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The growth-enhancement of clover by *Aspergillus*-treated sugar beet waste and *Glomus mosseae* inoculation in Zn contaminated soil

Almudena Medina, María Vassileva, José-Miguel Barea, Rosario Azcón *

Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada, Spain

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Abstract

In a microcosm experiment, using a Zn contaminated soil, we determined the effectiveness on plant growth of treatments such as *Aspergillus niger*-treated sugar beet waste (SBW, 50 g kg^{-1} soil), in presence or absence of rock phosphate (RP, 1.5 g kg^{-1} soil) and arbuscular mycorrhizal (AM) inoculation. An autochthonous, Zn adapted, strain of *Glomus mosseae* was used and its interaction with amendments was assessed on *Trifolium repens* growth, nutrition and symbiotic (AM colonization and nodulation) values. Treatments applied resulted alternative strategies for alleviating plant Zn toxicity and for Zn phytoextraction from Zn contaminated soils. *A. niger*-treated SBW + RP was the most effective amendment in improving plant growth and nutrition. The impact of such treatment on plant N, P and Zn concentration was particularly evident when associated with *G. mosseae*. Total growth (four harvests) of AM plants growing in SBW + RP-treated soil was about 28 times more than in non-mycorrhizal control plants. The effect of *G. mosseae* on shoot biomass ranged from 86% (without amendment) to 1192% (with treated SBW + RP). This growth improvement was the consequence of increased N, P and K nutrition and decreased Zn acquisition. Nevertheless, as consequence of an enhanced plant biomass, Zn phytoextraction by these plants increased by 1832% over untreated ones. Treatments applied also improved nodule formation by an inoculated *Rhizobium*, that were highly depressed in this Zn contaminated soil. Soil enzymatic activities, which are indicative of biological performance in the rhizosphere were also improved by treatments applied. Phosphatase activity was 1257% higher than in control soil by the application of SBW + AM fungus; following a similar trend, dehydrogenase and β-glucosidase activities reached the highest values in SBW + AM treatments. The application of AM fungus and treated SBW + RP amendment for decontaminating a Zn polluted soil can be regarded as a successful biotechnological tool for the recovery of polluted soils and as an important strategy for bioremediation purposes.
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Keywords: Agrowaste amendments; Arbuscular mycorrhiza; Plant Zn alleviation; Zn contamination; *Aspergillus niger*; Phytoextraction

1. Introduction

The establishment of plant species is considered as an effective strategy for reclaiming metal contaminated lands (Jeffries et al., 2003). The exploitation of natural resources causes important ecological problems. In polluted zones, decrease in the characteristic biodiversity of such area and the degradation of the natural ecosystem have been proved. Such unfavourable

Abbreviations: AM, arbuscular mycorrhizal; INT, 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride; INTF, iodo-nitrotetrazolium formazan; PNG, *p*-nitrophenyl-β-D-glucopyranoside; PNP, *p*-nitrophenol; PNPP, *p*-nitrophenyl phosphate disodium; RP, rock phosphate; SAR, specific absorption rate; SBW, sugar beet; TB, Trypan blue; THAM, tris–hydroxymethyl aminomethane

* Corresponding author. Tel.: +34 958 18 16 00x298; fax: +34 958 12 96 00.
E-mail address: rosario.azcon@eez.csic.es (R. Azcón).

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conditions for plant growth require applying methods for improving nutrient balance, microbial activity and soil quality (García et al., 1999). In polluted areas, plants are more dependent on microbial activity (Moreno-Ortego et al., 1999) since plant productivity is seriously limited in these areas, and consequently plant growth (Baker et al., 1994; Puppi et al., 1994). In fact, bioremediation has been defined as the use of microorganisms for the treatment of soil pollution (Leyval et al., 2002) and can be applied in association with different strategies of phytoremediation.

In metal polluted soils, mycorrhizal fungi can play an important role by improving plant establishment, promoting plant growth and nutrition and reducing metal translocation to shoots which alleviate metal toxicity (Colpaert and Vandenkoornhuyse, 2001; Vivas et al., 2003a,b). The tolerance and diversity of arbuscular mycorrhizal (AM) fungi in heavy metal contaminated soil are indicative of AM fungal adaptation to heavy metals (Colpaert and Vandenkoornhuyse, 2001; Joner et al., 2000; Leyval et al., 1995). Nevertheless, AM fungi have been selected from varied metal enriched soils but their role in plant tolerance to toxic metals is not well understood (Leyval et al., 2002). However, it has been demonstrated that the use of adapted microbial strains, in restoration and bioremediation studies, is more effective than applying non-adapted strains (Vivas et al., 2003a,b,c).

The deterioration of biological properties of metal contaminated soils is in part due to their progressive decrease in organic matter content (Turnau and Haselwandter, 2002); thus, the application of appropriate levels and kinds of organic amendments may be a valid choice to improve soil characteristics (Martens and Frankenberger, 1992).

Sugar beet waste is a lignocellulosic residue produced during sugar processing. This material is very cheap and is mainly used as animal feed; however, its use as an amendment has been recommended after microbial treatment with *Aspergillus niger* in medium added of rock phosphate (RP) (Rodríguez et al., 1999; Vassilev et al., 1996). During the fermentation process, *A. niger* is able to solubilize RP by its ability to excrete organic acids (citric acid mainly); moreover, SB waste is transformed in more simple sugars compounds by *A. niger* as a result of the production of hydrolytic enzymes of the cellulase complex (Hoshino et al., 1997). These simple sugars can be used as energy sources for heterotrophic microorganisms which require such compounds for growth and metabolic activities (Bowen and Rovira, 1999). In this way, the application of the transformed agrowaste residue could be interesting for improving physical and biological soil characteristics (Roldán et al., 1996).

On the other hand, physical immobilization of heavy metals in soil could be accomplished using amendments since organic matter makes strong complexes with heavy metals (Bolan and Duraisamy, 2003; Hartley et al., 2004). In fact, there are some reports of heavy metal biosorption in aqueous solution by sugar beet pulp (Dronnet et al., 1997; Reddad et al., 2002), these authors attributed the capacity of sugar beet pulp to bind metals to the carboxyl functions present in its constituents.

Previous studies have demonstrated that the fungal (*A. niger*) solubilization of rock phosphate on media based on agrowastes was compatible and interacted positively with AM fungi when supplied as amendments to the soil/plant system (Vassilev et al., 1996, 1998); nevertheless, to our knowledge, no information is available on the simultaneous use of amendments and AM inoculum for bioremediation purposes.

Soil microorganisms may play an important role in nutrient cycling in soils amended with organic materials and their activity can also alleviate the metal toxicity in the environment. Thus, measurements of this microbial activity in treated and/or inoculated soil provide information about the effect of AM symbiosis, the treated agrowaste or both, on some biochemical values in the contaminated rhizosphere soil (Caravaca et al., 2004).

The objectives of this study were to determine, in Zn contaminated soil, the effect of *A. niger*-treated sugar beet waste (SBW), with or without rock phosphate as soil amendments. In amended and non-amended soil, the effectiveness of mycorrhizal colonization, using a Zn adapted strain of *Glomus mosseae*, was tested on plant growth and nutrition, metal (Zn) accumulation and symbiotic values. Changes in soil biological properties (as dehydrogenase, β-glucosidase and phosphatase enzymatic activities) were also observed as affected by treatments applied to this artificially contaminated soil (600 $\mu g\ g^{-1}$ of Zn).

2. Materials and methods

2.1. Fermentation process

The strain of *A. niger* NB2 used throughout this study was maintained on potato-dextrose agar slants at 4 °C. It was shown to produce organic acids, mainly citric acid, when growing on complex substrates (Vassilev et al., 1986) and to mineralize lignocellulosic materials (Vassilev et al., 1998). For inoculum

preparation, *A. niger* was grown on a slant at 30 °C for 7 days and spores were scraped in sterile distilled water.

Sugar beet waste supplemented or not with rock phosphate was used as substrate in the fermentation trials. The SBW characteristics were: cellulose [29%], hemicellulose [23%] and lignin [5%].

The solid residue was dried in a 60 °C oven and then ground to pass a 2-mm-pore screen. Portions of 15 g of this solid substrate were placed in 250-ml Erlenmeyer flasks. Czapek-DOX mineral salt solution (0.01 g l^{-1}, $FeSO_4 \cdot 7H_2O$; 0.5 g l^{-1} Mg $SO_4 \cdot 7\ H_2O$; 0.5 g l^{-1} KCl; 3 g l^{-1} $NaNO_3$; 1.0 g l^{-1} $K_2\ HPO_4$; 30.0 g l^{-1} sucrose) was added (40 ml) to each flasks. Rock phosphate (Morocco fluorapatite, 12.8% soluble P, 1 mm mesh) was added when it was needed at a rate of 0.75 g/flask. These culture media were sterilized by autoclaving at 120 °C for 30 min. Three milliliters of spore suspension of *A. niger* (1.2×10^6 spores ml^{-1}) was spread carefully over the surface of the respective media.

The fermentation process was carried out at 30 °C for 20 days.

2.2. Soil–plant experiment

The treatments used in this experiment were as follows: (i) unamended soil: control; (ii) soil amended with *A. niger*-treated SBW; (iii) soil amended with *A. niger*-treated SBW + RP. These three treatments were inoculated or not with a Zn adapted AM fungus (*G. mosseae*). A calcareous loamy soil, sieved (2 mm), diluted with quartz-sand (<1 mm) (3:1, soil:sand, v/v) and sterilized by steaming (100 °C for 1 h for 3 days) was used as test soil. The soil had a pH 7.2 (water), 1.63% organic matter, nutrient concentrations (mg kg^{-1}): N-2.1; P-1.7 ($NaHCO_3$ extractable P); K-80. The soil texture was made up of 57.8% sand, 19% clay and 23.2% silt.

After sterilization, the soil/sand mixture was supplemented with 828 μg g^{-1} of Zn, in order to have an approximately final concentration in soil about 600 μg g^{-1}, which is two-fold the upper limit (300 μg Zn g^{-1} soil) estimated by EU to be toxic for the plants. The artificial contamination was performed by adding adequate amount of an aqueous solution of $ZnSO_4 \cdot 7H_2O$. After 2 weeks of soil incubation (for metal stabilization), the available Zn was determined according to Lakanen and Erviö (1971) methodology.

The fermentation products, prepared as described before, were mixed (5%) with the soil–sand mixture and left for equilibration for 2 weeks at room temperature.

Seeds (eight per pot and after emergence thinned to four seedlings) of *Trifolium repens* were planted in pots of 500 cm^3 capacity (five pots per treatment) and inoculated or not with mycorrhizal inoculum of *G. mosseae* strain belonging to EEZ collection (now is in process of identification by BEG). This *G. mosseae* strain was isolated from a Zn-treated long-term field experiment (10-year-old) at Nagyhörcsök (Hungary) and it resulted to be more Zn tolerant than a *G. mosseae* from collection. *G. mosseae* inoculum consisted of spores, mycelium and mycorrhizal root fragments (50% of fractional colonization). AM inoculum (5 g/pot) was applied to each of the corresponding pots at sowing time, just below the clover seeds.

Non-mycorrhizal treatments received the same amount of autoclaved inoculum and a filtrate (2 ml) of AM inoculum for adding the microbial population free of AM propagules.

The rhizobial inoculum consisted of 1 ml per pot of *Rh. trifolii* culture prepared following the standard procedures (Azcón et al., 1991) and contained 10^8 cells ml^{-1}.

T. repens plants were grown for 6 months in a greenhouse under a 16-h day:8-h night cycle, 21/15 °C and 50% relative humidity. Water loss was compensated by watering every day, after weighing pots.

2.3. Analytical methods

Plants were sequentially harvested after 60, 120, 180 and 240 days of sowing. After each harvest shoot fresh and dry (after drying at 70 °C) weight were recorded. At the last (fourth) harvest shoot and root were recorded.

Nodule numbers were estimated by direct observation using a binocular microscope. In shoot tissue collected from each harvest concentrations (mg g^{-1}) of N (micro-Kjeldahl), K and P (Olsen and Dean, 1965) as well as of Zn (μg g^{-1}) were determined [from three different measurements made on a pooled sample containing the five replicate shoot samples per treatment] after wet digestion of the air-dried plant samples with $HNO_3 + H_2O_2$ by inductively coupled plasma atomic emission spectrometry (ICP-AES), as described by Takács et al. (2001).

Specific absorption rate (SAR) is defined as the amount of nutrients or metal absorbed per unit of root biomass (Gray and Schlesinger, 1983). It was calculated as follows:

$$\mathrm{SAR} = \frac{\text{plant nutrient or metal(mg)}}{\text{root mass(g)}}$$

Roots were carefully washed and stained by the classical non-vital Trypan blue (TB) staining (Phillips and Hayman, 1970). Mycorrhizal development was

evaluated by the method of Trouvelot et al. (1986) and expressed as frequency of AM colonization in the sample (*F*%), intensity of AM colonization in the whole root system (*M*%) and relative and absolute arbusculum richness (*A*% and *a*%) referred to the analysed sample or to the calculated whole root system, respectively.

In rhizosphere soil samples enzymatic activities as well as percentage of stable aggregates were determined.

Dehydrogenase activity was determined following Skujins' method (1976), as modified by García et al. (1997). For this, 1 g of soil at 60% of its field capacity was exposed to 0.2 ml of 0.4% 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride (INT) in distilled water for 20 h, at 22 °C in darkness. The iodo-nitrotetrazolium formazan (INTF) formed was extracted with 10 ml of methanol, by shaking vigorously for 1 min and filtering through a Whatman No. 5 filter paper. INTF was measured spectrophotometrically at 490 nm.

Acid phosphatase activity was determined using *p*-nitrophenyl phosphate disodium (PNPP, 0.115 M) as substrate. Two milliliters of 0.5 M sodium acetate buffer adjusted to pH 5.5 using acetic acid (Naseby and Lynch, 1997) and 0.5 ml of substrate were added to 0.5 g of soil and incubated at 37 °C for 90 min. The reaction was stopped by cooling at 2 °C for 15 min. Then, 0.5 ml of 0.5 M $CaCl_2$ and 2 ml of 0.5 M NaOH were added, and the mixture was centrifuged at 4000 rpm for 5 min. The *p*-nitrophenol (PNP) formed was determined in a spectrophotometer at 398 nm (Tabatabai and Bremner, 1969). In controls, the substrate was added before the $CaCl_2$ and NaOH addition.

β-Glucosidase was determined using *p*-nitrophenyl-β-D-glucopyranoside (PNG, 0.05 M; Masciandaro et al., 1994) as substrate. This assay is also based on the release and detection of PNP. Two milliliters of 0.1 M maleate buffer (pH 6.5) and 0.5 ml of substrate were added to 0.5 g of sample and incubated at 37 °C for 90 min. The reaction was stopped with tris–hydroxymethyl aminomethane (THAM) according to Tabatabai (1982). The amount of PNP was determined in a spectrophotometer at 398 nm (Tabatabai and Bremner, 1969).

The percentage of stable aggregates was determined by the method of Lax et al. (1994). A 4-g aliquot of sieved (0.2–4 mm) soil was placed on a small 0.250-mm sieve and wetted by spray. After 15 min, the soil was subjected to an artificial rainfall of 150 ml with an energy of 270 J m^{-2}. The remaining soil on the sieve was put in a previously weighed capsule (*T*), dried at 105 °C and weighed (P_1). Then, the soil was soaked in distilled water and, after 2 h, passed through the same 0.250 mm sieve with the assistance of a small stick to break the remaining aggregates. The residue remaining on the sieve, which comprised of plant debris and sand particles, was dried at 105 °C and weighed (P_2). The percentage of stable aggregates with regard to the total aggregates was calculated by $(P_1 - P_2) \times 100/(4 - P_2 + T)$. The four soil samples of each treatment were analysed in triplicate for percentage of stable aggregates.

3. Results

At each harvest time, there were significant differences in growth response between plants treated with each amendment and control, non-amended plants. After 60 days of AM inoculation, the mycorrhizal effectiveness was only observed on SBW + RP-treated plants. This AM effect was eliminated at the third and fourth harvest meanwhile in control and SBW-treated plants, AM colonization was effective in increasing plant biomass in the fourth harvest (Fig. 1).

Total shoot biomass yielded was increased by AM colonization in amended and non-amended soil. The effect of treated SBW on shoot biomass ranged from 434% (without mycorrhiza) to 549% (with mycorrhiza). In unamended soil, the mycorrhizal effect on shoot biomass was 232% (Fig. 1). Treated SBW + RP increased shoot growth over control by 1006% (in absence of mycorrhiza) and by 1192% (in presence of *G. mosseae*).

Root biomass and nodule number were greater in mycorrhizal plants than in non-mycorrhizal plants growing in non-amended soil, but not in amended soil (Fig. 2a and b). Treated SBW + RP highly increased root development and the number of nodules formed.

Nodulation was zero in non-mycorrhizal plants but the number of nodules formed was stimulated by mycorrhizal colonization in soil without amendment (Fig. 2b). The effects of amendments (treated SBW and, particularly, treated SBW + RP) on nodule formation were evident and the beneficial effect of amendments maximized this symbiotic value to 210 nodule per plant (Fig. 2b).

Regarding N and K content in shoot, very low N and K acquisition by plants growing in soil without amendment was found. AM colonization increased N and K content in non-amended and amended soil. The dual treatments (AM inoculation and SBW + RP) substantially increased both nutrients [by 1672% (N) and by 1832% (K)] compared with plants growing in non-treated soil (Fig. 2c and d).

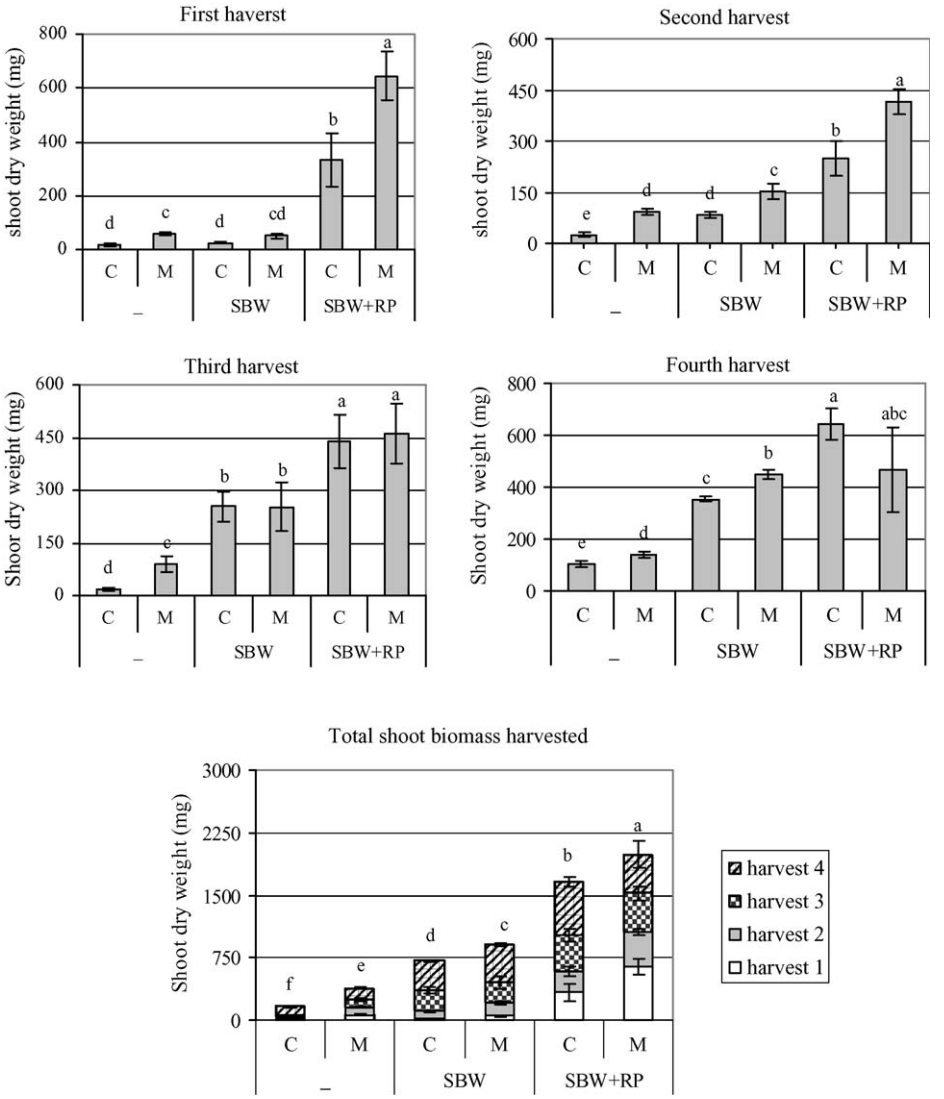

Fig. 1. Effects of amendments formed by *A. niger*-treated sugar beet waste (SBW, 50 g kg^{-1} soil) or SBW plus rock phosphate (RP, 1.5 g kg^{-1} soil) on shoot biomass production of non-mycorrhizal (C) or mycorrhizal (M) plants grown in Zn contaminated (600 μg Zn g^{-1}) soil. Plants were harvested after 60 days (first harvest), 120 days (second harvest), 180 days (third harvest) and 240 days (fourth harvest) of plant growth. Vertical bars represent standard errors.

Phosphorus and Zn concentrations in shoot were differently affected by the amendment and/or AM colonization (Fig. 3a and b). Each treatment and particularly the interactions of both (SBW + RP and AM fungus) increased P concentration in shoot (Fig. 3a) while, in opposite way, the same treatments acted decreasing Zn concentration (Fig. 3b). As a consequence, P/Zn ratio was increased by the amendment application and AM colonization (Fig. 3e). The interaction of these both treatments increased P/Zn ratio by 895% over control plants. Nevertheless, applied treatments also increased the total Zn plant acquisition, particularly in mycorrhizal SBW + RP-treated plants (Fig. 3d).

The percentage of AM colonized root in terms of *F%* (frequency) or *M%* (intensity) decreased with the treated SBW + RP. This effect was also found regarding relative and absolute arbusculum richness [*a%* and *A%*] in colonized root (Fig. 4).

The addition of amendment significantly increased all the enzymatic activities here determined in rhizospheric soil. The greatest increase in response to the application of SBW and AM fungus was observed in phosphatase activity (Fig. 5c) and it was about 1257%

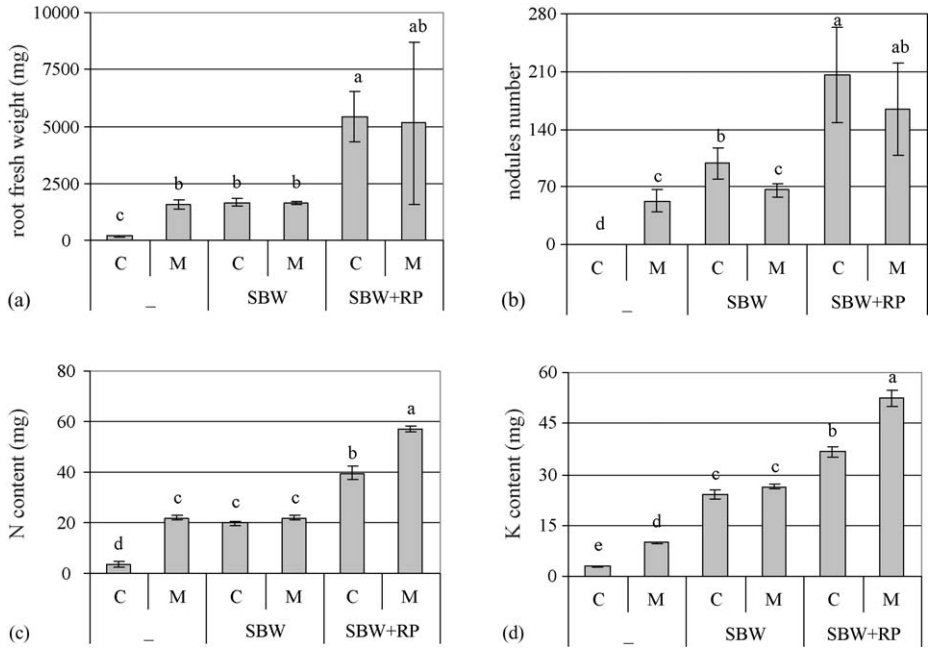

Fig. 2. Effects of amendments formed by *A. niger*-treated sugar beet waste (SBW, 50 g kg^{-1} soil) or SBW plus rock phosphate (RP, 1.5 g kg^{-1} soil) on root biomass (a), nodule number (b), N and K content (c and d, respectively) in shoot tissue of non-mycorrhizal (C) or mycorrhizal (M) plants grown in Zn contaminated (600 μg Zn g^{-1}) soil. Vertical bars represent standard errors.

higher than in non-amended rhizospheric soil. AM colonization increased phosphatase activity only in this SBW treatment while it depressed such activity in SBW + RP-treated rhizosphere (Fig. 5c). Following a similar trend, soil treated with SBW + AM fungus showed the highest dehydrogenase and β-glucosidase activities (Fig. 5a and b). Mycorrhizal inoculation was less effective increasing such enzymatic values on non-treated or on SBW + RP-treated soil (Fig. 5a–c).

Aggregate stability was not affected (SBW + RP) or negatively affected (SBW or unamended soil) by AM colonization (Fig. 5d).

When mycorrhizal plants were grown in amended medium, they showed greater specific N, P and K acquisition than non-mycorrhizal plants. However, specific for Zn absorption rate, there was no significant AM effect. In contrast, in non-amended soil, N, P, K and Zn were decreased by AM colonization. For P and K, the lowest SAR was determined in non-amended AM plants (Table 1).

4. Discussion

Contaminated sites well colonized by AM fungi support the idea that may help to the plant for surviving in these toxic soils and provide some benefit. Autochthonous AM fungal species, as *G. mosseae*, are particularly able to tolerate large metal concentrations being *Glomus* the predominant AM genus detected in the examinated sites (Vivas et al., 2003a,b,c). In this study, combining the addition of amendments (SBW or SBW + RP) and AM inoculation resulted in highest yield of plants growing in Zn contaminated soil. This effect was observed after successive harvests and it was related to a higher N, P and K plant acquisition and a lower Zn accumulation in these treatments.

Therefore, the improvement of shoot and root biomass here observed appeared to be due to a direct nutritional effect and metal sequestration of amendment and AM inoculation. The mycorrhizal effect on SBW + RP-treated plants was evidenced at whatever yield being more relevant at the first yield. In fact, this treatment enhanced P availability in soil increasing shoot P concentration particularly in AM plants and nodule production, that contributed to a greater N_2-fixation. Moreover, dual AM + SBW + RP treatments decreased Zn concentration in plant tissue. Effects of these treatments resulted in an increase of essential nutrients and in a decrease of the contaminant metal. In general, the applied treatments compensated the detrimental effect of Zn contamination on nutrients acquisition as consequence, in part, of decreasing Zn accumulation in plant. In spite of the amendment, SBW + RP had a negative influence on AM symbiosis

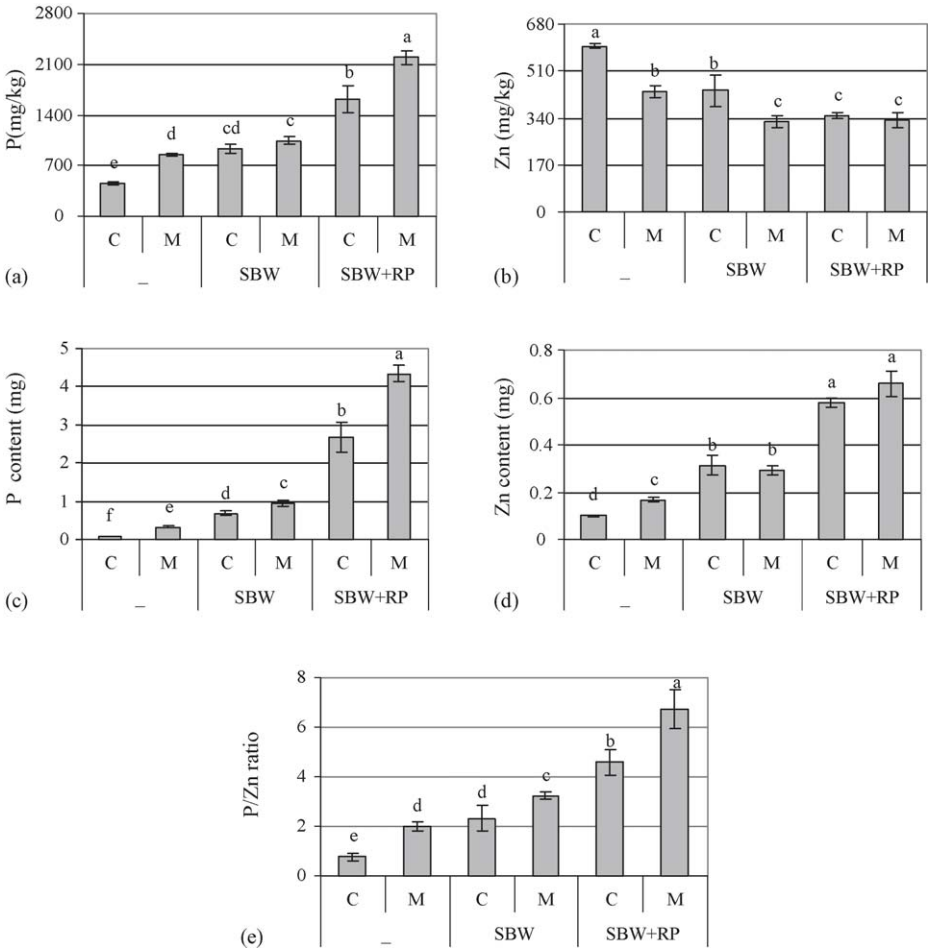

Fig. 3. Effects of amendments formed by *A. niger*-treated sugar beet waste (SBW, 50 g kg^{-1} soil) or SBW plus rock phosphate (RP, 1.5 g kg^{-1} soil) on P and Zn concentration (a and b, respectively); P and Zn content (c and d, respectively) and P/Zn ratio (e) in shoot tissue of non-mycorrhizal (C) or mycorrhizal (M) plants grown in Zn contaminated (600 μg Zn g^{-1}) soil. Vertical bars represent standard errors.

(as percentage), it was the most effective treatment increasing N, P and K uptake and enhancing tolerance to Zn stress in plant. Probably, a positive combination of effects is involved in the plant growth promotion observed by the applied treatments. As well, Zn phytoextraction from this contaminated soil was promoted by the combination of these treatments.

The mycorrhizal colonization in amended soils had an enhancing effect for specific absorption rate of nutrients (N, P and K) and a non-significant effect for the pollutant mineral studied (Zn). This selective and contrasting AM effect for nutrients and pollutant acquisition indicates the benefit of this symbiosis under contaminated conditions. Results suggest an altered root activity when it was AM colonized (Oudeh et al., 2002). In fact, mycorrhizal plants in amended soil have an increased amount of nutrients (N, P and K) absorbed per unit of root mass but a similar or lower amount of Zn was transported to the shoot in AM plants. Root/shoot transport changes have been attributed to the ionic regulation differences (Koide, 1993).

Regarding AM colonization, only 30% (*F*), 5% (*M*) and less than 2% (*A*) were observed in SBW + RP-treated plants. These AM colonization values were lower than those found in the other treatments (C or SBW). The presence of arbuscules within root cells indicates the nutrient benefit to the host since they are the fungal structures involved in bidirectional nutrient exchanges. In this study, arbuscular (*a*% or *A*%) values were lowest in AM plants supplied with SBW + RP which could be related with the highest P concentration in these treated plants. In fact, in SBW, there is no so high P concentration as in SBW + RP-treated plants that negatively affect carbohydrates in root (Schwab et al., 1991). These results are in contrast to that of Medina et al. (2004), who found an increase of AM colonization

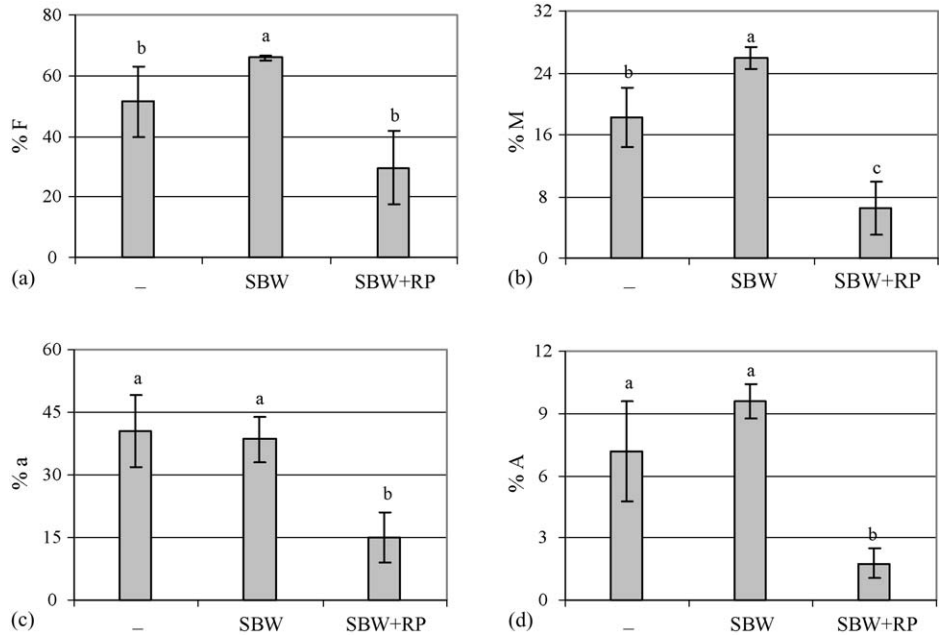

Fig. 4. Effects of amendments formed by *A. niger*-treated sugar beet waste (SBW, 50 g kg^{-1} soil) or SBW plus rock phosphate (RP, 1.5 g kg^{-1} soil) on AM colonization in terms of *F%* (mycorrhizal frequency in the sample), *M%* (intensity in the whole root system), and *a%* and *A%* (relative and absolute arbusculum richness respectively) (a–d, respectively). Plants were grown in Zn contaminated (600 μg Zn g^{-1}) soil. Vertical bars represent standard errors.

in roots of plants growing in *A. niger*-treated SB waste amended soils. In the present experiment, however, the environmental conditions and/or a possible enhanced growth of *A. niger*, could probably lead to a higher soluble level of P than expected, resulting in a decrease of AM colonization. Nevertheless, despite the low values of AM colonization in SBW + RP treatments, these plants reached the highest values of P

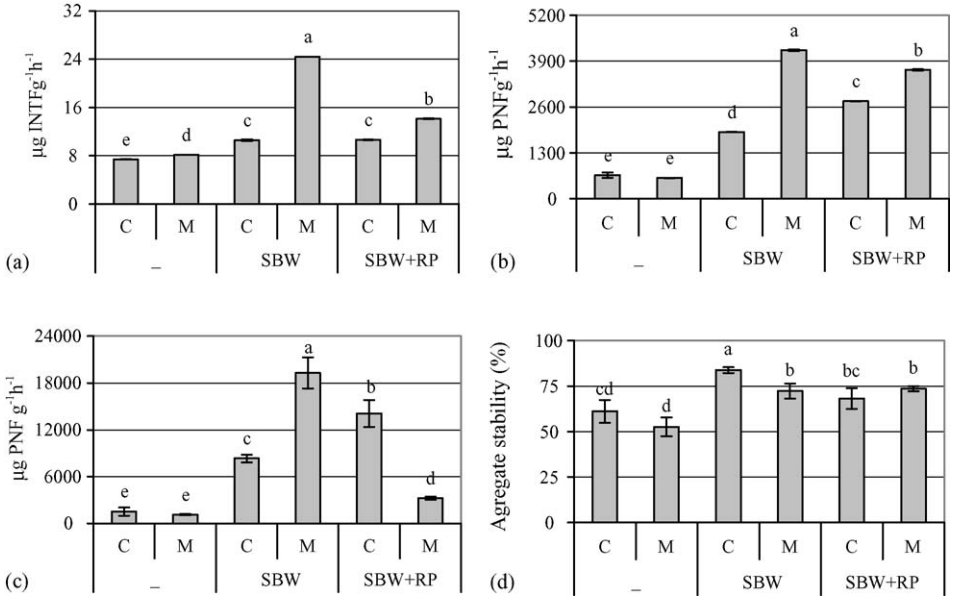

Fig. 5. Effects of amendments formed by *A. niger*-treated sugar beet waste (SBW, 50 g kg^{-1} soil) or SBW plus rock phosphate (RP, 1.5 g kg^{-1} soil) on dehydrogenase, β-glucosidase, phosphatase activities and aggregate stability in rhizosphere soil (Fig. 5a–d, respectively) of non-mycorrhizal (C) or mycorrhizal (M) plants grown in Zn contaminated (600 μg Zn g^{-1}) soil. Vertical bars represent standard errors.

Table 1
Effects of amendments on specific absorption rate (SAR) (mg g^{-1}) of N, P, K and Zn in Zn contaminated (600 μg Zn g^{-1}) soil

SAR	–		SBW		SBW + RP	
	C	M	C	M	C	M
N	19.3 ± 0	14.1 ± 0.00	12.0 ± 0.00	13.5 ± 0.00	7.3 ± 0.61	11.1 ± 0.01
P	0.4 ± 0	0.2 ± 0.01	0.4 ± 0.04	0.6 ± 0.04	0.5 ± 0.07	0.8 ± 0.04
K	16.1 ± 0	6.3 ± 0.24	14.6 ± 1.07	16.2 ± 0.50	6.0 ± 0.34	10.2 ± 0.62
Zn	0.5 ± 0	0.1 ± 0.01	0.2 ± 0.03	0.2 ± 0.02	0.1 ± 0.00	0.1 ± 0.01

Standard errors are given. SBW, sugar beet waste; RP, rock phosphate; SAR, specific absorption rate; C, non-mycorrhizal plant; M, mycorrhizal plant.

concentration in shoot when inoculated with AM fungus. This result indicates a positive interaction between *G. mosseae* and the amendment and consequently a more efficient AM colonization in presence of SB waste supplemented with RP. Muthukumar and Udaiyan (2000) proposed that not only the nutrient concentration but also their ratios in root can influence arbuscule formation. In agreement with that, Sylvia and Neal (1990) indicated that root colonization by AM fungi was unaffected by increasing tissue P when plants were N deficient, but increasing P inhibited AM colonization when plants were N sufficient. Thus, plant P/N ratios could explain the mycorrhizal infective values found according to the applied treatments.

Sainz et al. (1998) reported that organic amendments caused a reduction of AM activity when used in agricultural systems. Nevertheless, studies by others demonstrated that plants growing in organic systems showed higher percentage of AM infection than in conventional fertilized systems (Muthukumar and Udaiyan, 2000). Perhaps, P and organic matter (antagonistic effects) may have been compensated.

Mycorrhizal colonization represents an energetic cost to the host plant in the form of carbon supplied to the mycosimbiont (Douds et al., 2000). This cost could be compensated by the functionality of this symbiosis since AM fungus absorbs photosynthates but nutrients are supplied in return. Soluble C input by amendment, in addition to K that increases in SBW-treated plants and has an important role on root soluble carbohydrates, could explain the positive impact of SBW (without RP) on AM development.

The quality and productivity of soils can be improved by the addition of organic amendments to soil (Roldán et al., 1994). Many studies, both in the field and under controlled conditions, have pointed to the important role of organic matter in the formation and stabilization of soil aggregates. Many authors suggest that it is the polysaccharides which play the most important part in improving soil structure (Bearden and Petersen, 2000). On the other hand, the microbiological origin of the improvement of physical soil properties has been shown as well (Lynch and Bragg, 1985). The microorganisms participate mechanically (union by hyphae) or by the excretion of polysaccharides into the medium (Reinersten et al., 1984). The symbiosis between AM fungi and plants has been shown to increase the stability if soil aggregates (Bearden and Petersen, 2000). Our results show an increase in aggregate stability by the addition of the amendments (SBW or SBW + RP) to the soil, but no effect of AM inoculation was observed. Barea et al. (2005) found that the effect of AM fungi on aggregate stability was recorded 3 years after plants establishment. Since in our experiment plants were harvested after 240 days of sowing, the contribution of *G. mosseae* on soil aggregates was not here evidenced; however, microbial population which developed after the addition of the wastes (SBW or SBW + RP) could be responsible for soil aggregate formation and stabilization, as Roldán et al. (1994) suggested.

Degradation of SBW agrowaste of lignocellulosic composition by biological processes, as *A. niger* treatment, provides an organic amendment rich in polysaccharide compounds and available P (when RP was applied in the fermentation process). This organic matter can be used as C and energy sources for activities of soil microorganisms. This effect was confirmed by the enhancement of the dehydrogenase, β-glucosidase and phosphatase values which are indicative of greater microbial activities (Alef et al., 1995; Ceccanti and García, 1994; García et al., 1997). Enzymatic activities are considered as major factors contributing to overall soil microbial activity, and soil quality (Nannipieri, 1994). The increase of enzymatic activities in soils is involved in an increase in the availability of nutrients to plants, which in turn have a positive influence on soil fertility (García et al., 1997). Values of β-glucosidase activity, that indicates carbohydrates transformation, showed that SBW + AM fungus increased this hydrolytic activity which is important as energy source for rhizospheric microorganisms. As well, dehydrogenase

and phosphatase activities (indexes of microbial activity and phosphorus mineralization, respectively) were maxima when SBW and AM fungus were applied, which is an indication of the effect of each treatment on nutrient cycling and energy flow. Both are biological processes determining nutrient availability for plant growth (Speir, 1977).

In this study, as previously was reported by García et al. (1998), soil hydrolase activities were increased by the application of organic products. Nevertheless, phosphatase activity was highly reduced in the rhizosphere of plants growing in substrate with the highest P content (AM plants in SBW + RP amendment). These results suggest that P-ase activity functioned as an alternative mechanism for increasing P in plant tissues (Azcón and Barea, 1997).

In general, the highest enzymatic activities were related to the stimulating effect of root exudates. But according to these results, the quality of such exudates seems more important than their quantity (regarding root development).

Root exudates have a variety of roles (Marschner, 1995) including that of metal chelators that may reduce the plant uptake of certain metals. The range of compounds exuded is wide and could play a role in plant metal tolerance (Hall, 2002). These compounds are greater in rhizosphere of plants with a more developed root system as in this study occurred with SBW + RP-treated and AM inoculated plants. Thus, the role of such treatments alleviating Zn toxicity in plant, possibly via exudates chelation, may be important but it was not checked. An indication related to this is the fact that the metabolic (enzymatic) activity of particular groups of rhizosphere microorganisms, involved in nutrient cycling, increased with the specific amendments applied to this Zn-contaminated soil (García et al., 2000).

According to Zhu et al. (2001), the level of Zn contamination in the soil has definitive influence on the AM role increasing or decreasing this metal in shoot tissues being determinant for the AM effectiveness. If AM colonization protects the host plant against metal toxicity, the AM colonization and plant-available metal may be correlated (Whitfield et al., 2004). Nevertheless, the effects of AM colonization on host concentration of metals have been shown to vary with the host plant (Díaz et al., 1996; Hildebrandt et al., 1999) and fungal species involved as Kaldorf et al. (1999) reported. All these factors may explain why Whitfield et al. (2004) did not evidence that AM fungi reduced plant uptake of heavy metals but increased Zn uptake, while results from Zhu et al. (2001), in agreement with those here reported, indicate that AM colonization exerted protective effect against plant Zn accumulation in a range from 0 to 400 mg Zn kg^{-1}. These authors, as we observed in this study, reported that mycorrhizal effect cannot be explained simply by tissue dilution. In fact, recently Burleigh et al. (2003) reported that the expression of MtZIP2 gene (a plant Zn transporter) was down-regulated in the roots of mycorrhizal plants and associated with a reduced level of Zn within the host plant tissues.

Concluding, our findings illustrate about the complexity of soil/plant interactions with regards to metal accumulation. The amendments and AM colonization had the expected effect, reducing metal concentration in shoot biomass that allowed an enhancement of plant growth and, consequently, a higher phytoextraction of this metal from contaminated soil. Thus, these treatments can be considered as successful biotechnological tools for the recovery of polluted soils.

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