

EFFECT OF ARBUSCULAR MYCORRHIZAL FUNGAL INOCULATION ON *Eucalyptus globulus* SEEDLINGS AND SOME SOIL ENZYME ACTIVITIES UNDER APPLICATION OF SEWAGE SLUDGE AMENDMENT

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ABSTRACT

The sewage sludge (SS) represents a source of organic matter although its increasing accumulation need a suitable environmental treatment. Our study analyzed the effect of the interaction between sewage sludge (doses 0, 2, 4, 6 and 8 g per 100 g of soil) and *Eucalyptus globulus* nursery seedlings inoculated with arbuscular mycorrhizal (AM) fungi as a fertilizers alternative and evaluate its effect on some soil enzyme activities. The seedlings were inoculated with *Glomus claroideum*, *Glomus viscosum*, *Glomus intraradices* and *Glomus constrictum*, establishing a uninoculated control. The sewage sludge application increased the shoot dry weight and the inoculation with *G. viscosum* was more effective at 6 g of SS in 100 g soil. The root colonization and succinate dehydrogenase activity was decreased by all doses of SS used. The Fluorescein diacetate activity was increased by the AM inoculation but not by sewage sludge. The β -glucosidase activity was increased in presence of SS at 6 g 100 g⁻¹. The N and P uptake by the *E. globulus* plants was higher in presence of sewage sludge and more effective with *G. viscosum* plant inoculation. In conclusion the application of these amounts of SS promoted the plant growth and can be regarded as a successful biotechnological tool for the greenhouse plant production.

Keywords: Biological fertilisers, *Glomus*, sewage sludge, soil enzymes, symbiosis

INTRODUCTION

The sewage sludge (SS) as a product of wastewater treatment is considered beneficial because improve soil fertility, biological parameters and plant nutrition. These residues with high nitrogen content enhance the soil productivity and can be

used as fertilizer (Banerjee *et al.*, 1997). In addition, the SS application improve the soil structure and fertility (Cuevas *et al.*, 2000; Mata-González *et al.*, 2002), but changes in soil microbial community in response to this application are not well

known (Sullivan *et al.*, 2006). Several authors show diverse responses of soil microorganisms to SS (Cuevas *et al.*, 2000; García-Gil *et al.*, 2004; Mata-González *et al.*, 2002). On the other hand, the SS can contribute to the rehabilitation of mineral sand mines (Rate *et al.*, 2004) and the application to land offers an environmentally acceptable and agronomically favourable means of waste disposal (Oudeh *et al.*, 2002).

Soil microorganisms are important in the recovery of disturbed and potentially toxic environments because they produce plant growth stimulating substances such as hormones and vitamins, bind soil particles into stable aggregates which improve soil structure, reduce erosion potential and can contribute to nutrient availability to plants (Shetty *et al.*, 1994). The arbuscular mycorrhizal (AM) fungi are an important component of the soil microbial biomass. The symbiosis is mutualistic based on bidirectional nutrient transfer between the symbionts. The plant benefits particularly through enhanced phosphorus (Pearson and Jackobsen, 1993; Baon *et al.*, 1992), nitrogen (Cliquet and Stewart, 1993), other mineral nutrients and water uptake, which often results in better growth. On the other hand, plants colonized by AM fungi also show an enhanced resistance to plant against several biotic and abiotic factors (Feng *et al.*, 2002; Arriagada *et al.*, 2004). It was reported that the AM fungi *Glomus intraradices* and *Glomus deserticola* and the addition of composted SS improve the growth and the N and P contents in shoot tissues of *Juniperus oxycedrus* (Alguacil *et al.*, 2006). On the other side, the analyses of some biological parameters give more complete information on soil quality and health as affected by agricultural practices (Avidano *et al.*, 2005). Research shows that fluorescein

diacetate (FDA) hydrolysis and β -glucosidase are good indicators of soil biogeochemical processes. In fact, several authors used the hydrolysis of FDA to estimate microbial activities in soil (Schnürer and Roswall, 1982; Gumprecht *et al.*, 1995; Bandick and Dick 1999). The β -glucosidase activity plays a key role in the C cycle, being positively related to total soil carbon and it was closely associated with microbial biomass (Alvear *et al.*, 2005).

The aim of the present study was to determine the effect of SS application on the plant growth promotion by the AM fungi inoculation and measure the influence of this interaction on the biochemical parameters (fluorescein diacetate and β -glucosidase) in the rhizosphere soil.

MATERIAL AND METHODS

Experimental design

A design 5 x 5 full factorial randomized experimental design was used, including five mycorrhizal treatments: (1) uninoculated controls, (2) soil inoculated with *Glomus claroideum*, or (3) *Glomus viscosum*, or (4) *G. intraradices*, or (5) *Glomus constrictum*; and five SS doses: 0, 2, 4, 6 and 8 g per 100 g of soil. Ten replicate pots per treatment were used.

Soil and sewage sludge

The soil used in this study was an Andisol (Acruoxic Hapludands) and was sampled from a 5- to 20-cm depth (Ñielol soil series) at the relict native forest Rucamanque, (38° 39' S, 72° 35' W), Temuco, Chile (Table 1). The samples were brought to the laboratory, sieved (2 mm), and visible leaves branches and roots were eliminated. Field moistens soil

samples were kept at 4°C in plastic containers until analysis.

The sewage sludge (SS) used was collected from municipal wastewater at Vilcún, Araucanía Region, Southern of Chile. The main characteristics of the SS are showed in Table 2.

Biological material description

Eucalyptus globulus Labill seeds previously surface-sterilised (HgCl₂ for 10 min), and thoroughly rinsed with sterilized water, were sown in moistened sand. After germination, uniform seedlings were planted in 0.3 L pots filled with a mixture of sterilized sand:soil 1:4 v:v.

The arbuscular mycorrhizal (AM) fungi used in the experiment were *G. laroideum* (Schenck and Smith) Walker and Vestberg; *G. viscosum* T.H. Nicolson, *G. intraradices* Schenck & Smith and *G. constrictum* Trappe, obtained from the culture collection at the Bioremediation laboratory of the University of La Frontera, Temuco, Chile.

Arbuscular mycorrhizal inoculation of seedlings

The AM fungal inoculum was prepared with a root and soil mixture consisting of rhizosphere soil containing spores and colonized root fragments of *Medicago sativa* L. in amounts of 8 g per pot, which were predetermined to achieve high levels of root colonization. A water filtrate (Whatman N° 1 paper) of the inoculum was applied to the uninoculated pots containing common soil microflora but free of AM fungal propagules. Plants were inoculated with AM fungi at transplantation (after 4 weeks of growth).

Growth conditions

Plants were grown in a greenhouse with supplementary light provided by Sylvania incandescent and cool-white lamps, 400 E m⁻² s⁻¹, 400–700 nm, with a 16/8 h day/night cycle at 25/19°C and 70% relative humidity.

Harvests and analyses

Plants were harvested after 16 weeks and dry biomass of shoots and roots and nutrient concentration were determined. The P and N contents analysis were determined as described by Jackson (1973), after grinding the plant material to pass through a 0.5-mm screen and digested in a H₂SO₄-H₂O₂ mixture.

After the harvest, two samples of fresh roots were randomly taken from the entire root system. One of the samples was cleared and stained with trypan blue (Phillips and Hayman, 1970) and the percentage of root colonized by AM fungi was calculated by the gridline intersect method (Giovannetti and Mosse, 1980). In a second sample, succinate dehydrogenase (EC 1.3.99.1) (SDH) activity was measured in fungal mycelia through the reduction of tetrazolium salts at the expense of added succinate (MacDonald and Lewis, 1978). The percentage of AM fungal mycelia with SDH activity was determined under a compound microscope (Ocampo and Barea, 1985).

Biochemical determinations

The fluorescein diacetate activity (FDA) was assessed as described by Adam and Duncan (2001) and expressed as µg fluorescein released g⁻¹ dry soil. β-glucosidase was determined by measuring

Table 1: Chemical characteristics of soil sample

Depth	pH	MO %	C %	N mg kg ⁻¹	Olsen P mg kg ⁻¹	K mg kg ⁻¹	Al mg kg ⁻¹
5-20	5.26	19.7	14.27	23.1	6.9	433	1,794.2

OM: Organic matter

Table 2: Chemical characteristics of Sewage Sludge

Parameters	Units
Organic matter (%)	49.27
Electrical conductivity (dSm ⁻¹)	3.71
pH (CaCl ₂)	6.98
pH (water)	7.14
Olsen P (mg kg ⁻¹)	1.21
Available K (mg kg ⁻¹)	2.91
Available S (mg kg ⁻¹)	381
N (Kjeldhal) (g kg ⁻¹)	38.9
Cation exchange capacity (cmolc 100 g ⁻¹)	94.9
Al-saturation (%)	0.02
Al-extractable (mg kg ⁻¹)	90

p-nitrophenol released from *p*-nitrophenyl-beta-D-glucopyranoside according to Eivazi and Tabatabai (1990). One unit of enzyme activity was defined as 1 μmol of product released per min (IU). The average and standard deviations of enzymatic activities from triplicate culture are reported.

Statistical analysis

The values were arcsine transformed before statistical analysis. The data were analysed by factorial analysis of variance with AM fungi treatment, SS treatment and their interaction as sources of variation.

Statistical procedures were carried out with the SPSS software, version 11.0 (SPSS Inc., 1989–2001). Statistical significance was determined at $P < 0.05$.

RESULTS

The results of factorial ANOVA are given in Table 3. FDA activity was significantly increased by AM treatments and the interaction between AM and SS. The β -glucosidase activity was significantly increased by AM and SS treatments. All the treatments and their interactions (AMxSS) increased shoot dry weight. At the end of the study the mycorrhiza-inoculated seedlings were significantly larger. The biomass of inoculated plants with all AM fungi was greater than uninoculated plants (Figure 1). This beneficial effect was more evident with the *G. viscosum* inoculation. In the other hand, the addition of SS also increased the shoot dry weight of *E. globulus*. In fact, the shoot biomass was significantly increased at 6 g of SS treatment. In addition, this beneficial effect was more evident and significantly increased with the interaction between *G. viscosum* inoculation plants and the application of 4 g of SS to 100 g⁻¹ of soil. The root dry weight of *E. globulus* was not stimulated in soil inoculated or not with all the AM fungi (Figure 1). The application of SS at 8 g to 100 g of soil decreased the root dry weight inoculated or not with the AM fungi.

The effects of the interaction between SS and *E. globulus* inoculated with AM fungi on the N contents in plant shoot are illustrated in Figure 2A. The AM fungi *G. claroideum* and *G. viscosum* have a significant increase in the N contents of

the plant tissues. *G. intraradices* and *G. constrictum* did not produce differences in N contents on plant shoot. The N content was also higher when *E. globulus* were inoculated with *G. viscosum* and SS were applied in the same treatment. The effects of SS and *E. globulus* inoculated with AM fungi on the P contents are illustrated in Figure 2B. The P contents on plant shoot were significantly higher in all treatment with SS application. This effect was also observed on plants inoculated with the all AM fungi. In this case, the AM fungi *G. claroideum*, *G. viscosum* and *G. intraradices* showed an increased of 2 to 8 g of SS to 100 g of soil in P content on plant shoot.

The average mycorrhizal root colonization of plants inoculated with *G. claroideum*, *G. viscosum* and *G. intraradices* was 67%, 59% and 67% of total root length, whereas the AM colonization was unaffected (8%) in plants inoculated with *G. constrictum* (Fig. 3A). The effect of SS in mycorrhizal root colonization of *E. globulus* decreased in presence of 2, 4, 6 and 8 g of SS to 100 g⁻¹ of soil applied. The interaction between SS and *E. globulus* inoculated with *G. intraradices* increased the AM colonization in presence of 2, 4 and 6 g of SS to 100 g of soil.

The metabolic activity of the AM fungi, measured as SDH activity of the fungal mycelium inside the root of *E. globulus* showed the same effect for 0 and 2 g of SS to 100 g of soil applied (Fig. 3B). The sewage sludge decreases the metabolic activity of the AM fungi. Nevertheless, 4g of SS to 100 g⁻¹ of soil applied shows unaffected the SDH activity in plants inoculated with *G. claroideum*, *G. viscosum* and *G. intraradices*.

Table 3: F-values and significance for the main treatment effects and their interactions, based on a bifactorial ANOVA

Variables	AM	SS	AM*SS
Shoot dry weight	53.21**	275.28 **	11.03***
Root dry weight	21.33 n.s.	197.41 n.s.	4.17 n.s.
FDA	47.58**	164.35 n.s.	10.06 **
β -glucosidase	24.27**	239.48 2**	12.73 n.s.

AM: Arbuscular mycorrhiza; SS: Sewage sludge. Significance conventions: ns=not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Fluorescein diacetate activity was increased by the inoculation with all AM fungi and this effect was more evident in treatment with the arbuscular mycorrhizal fungus *G. viscosum* (Figure 4A). The application of this sewage sludge at different doses did not affect the FDA hydrolysis in *E. globulus* plants. In contrast, the inoculation with *G. viscosum* in presence of sewage sludge increased FDA activity.

The β -glucosidase activity in the rhizosphere of *E. globulus* plant was increased only in the presence of SS to 2-6 g to 100 g of soil (Figure 4B). The most important increased of β -glucosidase was the inoculation with *G. viscosum* in presence of sewage sludge to 4 g of SS to 100 g⁻¹ of soil but significantly higher at 6 and 8 g of SS to 100 g of soil.

DISCUSSION

The aim of this study was to investigate determine the effect of SS and mycorrhizal inoculation on the plant growth promotion and how could affect the plant nutrition and some soil biological properties. Our results confirm,

in general, as was previously described, a positive plant growth response to organic amendments (Muthukumar and Udaiyan, 2000). Increases in growth may be attributable in part to higher net mineralization of adequate nutrients (Antolin *et al.*, 2005) in particular the N mineralization from this SS (Khan and Scullion, 2000).

The shoot dry weights of *E. globulus* colonized by all AM fungi assays and with application to SS, in general, were significantly increased. Bestel-Corre *et al.* (2004) found that the application of municipal SS in plants of *Medicago truncatula* non-inoculated with *G. mosseae* improved the plant biomass more than those inoculated with this mycorrhizal fungus. Sullivan *et al.* (2006) reported a decrease of AM associations with an increase of the application rate of SS due directly to nutrient content or indirectly through the effects of SS on plant community species richness and structure. In fact, in our experiments we observed a decrease of AM colonization and SDH activity of the intraradical fungal mycelium of *E. globulus* by all the treatments with SS application. In the other hand, Alguacil *et al.* (2006) found

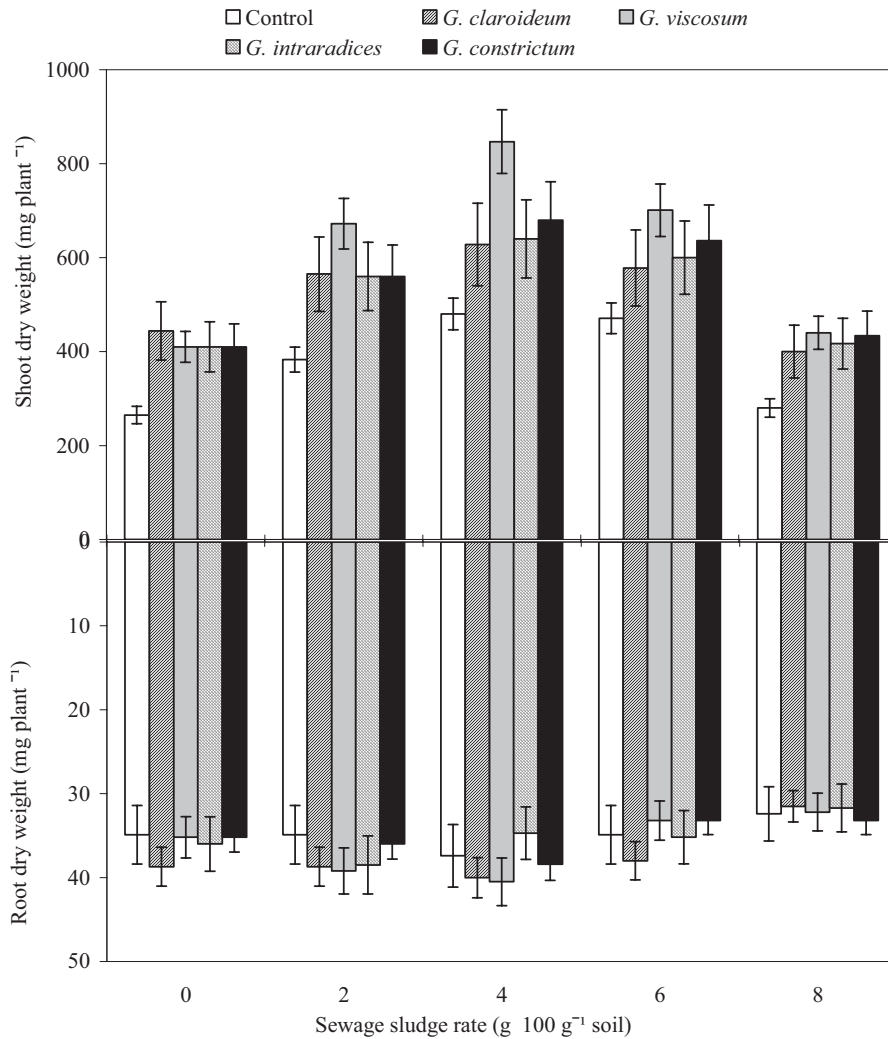


Figure 1: Shoot and root dry weight (mg plant⁻¹) of *Eucalyptus globulus* inoculated with the arbuscular mycorrhizal fungi *Glomus claroideum*, *G. viscosum*, *G. intraradices* and *G. constrictum* in soil amended with sewage sludge. The data are the means \pm standard error (n = 10)

that the inoculation with AM fungi and the addition of SS significantly stimulated growth of *Juniperus oxycedrus* (shoot and root dry weights) and the N and P contents in shoot tissues, with respect to the non-inoculated and non-treated with

SS plants. The FDA activity was not stimulated for the SS application to control treatments (without the inoculation with AM). It has been reported that large fertilizer additions have led to reductions in microbial

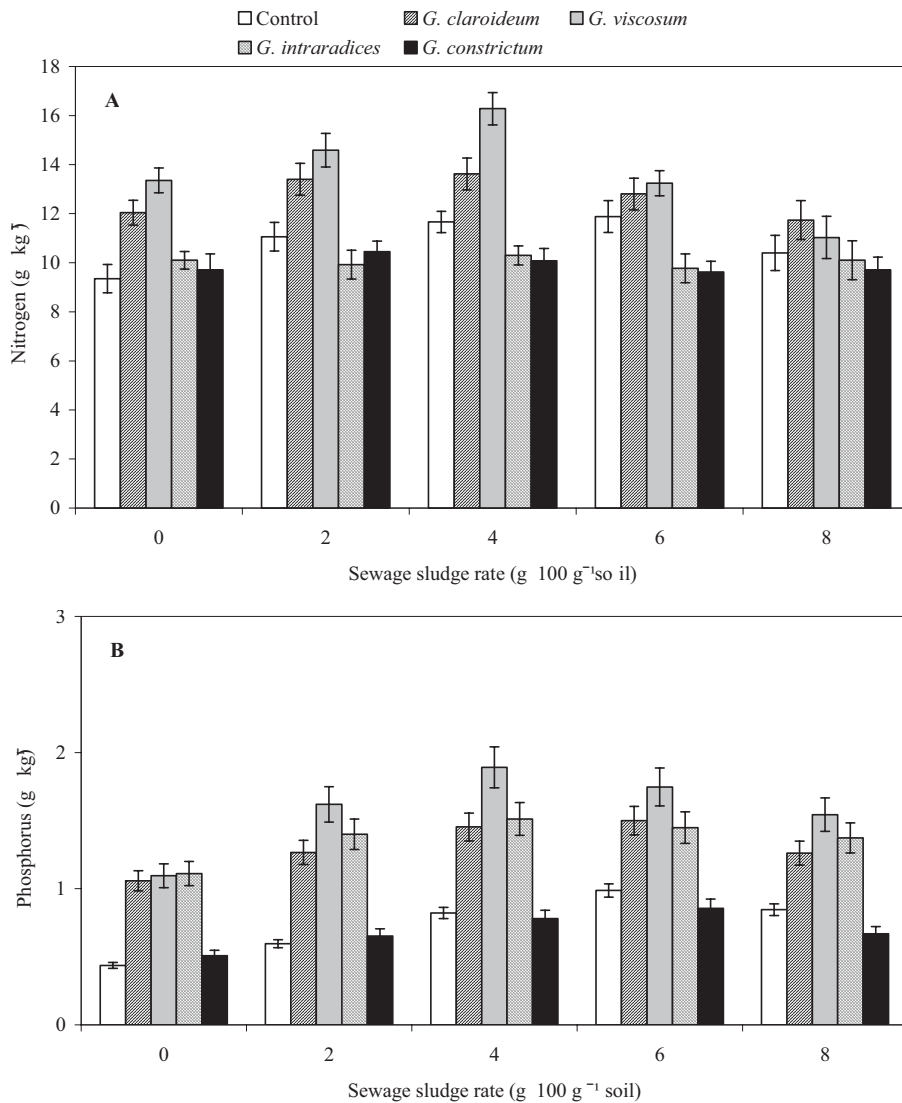


Figure 2: Nitrogen (A) and phosphorus (B) in shoot of *Eucalyptus globulus* plants (g kg⁻¹) inoculated with the arbuscular mycorrhizal fungi *Glomus claroideum*, *G. viscosum*, *G. intraradices* and *G. constrictum* in soil amended with sewage sludge. The data are the means \pm standard error (n = 10)

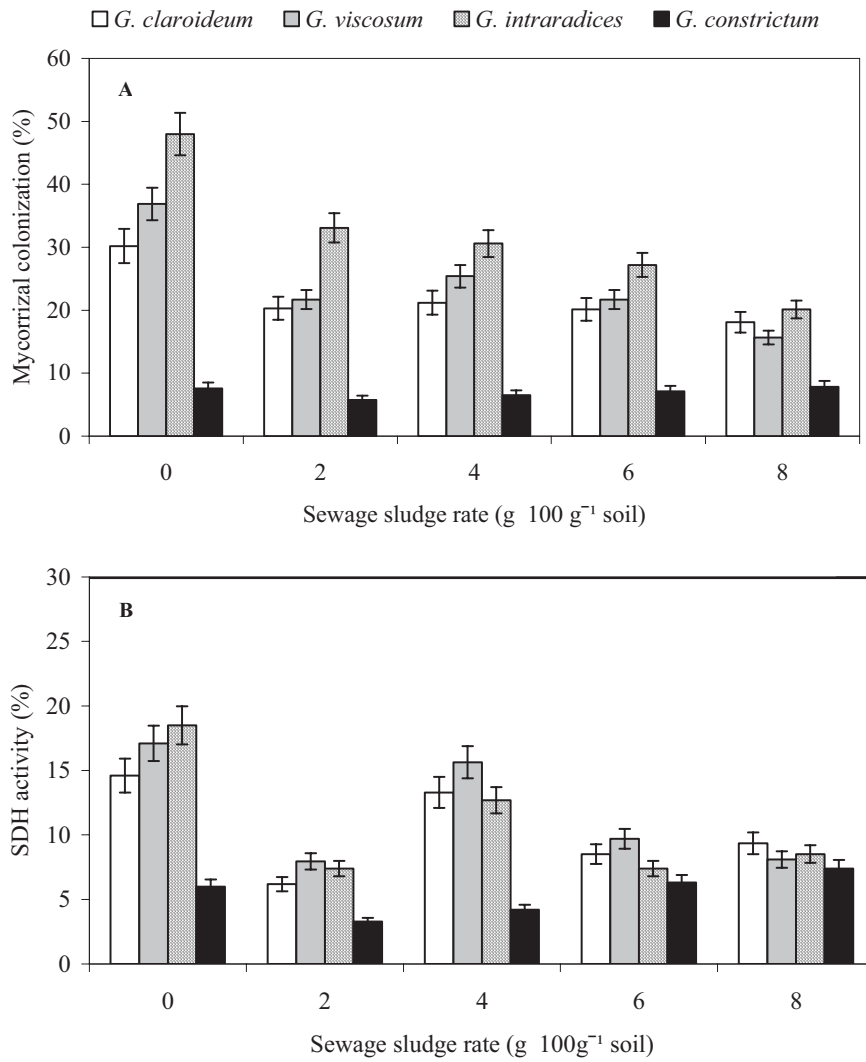


Figure 3: Percentage of mycorrhizal root colonization (A) and Arbuscular mycorrhizal intraradical mycelium with succinate dehydrogenase (SDH) activity (B) of *Eucalyptus globulus* inoculated with the arbuscular mycorrhizal fungi *Glomus claroideum*, *G. viscosum*, *G. intraradices* and *G. constrictum* in soil amended with sewage sludge. The data are the means \pm standard error (n = 10)

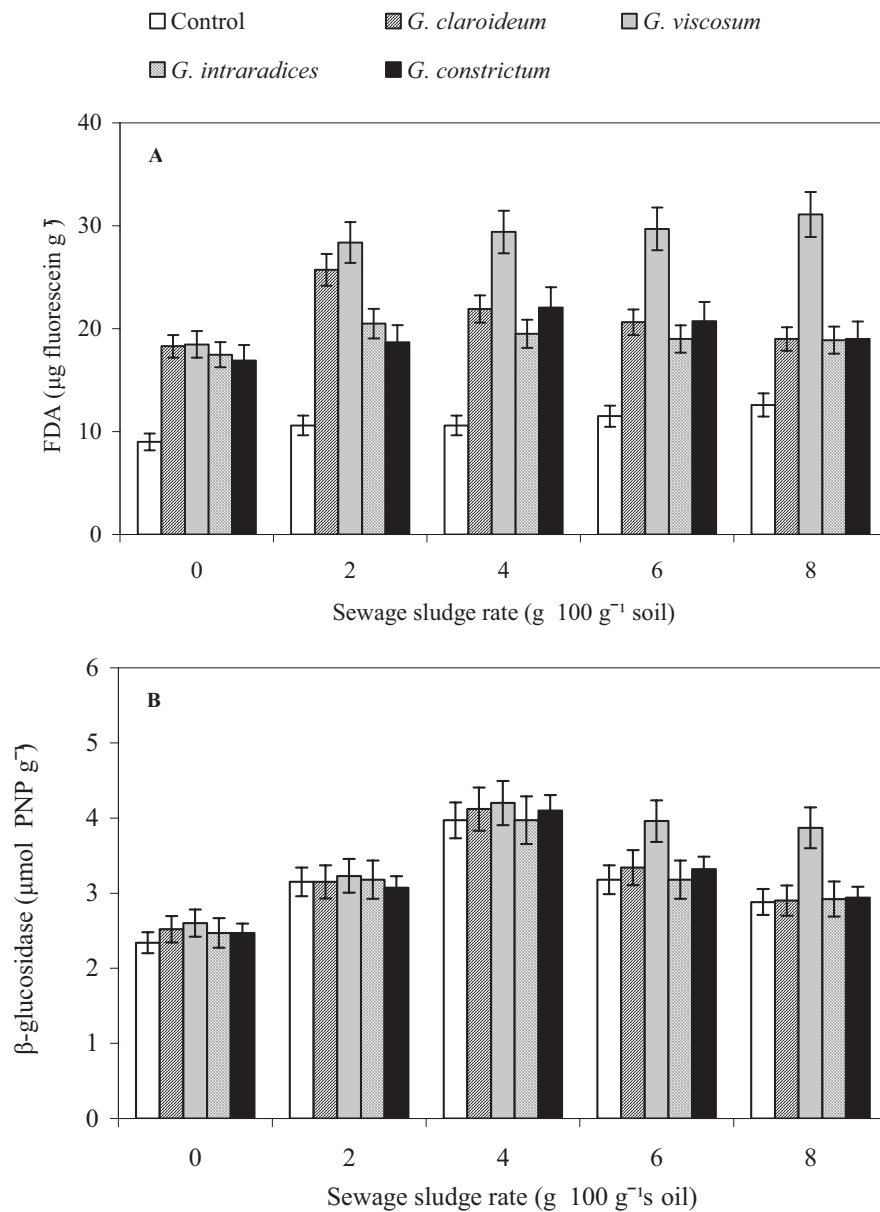


Figure 4: Fluorescein diacetate (FDA) ($\mu\text{g fluorescein g}^{-1}$) (A) and β -glucosidase ($\mu\text{mol PNP g}^{-1}$) (B) activities in the rhizosphere soil of *Eucalyptus globulus* plants inoculated with the arbuscular mycorrhizal fungi *Glomus claroideum*, *G. viscosum*, *G. intraradices* and *G. constrictum* in soil amended with sewage sludge. The data are the means \pm standard error ($n=10$)

activity, which have been attributed, at least partially, to changes in pH (Insam and Palojärvi, 1995). It is necessary to consider that soil pH is 5.26. This pH causes an increase of the proton and chloride presence, which undoubtedly causes an initial decrease of the bacterial biomass, which is better developed at alkaline pH, it seems unlikely that these pH variations would be sufficiently great to cause differences in biological activity (García *et al.*, 1994).

Pascual *et al.* (2007) reported that after addition of SS to soil significantly enhanced the β -glucosidase activity, probably because SS has a high amount of utilizable substrates for microbial growth (Ros *et al.*, 2003). Medina *et al.* (2006) observed an increased of β -glucosidase activity by inoculation with *Glomus mosseae* and agrowaste amendment. According to our experiments, in the soil with application of SS and inoculated with *G. viscosum* we observed an important enzymatic activity, thus suggesting the important role of this fungus in the soil organic matter mineralization.

Many authors suggest that increasing on plant biomass to organic amendments can be attributed to enhanced nutrient status (Muthukumar and Udaiyan, 2000; Alguacil *et al.* 2006; Medina *et al.*, 2006). In order to our experiments, the inoculation with *G. viscosum* increased N and P in plants. The application of SS also increased soil fertility and rapidly increased the shoot N and P contents of *E. globulus*. Therefore, improved plant growth appears to be due to a direct nutrient supply by organic mineralization of SS (Antolin *et al.*, 2005), and by the beneficial effects of AM inoculation (Alguacil *et al.*, 2006; Medina *et al.*, 2006).

CONCLUSION

In conclusion the SS from municipal wastewater used in this study improves the soil fertility due to high content of organic and inorganic plant nutrients. The application of SS to *E. globulus* inoculated with AM fungi showed results very variable in the plant biomass production and the enzymes activities. Sewage sludge application improved some soil enzymes activities and plant nutrition. In particular conditions, some authors indicated that the application amounts of SS could be used for several years to maintain crop production. In this study, the application of certain amounts of SS does not cause problems to *E. globulus*, but the applied amounts have to be taken into account in future bioassays planning with other plants. These bioassays can be a suitable tool to evaluate the beneficial effects of SS as soil amendments with beneficial impacts on *E. globulus* plant growth.

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