Application of *Aspergillus niger*-treated agrowaste residue and *Glomus mosseae* for improving growth and nutrition of *Trifolium repens* in a Cd-contaminated soil

A. Medina, N. Vassilev, J.M. Barea, R. Azcón

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

Keywords: Amendments, Cd contamination, *Glomus mosseae*, Rhizobium

1. Introduction

Elevated concentrations of heavy metals in soil from anthropogenic sources (fertilizers, amendments) or mining activities (Shetty et al., 1995) poses long-term risk to environmental and sustainable production.
The deteriorated physical and biological characteristics of contaminated soils need to be improved to establish a vegetation cover. To reach the objectives based on recovering soil quality properties, the application of transformed organic agrowaste residues has interest since it is a meaning of increasing soil organic matter content and improving physical–biological properties (Medina et al., 2004a, b; Caravaca et al., 2004).

The toxicity of metals in soil depends on their bioavailability that is considered as the ability to be transferred from the soil solution to the plants growing on it. Nevertheless, metal bioavailability is not only dependent on metal concentration in the soil; factors such as organic matter and soil microorganisms are involved, as well.

In degraded soil, the activity of microbiota is low because of the lack of suitable organic substrates. The lack of availability of simple sugars normally limits the development of heterotrophic organisms. Nevertheless, this is not a limiting step if agrowaste amendments are incorporated into the unfertile soil. Soil enzymes play an important role in the mineralization of organic products, making fundamental ions available for plant growth (Díaz-Ravina et al., 1992). The microbial activity is of great importance in nutrient cycling and energy flow, and Alexander (1967) determined that microbial metabolism releases 1–4% of the soil organic N in available form for plants during the growth period.

Microbial metal tolerance has been demonstrated and it has interest from an ecological and practical point of view. Mycorrhizal fungi able to colonize polluted soil suggest the metal tolerance of these fungi. But in polluted soils the mycorrhizal component may disappear or, at least, be severely depleted (Bářík, 1989).

There is evidence that mycorrhizal fungi help the plant to thrive under polluted conditions (Shetty et al., 1994a, b). The fungi involved in arbuscular mycorrhizal (AM)-colonization provide a direct link between soil and plant, which increases the supply of nutrients to the plant.

Some AM fungi have the capacity to decrease Cd concentration in plant growing in Cd-polluted soil (Joner et al., 2000b; Lévyal et al., 1997; Vivas et al., 2003a, b). These findings corroborate that soil fungi and bacteria represent important soil components of Cd immobilization (Speir et al., 1999). On the other hand, organic matter addition, as amendment, can improve AM development (Douls et al., 1997; Gaur and Adholeya, 2000) and metal sequestration (Oudeh et al., 2002). Thus, it can be considered as an alternative strategy to prevent Cd toxicity.

The use of sugar beet (SB) agrowaste of lignocellulosic composition as a 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). A. niger grown on SB residue was able to solubilize RP by its ability to excrete organic acids (citric acid mainly). As a result of such fermentative process, RP is solubilized and lignocellulosic (SB) material is transformed in more simple sugars compounds. The application of such product to soil as amendment was effective in improving physical, chemical and microbiological properties of soil (Caravaca et al., 2004). Nevertheless, no information exists on the use of such materials in a bioremediation program.

In this study, we used an artificially Cd-contaminated soil, for assessing the effectiveness of A. niger-treated SB in presence and in absence of RP, in interaction with a Cd-adapted Glomus mosseae strain, in reducing Cd plant toxicity and in promoting plant establishment. Biological characteristics of amended and non-amended Cd-polluted soils were determined in presence or absence of AM-colonization.

2. Materials and methods

2.1. Fermentation process

NR2 strain of A. niger was used in this study. It had previously been selected as producing citric acid on complex substrates (Vassilev et al., 1986).

Sugar beet waste, a lignocellulosic material, was ground in an electrical grinder to 1 mm fragments. It was mixed at a concentration of 10% with 50 ml Czapek’s solution (described in Fluka Chemica, catalogue no. 70185) contained (g l⁻¹ of distilled water): FeSO₄, 0.01; MgSO₄·7H₂O, 0.5; KC1, 0.5; NaNO₃, 3.0; sucrose, 30; K₂HPO₄, 1.0 and a final pH of 7.3 ± 0.2 for static fermentation in 250 ml Erlenmeyer flasks. The latter were inoculated with 3 ml of A. niger spore suspension (1.2 × 10⁶ spores). RP at a concentration of 1.5 g l⁻¹ was added when appropriate. Static fermentation was performed at 28 °C for 20 days.
2.2. Soil–plant experiment

The experiment consisted of a completely randomized factorial block with two factors: (1) mycorrhizal inoculation with autochthonous G. mosseae strains and (2) A. niger-treated SB amendment supplemented or not with RP before fermentation process. Non-SB-treated control was also used. All treatments were replicated five times with a total of 30 pots.

The soil used for the experiment was from the Granada province (Spain), with pH 7.2 (water), 1.6% organic matter, nutrient concentrations: 2.1 mg kg$^{-1}$ N; 1.7 mg kg$^{-1}$ P (NaHCO$_3$-extractable P); 0.8 mg kg$^{-1}$ K. The soil texture was made up of 57.8% sand, 19% clay and 23.2% silt. The soil was air-dried, sieved to less than 2 mm, mixed with quartz-sand (<1 mm) at a ratio of soil:sand of 2:1 (v/v) and sterilized by steaming for three sterilization cycles (100 $^\circ$C for 1 h for 3 days).

After sterilization, the soil was supplemented with Cd by adding adequate amount of an aqueous solution of CdSO$_4$. After 2 weeks of incubation (for metal stabilization), the available amount of Cd was 5 $\mu$g g$^{-1}$ determined using EDTA as extractant (Lakanen and Erviö, 1971).

The amendment was mixed (when necessary) at a rate of 5% with the soil–sand mixture and left for equilibration for 3 weeks at room temperature.

Rock-phosphate (0.75 g per pot) was applied to the pots that had not received the RP-supplemented amendment.

Ten seeds of Trifolium repens were planted in each pot (500 ml capacity) inoculated or not with indigenous AM inoculum of a Cd-adapted G. mosseae strain, isolated from Cd-treated long-term field experiment (10 years old) at Nagyhorcsok (Hungary) (Vörös et al., 1998).

The G. mosseae strain was bulked in a open-pot culture of red clover and consisted of spores, mycelium and mycorrhizal root fragments. Ten grams of inoculum were applied to each one of the corresponding pots in the bottom of a 5-cm-deep hole. The seedlings were thinned to 4 per pot, 2 weeks after emergence.

Rhizobial inoculum consisted in a suspension (1 ml, $10^8$ cfu per pot) of Rhizobium leguminosarum var trifolii prepared following standard procedure.

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

The plants were grown in a greenhouse under a day/night cycle of 16–8 h, 21–15 $^\circ$C and 50% relative humidity. Photosynthetic photon flux density (PPFD) was 500 $\mu$mol m$^{-2}$ s$^{-1}$ as measured with a light meter (LICOR, model LI-188B). Water loss from field capacity was replaced daily by top watering (tap water).

2.3. Determination of growth and symbiotic parameters

After 3 months, plants were harvested and the dry biomass of shoots, and roots, nutrients and metals concentrations in shoots, and symbiotic development (mycorrhizal infection and nodulation) were determined. Shoot concentrations (mg g$^{-1}$) of N (micro-Kjeldahl) K and P, as well as of Cd (mg g$^{-1}$) were determined.

Three different measurements made on a pooled sample containing the five replicate pots per treatment. Analyses were clone after wet digestion of the air-dried plant samples with HNO$_3$ + H$_2$O$_2$ by atomic absorption spectrometer (A.A.S.) model 5000 of Perkin-Elmer, Germany.

Roots were carefully washed and then divided into two batches: one was stained by the classical non-vital trypan blue (TB) staining (Phillips and Hayman, 1970) and the other was used for histochemical vital staining succinate dehydrogenase (SDH) in order to measure total (TB) and living (SDH) AM fungal development.

Succinate dehydrogenase activity was revealed according to the procedure described by Smith and Gianinazzi-Pearson (1990). The roots were immersed in a freshly made solution containing 0.2 M Tris–HCl pH 7.0, 2.5 M sodium-succinate hexahydrate, 4 mg ml$^{-1}$ nitro blue tetrazolium, 5 mM MgCl$_2$. Root fragments were stained overnight at room temperature and then cleared for 15–20 min in a 3% active chlorine solution of sodium hypochlorite.

Mycorrhizal development, after either non-vital or vital staining procedures, was evaluated by the method of Trouvelot et al. (1986) (for more information, see http://www.dijon.inra.fr/bbcceipt/Mychintec/Mycocalc-prg/). An estimate of the length of root colonized by the fungus (the colonization frequency, $F$%) is given as the ratio between colonized root fragments and the total number of root fragments observed. The colonization intensity (% of root) is an estimate of the amount
of root cortex that became mycorrhizal, relative only
to the mycorrhizal root fraction, while $M\%$ is the col-
onization intensity relative to the whole root system.
Arbuscule abundance ($a\%$ and $A\%$) is an estimate of
arbuscule richness in the mycorrhizal root fraction and
in the whole root system, respectively.

Nodulation formation was determined in clean roots by
direct observation using a binocular microscope.

2.4. Soil analyses

In rhizospheric soil samples, enzymatic activities
as well as indole acetic acid (IAA) production 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% INT (2-$p$-iodophenyl-
3-$p$-nitrophenyl-5-phenyltetrazolium chloride) in dis-
tilled water for 20 h, at 22°C in darkness. The iodo-
nitrotetrazolium formazan (INTF) formed was ex-
tracted 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 re-
action 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
(1780 × g) for 5 min. The $p$-nitrophenol (PNP) formed
was determined in a spectrophotometer adjusted to a wavelength of 490 nm.

$\beta$-Glucosidase was determined using $p$-nitrophenyl-
$\beta$-$D$-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 sub-
strate 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 deter-
mined in a spectrophotometer at 398 nm (Tabatabai and
Bremner, 1969).

The indole acetic acid in rhizosphere soil was deter-
mimed by a colorimetric method developed by Mitchell
and Brunstetter (1939) and Gordon and Weber (1950).
For that, 2.0 g of air-dried soil (on an oven-dry ba-
sis) were placed in a 50 ml Erlenmeyer flask and 6 ml
of phosphate buffer (pH 7.5) with glucose (1 g glu-
cose/100 ml phosphate buffer) and 4 ml of $L$-trytophan
(1 g tryptophan/100 ml H$_2$O) were added. These soil
solutions were mixed, stopped and incubated at 37°C
for 24 h in the dark. For the extraction, 2 ml of 5%
trichloroacetic acid solution were added to inactivate
the enzymes involved in the bioassay of auxin, then
1 ml of 0.5 M CaCl$_2$ solution were added. The soil so-
lution was filtered (Whatman No. 2). Three milliliters
of the filtrate were put in a test tube, and to this 2 ml of
salper solution (2 ml 0.5 M FeCl$_3$ and 98 ml 35% per-
chloric acid) were added. This mixture was incubated
for 30 min at 25°C in the dark. Then, the absorbance
of the red solution was measured with a spectropho-
tometer adjusted to a wavelength of 535 nm (Wöhler,
1997).

2.5. Statistics

The results (except concentration and content of ele-
ments on shoot) were statistically evaluated by factorial
analysis of variance with bacterial treatment, mycor-
rhizal treatment and bacterial treatment–mycorrhizal
treatment interaction as sources of variation. Percent-
age values were arcsine-transformed before statistical
analysis.

3. Results

_Tritium_ plants failed to establish when Cd-
contaminated soil was non-amended and lacking of
AM inoculum (Fig. 1).

The application of amendments and AM inocula-
tion significantly increased shoot biomass at whatever
harvest time. SB + RP amendment was the most effec-
tive treatment increasing plant growth in absence of
AM-colonization while AM-colonized plants reached
the highest growth in SB-amended soil (Fig. 1).

In non-amended Cd-polluted soil, plant growth
was seriously limited but AM-colonization greatly
improved plant biomass. In the first harvest SB + RP amendment affected plant growth in a similar extent than AM inoculum (Fig. 1a); but in the second harvest, the amendment (SB + RP) had a higher effectiveness in increasing shoot biomass than G. mosseae colonization without amendment (Fig. 1b). The mycorrhizal colonization in amended soil increased shoot growth by 1089% (SB) and by 192% (SB + RP) over non-treated plants. These results show that not only AM-colonization but also SB amendment were critical for plant growth in Cd-polluted soil. Mycorrhizal responses were different depending on the amendment applied. AM-colonization was particularly effective increasing shoot and root growth in soil amended with SB (Figs. 1 and 2).

The effect of AM fungus on root development was particularly marked in SB-amended soil which resulted the best treatment for enhancing such value; this was
more important on root growth than on nodule production (Fig. 2 a and b). While root growth was strongly increased by amendments or AM-colonization (control or SB-treated plants) the nodule number did not respond to AM inoculum in SB or SB + RP-amended soil (Fig. 2 b). Nodule number was nearly zero in non-mycorrhizal non-amended plants (Fig. 2).

Regarding plant nutrient acquisition, AM-colonization or SB amendments increased shoot P concentration. The highest P concentration was analyzed in AM inoculated plants growing in SB + RP-amended soil (Fig. 3 b). In contrast, the lowest Cd concentration in shoot tissues was observed in mycorrhizal plants growing in SB + RP-amended soil (Fig. 3 a). Mycorrhizal colonization was particularly effective increasing P and decreasing plant Cd concentration in SB + RP-amended soil. In mycorrhizal plants, Cd transferred from soil to plant biomass ranged from 0.09 μg Cd g⁻¹ (SB + RP-amended soil) to 0.6 μg Cd g⁻¹ (non-amended soil). These results are a confirmation of the effective interaction of these treatments (AM inoculation and SB amendments) improving nutrients and decreasing Cd in shoot tissues resulting in an increased plant tolerance to Cd contamination.

There were significant differences in foliar N and K content between mycorrhizal plants growing in non-amended or growing associated with each one of the amendments (Fig. 4 a and b). The highest N or K plant acquisition were observed in AM-colonized plants but the interaction of G. mosseae with SB was more effective in enhancing N and K content than with SB + RP. Nevertheless, SB amendment increased N and K content in a lesser extent than SB + RP (Fig. 4).

With regard to AM-colonization (Table 1), all the inoculated plants, irrespective of amendments, showed a high frequency (%F) of colonization and nearly the totality remained alive (SDH staining). The intensity of mycorrhizal colonization (%M and %m) increased as affected by amendments, but colonization intensity was particularly stimulated by SB. Nevertheless, the highest differences between treatments were found regarding the arbuscule richness (%A and %a). Arbuscule formation was totally inhibited in absence of amendments. But the detrimental effect of Cd on arbuscule formation seems to be compensated by amendments, particularly SB + RP. The arbuscule richness in the mycorrhizal root fraction (%a) estimated after the vital staining (SDH) was highly stimulated in SB + RP-amended soil, while %A was similar for the two amendments.

Amended soil showed higher enzymatic activities in the rhizosphere than non-amended soils (Fig. 5). Some changes on these hydrolytic activities were also induced by AM-colonization. The strongest effect of amendments application was observed on β-glucosidase activity that was very low in rhizosphere of non-amended soil (Fig. 5 b). The highest dehydrogenase and phosphatase activities were tested in mycorrhizal and SB + RP-amended soils (Fig. 5 a).

Amendments increased IAA production in rhizosphere soil more than AM-colonization (Fig. 5 d).

4. Discussion

A relevant effect of amendments was the improvement of the arbuscular colonization, which was not observed in highly colonized plants in absence of amendment. This effect may contribute to a higher performance of AM-colonized plants in Cd-polluted soil.

No previous information of the influence of SB or SB + RP amendments and AM-colonization on plant Cd tolerance has been reported. The effectiveness of AM-colonization, using a Cd-adapted G. mosseae strain, particularly when associated to SB amendment is demonstrated in this study. SB + RP amendment was the most efficient in decreasing Cd concentration in shoot tissue of AM plants, probably due to the fact that P concentration in the medium was the highest when RP was applied together with SB at the fermentation process. The phytotoxicity caused by Cd seems to be alleviated by a minor absorption of Cd and to an indirect effect of increased P nutrition (Sieverding, 1991).

If Cd bioavailability in the experimental soil is regarded as the amount of Cd transferred from the soil solution (5 μg g⁻¹) to the plant growing in such soil, it was reduced by the treatments applied. In SB + RP-amended soil, only 0.09 μg Cd g⁻¹ was transferred from the soil to mycorrhizal plant biomass while in non-amended soil the Cd amount transferred to the shoot of mycorrhizal plants was 0.6 μg Cd g⁻¹. The combination of SB + RP and G. mosseae highly decreased Cd bioavailability. Thus, plant Cd toxicity resulted to be more related with the treatment applied than with the Cd concentration in the soil.
Fig. 3. Effect of amendments (SB or SB + RP) and mycorrhizal inoculation (M) on Cd and P concentration (a and b, respectively) in shoot tissue of *Trifolium* plants growing in Cd (5 μg g⁻¹)-contaminated soil. Vertical bars represent standard errors.

The effect of SB + RP amendment in addition to AM-colonization decreasing Cd transfer from soil to plant can be regarded as an interesting mechanism for alleviating metal toxicity in plants. AM-colonization is able to decrease Cd concentration in plants growing in Cd-polluted soil (Joner et al., 2000) and the amendment itself, apart from the metal sequestration ability, improved AM development and function. The dual application of these treatments resulted an efficient strategy capable of removing Cd from contaminated soil and thus of preventing Cd toxicity in plant.

The absence of nodule in non-mycorrhizal and non-amended plants indicate that inoculated *Rhizobium* was highly sensitive to the high available Cd concentration in soil (Biró et al., 1995) and it was not able to colonize roots in non-treated soil. But this detrimental effect of Cd on nodule formation was compensated by amendments or AM-colonization. The highest nodule production in SB + RP added medium may be due to the greatest P amount in this amendments as result of RP solubilizing ability of *A. niger* on this cellulosic substrate (Rodríguez et al., 1999; Vassilev et al., 1996).

The AM-colonized plants grown in amended soils showed the highest percentages of AM-colonization in terms of frequency (%F). Nevertheless, infective parameter as %m or %M and, in a higher extent, %a or %A showed a high sensitivity to the Cd-polluted conditions used. In unamended soils, intracellular mycelium development (%m or %M) was highly reduced and no arbuscules (%a and %A) were formed. The importance of arbuscules in the exchange of nutrients between the plant and the AM fungus is determinant in the symbiotic relationship. Thus, the important role of SB + RP...
Table 1

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<tr>
<td></td>
<td>%F</td>
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<tr>
<td>No-SB</td>
<td>91 ± 2.90</td>
<td>100 ± 0.00</td>
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<tr>
<td>SB</td>
<td>20 ± 5.34</td>
<td>18 ± 3.39</td>
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<tr>
<td>SB + RP</td>
<td>0 ± 0.00</td>
<td>19 ± 5.07</td>
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<td>% A</td>
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Mycorrhizal values were—% F: percentage of root fragments with fungal colonization; % M: percentage of the fractional colonization extent in the root system; % m: percentage of colonization in the root fraction of 1 cm; % a: percentage of arbuscule abundance in the root fraction of 1 cm, % A: percentage of root cortex with arbuscules. Standard errors of the means are given.

Increasing arbuscule colonization and its vitality in the root fraction (%a).

Surprisingly, the highest symbiotic developments (nodule formation or AM-colonization) on SB + RP-amended soil did not result in the highest N plant content or in the greatest plant biomass. This lack of correspondence must be due to some nutrient disequilibria here not determined.

The inoculation with *G. mosseae* increased nutrients in plants, particularly when associated to the amendments (SB or SB + RP), probably due to several mechanisms. The application of fermented SB (with or without RP) agrowaste also increased soil fertility. Additive and interactive effects of applied treatments (enhancing major nutrients and C sources, symbiotic developments and depressing Cd concentration in

Fig. 5. Dehydrogenase, β-glucosidase, phosphatase activities and indole acetic acid (IAA) production (a–d, respectively) in rhizosphere soil in *Trifolium* plants growing in Cd (5 μg g⁻¹)-contaminated soil. Verticals bars represent standard errors.
shoot tissue) significantly enhanced growth and nutrition of Trifolium plants growing in Cd-polluted soil.

Measurements in microbial activities in the rhizosphere of this contaminated soil provide a direct information about the functioning of the system. β-Glucosidase is a hydrolytic enzyme involved in the C cycle was totally depressed in non-amended soil, without C substrate (Cecconi and Garcia, 1994).

The phosphatase activity was also stimulated by AM inoculation; it is surprising that this activity was greater in SB + RP-amended soil than in the rest of treated soils, since soluble P [highest in this amendment as previously determined by Rodríguez et al. (1999)] leads to a decrease of this activity in soil (Azcón and Barea, 1997). Phosphatases are enzymes with relatively broad specificity and capable of hydrolysing various organo-phosphate esters (Alfè et al., 1995). The highest activity in AM inoculated and SB + RP-amended soil is in concordance with the highest microbial activity as dehydrogenase values show (García et al., 1997). Dehydrogenase and indole acetic acid accumulation in rhizosphere soil is considered a marker of soil activity. The application of SB amendments here used as substrates was able to promote most of the enzyme synthesis (García et al., 2000) and this phytohormone. Mycorrhizal colonization did not affect IAA production but it was effective on root growth of non-treated and SB-treated plants.

We can conclude that the success of revegetation in Cd-contaminated soils is highly dependent on the mycorrhizal inoculation using Cd-adapted AM endophytes as well as on the application of fermented agrowaste residue as SB (with or without RP). The effect of these combined treatments not only increased plant growth but also allowed an enhancement of biological and biochemical values in the rhizosphere soil, improving soil quality.

Acknowledgements

This research was supported by the Plan Nacional CICYT AGL2003-05619-C02-02. We thank A. Strauss-Creda, C. Alessandro, P. Trasar-Cepeda, and C. Masciandaro for providing the sugar beet residue.

We would like to thank “Azucarera de Jaén” (Spain) for supplying the AM fungus inoculum and Pilar Fuentetaja Casado for technical assistance. Almudena Medina is grateful to Junta de Andalucía for the grant. We thank Astrid Vi-


