GRSP content and SOC ($r=0.98$, $p<0.001$) was found, as documented previously (Rillig et al., 2001; Morales et al., 2005; Nichols and Wright, 2006; Bedini et al., 2007; Schindler et al., 2007; Vodnik et al., 2008). In this study, the contribution of C-GRSP to SOC ranged between 16% and 89%, which probably is the highest value reported under field conditions to date. Although the methodology used for GRSP extraction and quantification may overestimate its content in soil (Rosier et al., 2006), in our study the non-specificity in the detection was presumably reduced because the SOC contents were very low (Fig. 1). Specifically, near *P. viridis* the non-GRSP C fraction was significantly lower than in the other soils, despite having significantly higher SOC.

On the other hand, highly significant correlations were found between SOC and GRSP content relative to the formation of WSA ($r=0.57$, $p<0.01$ and $r=0.60$, $p<0.001$, for SOC and GRSP, respectively). Using structural equation modeling, Rillig et al. (2002) have inferred that a direct effect of GRSP on WSA may be higher than the (residual) contribution of AMF hyphae. This effect is ecologically very important, since even under extreme conditions the deposition of large amounts of GRSP could promote WSA formation/stabilization, helping to prevent erosion processes, especially when HM accumulation is mostly superficial as a result of metal smelter activity (Ginocchio et al., 2004), which is the case in the present study.

4.3. *Microsite variation of soil characteristics and plant community*

The parameters studied were highly spatially variable, consistent with previous studies, which have found highly heterogeneous chemical changes in soils at small spatial scales (Ginocchio et al., 2004; Leung et al., 2007). In our case, the formation of microhabitats was heavily dependent on soil acidity and HM concentration (Fig. 2). Ginocchio (2000) reported that the HM levels in this ecosystem were caused by the deposition of pollutants since 1964, especially during the first 30 years, because only since 1992 are environmental regulations in Chile in effect. The area was also exposed to gaseous emissions of SO_2 and the subsequent acid rain, which contributed to the acidification of soils and the increase in HM availability, accelerated by the low buffering capacity of soils in the studied area (Ginocchio, 2000). It should be noted that the contents of GRSP were also variable at the microsite level, among different plants and survey areas, consistent with previous observations in semi-arid Mediterranean environments (Rillig et al., 2003).

Our results, especially considering PCA (Fig. 2), suggest a high level of interrelation of soil conditions and the presence of certain plant species (variables strongly correlated with PC1 explained 67% of the total variance), with highly uniform characteristics for each plant and rhizospheric zone in particular. Moreover, observations made by this research group showed marked effects of the variables here studied on the composition of AMF communities associated with the plant species studied here (Cornejo et al., unpublished data). Therefore, not only are further studies required on the conservation and technological utilization of these plants (Whiting et al., 2004), but also on their associated rhizospheric organisms, particularly AMF.

5. Conclusion

In conclusion, our study provides new evidence on the contribution of GRSP in HM sequestration in polluted soils. Based on our results, we can conclude that the GRSP-Cu can be a substantial pool in the soil, and could represent one of the main forms of "immobilized" Cu, contributing to the chemical stabilization of contaminated soils through the deposition of enriched-Cu particles. Furthermore, the accumulation of high quantities of GRSP may contribute to the formation of soil aggregates, even under extreme conditions of acidity and HM availability. There is undeniably the need to characterize the biochemical nature of GRSP to understand its contribution to HM sequestration and the chemical mechanisms involved. Finally, from a biotechnological perspective, further studies on the characterization of metallophytes and their associated AMF communities are needed, to select the most suitable ecotypes to use in site remediation in large areas of central Chile and other soils subjected to the effect of copper mining activities.

Acknowledgements

This work was supported by Fondecyt 3070052 and 1030702 Grants. The authors also acknowledge financial support given to Matthias Rillig for travel and expenses while staying in Chile through Fondecyt CI 7070333 Grant. We also thank Alexander Neaman and Isabel González (Universidad Católica de Valparaíso, Chile) for their help in soil sampling and Paulina Etcheverría and Carlos Castillo (Universidad de La Frontera, Chile) for technical assistance.

REFERENCES

Adriano DC. Trace elements in terrestrial environments: biogeochemistry, bioavailability, and risk of metals. New York: Springer-Verlag; 2001. 867 pp.

Allen M. The ecology of arbuscular mycorrhizas: a look back into the 20th century and a peek into the 21st. Mycol Res 1996;100:769–82.

Barea JM, Azcón-Aguilar C, Azcón R. Interactions between mycorrhizal fungi and rhizosphere microorganism within the context of sustainable soil–plant systems. In: Gange, AC, Brown, VK, editors. Multitrophic interactions in terrestrial systems. Cambridge: Cambridge University Press; 1997. p. 521–59.

Bedini S, Avio L, Argese E, Giovannetti M. Effects of long-term land use on arbuscular mycorrhizal fungi and glomalin-related soil protein. Agric Ecosyst Environ 2007;120:463–6.

Chern EC, Tsai DW, Ogunseitan OA. Deposition of glomalin-related soil protein and sequestered toxic metals into watersheds. Environ Sci Technol 2007;41:3566–72.
Dai J, Becquer T, Rouiller H, Reversat G, Bernhard-Reversat F, Lavelle P. Influence of heavy metals on C and N mineralization and microbial biomass in Zn-, Pb-, Cu-, and Cd-contaminated soils. Appl Soil Ecol 2004;25:99–109.
De Gregori I, Fuentes E, Rojas M, Pinochet H, Potin-Gautier M. Monitoring of copper, arsenic and antimony levels in agricultural soils impacted and non-impacted by mining activities, from three regions in Chile. J Environ Monit 2003;35:287–95.
Del Val C, Barea JM, Azcón-Aguilar C. Diversity of arbuscular mycorrhizal fungus population in heavy-metal contaminated soil. Appl Environ Microbiol 1999;65:718–23.
Driver JD, Holben WE, Rillig MC. Characterization of glomalin as a hyphal wall component of arbuscular mycorrhizal fungi. Soil Biol Biochem 2005;37:101–6.
Ellis R, Morgan P, Weightman A. Cultivation-dependent and independent approaches for determining bacterial diversity in heavy-metal-contaminated soil. Appl Environ Microbiol 2003;69:3223–30.
Gadkar V, Rillig M. The arbuscular mycorrhizal fungal protein glomalin is a putative homolog of heat shock protein 60. FEMS Microbiol Lett 2006;263:93–101.
Giller K, Witter E, McGrath S. Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. Soil Biol Biochem 1998;30:1389–414.
Ginocchio R. Effects of a copper smelter on a grassland community in the Puchuncavi Valley, Chile. Chemosphere 2000;41:15–23.
Ginocchio R, Carvallo G, Toro I, Bustamante E, Silva Y, Sepúlveda N. Micro-spatial variation of soil metal pollution and plant recruitment near a copper smelter in Central Chile. Environ Pollut 2004;127:343–52.
González S, Ite R. Acumulación metálica en suelos del área bajo influencia de las chimeneas industriales de Ventanas (provincia de Valparaíso, V Región). Agric Tec 1992;50:214–9.
González-Chávez M, Carrillo-González R, Wright S, Nichols K. The role of glomalin, a protein produced by arbuscular mycorrhizal fungi, in sequestering potentially toxic elements. Environ Pollut 2004;130:317–23.
Jeffries P, Gianinazzi S, Perotto S, Turnau K, Barea JM. The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. Biol Fertil Soils 2003;37:1–16.
Kemper WD, Rosenau RC. Aggregate stability and size distribution. In: Klute, A, editor. Methods of soil analysis, Part I. 2nd ed. Madison: American Society of Agronomy; 1982. p. 425–44. Agronomy 9.
Khan A. Role of soil microbes in the rhizospheres of plants growing on trace metal contaminated soils in phytoremediation. J Trace Elem Med Biol 2005;18:355–64.
Leung H, Ye Z, Wong M. Survival strategies of plants associated with arbuscular mycorrhizal fungi on toxic mine tailings. Chemosphere 2007;66:905–15.
Lovelock C, Wright S, Clark D, Ruess R. Soil stocks of glomalin produced by arbuscular mycorrhizal fungi across a tropical rain forest. J Ecol 2004;92:278–87.
Morales A, Castillo C, Rubio R, Godoy R, Rouanet JL, Borie F. Glomalin levels in soils from two ecosystems of Southern Chile. J Soil Sci Plant Nutr 2005;5:37–45.
Nelson DW, Sommers LE. Total carbon, organic carbon and organic matter. In: Page, AL, Miller, RH, Keemey, R, editors. Methods of soil analysis, Part II. 2nd ed. Madison: American Society of Agronomy; 1982. p. 539–79. Agronomy 9.
Nichols, K. Characterization of glomalin, a glycoprotein produced by arbuscular mycorrhizal fungi. Doctor of Philosophy Thesis. University of Maryland, College Park, Maryland 2003, 285 pp.
Nichols KA, Wright SF. Carbon and nitrogen in operationally-defined soil organic matter pools. Biol Fertil Soils 2006;43:215–20.
Purin S, Rillig MC. The arbuscular mycorrhizal fungal protein glomalin: limitations, progress, and a new hypothesis for its function. Pedobiologia 2007;51:123–30.
Reinhardt D. Programming good relations development of the arbuscular mycorrhizal symbiosis. Plant Biol 2007;10:98–105.
Rillig MC. Arbuscular mycorrhizae, glomalin, and soil aggregation. Can J Soil Sci 2004;84:355–63.
Rillig M, Steinberg P. Glomalin production by an arbuscular mycorrhizal fungus: a mechanism of habitat modification? Soil Biol Biochem 2002;34:1371–4.
Rillig MC, Mummey DL. Mycorrhizas and soil structure. New Phytol 2006;171:41–53.
Rillig MC, Wright SF, Eviner VT. The role of arbuscular mycorrhizal fungi and glomalin in soil aggregation: comparing effects of five plant species. Plant Soil 2002;238:325–33.
Rillig MC, Ramsey PW, Morris S, Paul EA. Glomalin, an arbuscular-mycorrhizal fungal soil protein, responds to land-use change. Plant Soil 2003;253:293–9.
Rillig MC, Caldwell BA, Wösten HAB, Sollins P. Role of proteins in soil carbon and nitrogen storage: controls on persistence. Biogeochemistry 2007;85:25–44.
Rosier CL, Hoye AT, Rillig MC. Glomalin-related soil protein: assessment of current detection and quantification tools. Soil Biol Biochem 2006;38:2205–11.
Schindler F, Mercer E, Rice J. Chemical characteristics of glomalin-related soil protein (GRSP) extracted from soils of varying organic matter content. Soil Biol Biochem 2007;39:320–9.
Soil Survey Division Staff. Soil survey manual. USDA handbook 18. Washington: U.S. Government Printing Office; 1993. 503 pp.
Steinberg PD, Rillig MC. Differential decomposition of arbuscular mycorrhizal fungal hyphae and glomalin. Soil Biol Biochem 2003;35:191–4.
Vodnik D, Grčman H, Maček I, van Elteren JT, Kovačevič M. The contribution of glomalin-related soil protein to Pb and Zn sequestration in polluted soil. Sci Total Environ 2008;392:130–6.
Whiting SN, Reeves RD, Richards D, Johnson MS, Cooke JA, Malaisse F. Research priorities for conservation of metallophyte biodiversity and their potential for restoration and site remediation. Rest Ecol 2004;12:106–16.
Wright S, Upadhyaya A. Extraction of an abundant and unusual protein from soil and comparison with hyphal protein of arbuscular mycorrhizal fungi. Soil Sci 1996;161:575–86.
Wright S, Upadhyaya A. A survey of soils for aggregate stability and glomalin, a glycoprotein produced by hyphae of arbuscular mycorrhizal fungi. Plant Soil 1998;198:97–107.