

EFFECTIVENESS OF THE APPLICATION OF ARBUSCULAR MYCORRHIZA FUNGI AND ORGANIC AMENDMENTS TO IMPROVE SOIL QUALITY AND PLANT PERFORMANCE UNDER STRESS CONDITIONS

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ABSTRACT

Plant growth is limited in arid and/or contaminated sites due to the adverse conditions coming from heavy metal (HM) contamination and/or water stress. Moreover, soils from these areas are generally characterised by poor soil structure, low water-holding capacity, lack of organic matter and nutrient deficiency. In order to carry out a successful re-afforestation, it is necessary to improve soil quality and the ability of plants species to resist this harsh environment. The symbiosis with arbuscular mycorrhizal (AM) fungi has been proposed as one of the mechanisms of plant heavy metal tolerance and water stress avoidance. On the other hand, addition of organic amendments to the soil can reverse degradation of soil properties. Agro-waste residues such as dry olive cake (DOC) and sugar beet waste (SB) supplemented with rock phosphate (RP) can be used as organic amendments after fermentation by *Aspergillus niger*. The application of *A. niger*- treated DOC and/or SB to semi-arid soils and/or HM-contaminated soils increased aggregate stability, soil enzymatic activities, water soluble C and water soluble carbohydrates as well as nutrient availability, especially P. AM inoculation, using adapted endophytes, was more efficient with respect to increasing plant nutrition and growth as well as plant tolerance to drought or HM-stress conditions. The combined treatments involving mycorrhiza fungi inoculation and addition of the amendments into the soil can be proposed as a successful revegetation strategy for plant performance in P-deficient soils under semiarid Mediterranean conditions. The beneficial effectiveness of this symbiosis with suitable AM fungi in *A. niger*-treated agro-waste residue-amended soil can also be regarded as a successful biotechnological tool for reclamation of HM-contaminated soils.

Keywords: Arbuscular mycorrhiza (AM) fungi, organic amendment, heavy metal (HM) contamination, water stress, drought, organic matter, rock phosphate, revegetation, bioremediation, *Aspergillus niger*, phosphate solubilization.

INTRODUCTION

Plant productivity can be limited seriously in heavy metal and/or semi-arid sites. In this respect, microbial inoculants can help plants to cope with adverse conditions and

arbuscular mycorrhizal (AM) fungi have an extraordinary importance since they increase nutrient acquisition by the plant as well as resistance to biotic and abiotic stress (Barea and Jeffries, 1995; Barea *et al.*, 2002a, b, c, d). In fact, the symbiosis with AM fungi has been proposed as one of the mechanisms of heavy metal plant tolerance (Hildebrandt *et al.*, 2007) and water stress avoidance (Augé, 2004; Ruiz-Lozano and Azcón, 1996; Ruíz-Lozano *et al.*, 1995). Nonetheless, the mycorrhizal component may disappear or, at least, be severely depleted in degraded soils, so it may be necessary to reinforce or replace it by appropriate inoculation (Requena *et al.*, 1996).

These arid and/ or contaminated soils are generally characterised by poor soil structure, low water-holding capacity, organic matter lack and nutrient deficiency. Thus, the inoculation of mycorrhizal fungi may not be enough to assure the establishment of plant cover. Therefore, in order to carry out successful reforestation, it is necessary to improve soil quality and the ability of the plant species to resist these harsh environments. In this respect, the application of organic amendments to the soil, prior to the inoculation of AM fungi, has been recommended (Medina *et al.*, 2004a, b). The beneficial effects of organic amendments include provision of plant nutrients, increased humus content and thereby increased water-holding capacity, improved soil structure, and increased microbiology activity (Caravaca *et al.*, 2002).

One attractive approach is the use of agro-wastes as organic amendments. Large amounts of dry olive cake waste (DOC) is produced when oil is extracted from olive fruit. On the other hand, sugar beet waste (SB) is a lignocellulosic residue produced during sugar processing, which is abundant and cheap. However, such material, because of its

lignocellulosic composition, has been shown to have a detrimental effect on plant growth (Vassilev *et al.*, 1986; Vassileva *et al.*, 1998). The complete bio-recycling of these agroindustrial wastes is now accepted as an important element of sustainable agriculture (Vassilev *et al.*, 2006). Such lignocellulosic materials can be used as organic amendments after biotransformation processes, (Vassilev *et al.*, 1997, 2006, 2007).

In addition, phosphorus is a limiting nutrient for plant growth in degraded soils and the possibility of using rock phosphate as a fertilizer has received considerable interest in recent years (Barea *et al.*, 2002d). Rock phosphates (RP) are natural, inexpensive and easily obtainable fertilizers, but their solubility is very low in non-acidic soils.

The use of fermentation processes for RP solubilization by *Aspergillus niger* grown on DOC and SB agro-wastes has been proposed (Vassilev *et al.*, 1998, 2002, 2006). During fermentation, at least three simultaneous activities occur: mineralization of the lignocellulosic substrates, biosynthesis of organic acids and, as a consequence, a solubilisation of RP with a strict correlation between both last processes (Vassilev *et al.*, 1995). The percent of substrate mineralization ranged from 16% in experiments with DOC to 69% in experiments with SB (Vassilev *et al.*, 1998).

A series of microcosm experiments has been performed in greenhouse to evaluate the effectiveness of the resulting fermented products. All amendments improved plant growth and P acquisition, which were further enhanced by mycorrhizal and/or bacterial inoculations (Rodríguez *et al.*, 1999; Vassilev *et al.*, 1998, 2006). Moreover, it has been reported that mycorrhizal plants benefited from P solubilized from RP by *A. niger* (Vassilev *et al.*, 2002) by the use of isotopic ^{32}P dilution technique.

The aim of the present review paper is to summarize the achievements reached by the application of these two organic amendments together with the inoculation of beneficial soil microorganisms, such as arbuscular mycorrhiza fungi in improving soil properties and plant performance in semiarid and/or heavy metal contaminated sites.

USE OF BIOTRANSFORMED AGROWASTES AND AMF FOR RECLAMATION OF DESERTIFIED AREAS

Stressed arid areas may be the result of progressive degradation of vegetation cover (species diversity) and soil quality, both of which involve soil structure, nutrient availability and microbial activity (Barea and Jeffries, 1995).

The establishment of a plant cover based on autochthonous plant species constitutes the most effective strategy for reclaiming degraded lands in semiarid Mediterranean areas. Revegetation programmes based on planting drought tolerant, native shrub species would assist in the conservation of biodiversity and help to prevent the process of erosion and desertification (Caravaca *et al.*, 2003b). The vegetation cover helps avoid soil losses, together with improving soil physical properties. However, in these semiarid areas the low productivity and fertility of the soil and the severe water deficits seriously limit plant growth. To carry out successful re-afforestation, it is necessary to improve soil quality and the ability of the plant species to resist the semiarid environment, (Alguacil *et al.*, 2003b, Caravaca *et al.*, 2005a). The establishment of a mycorrhizal symbiosis can improve the performance of the shrubs. Mycorrhiza represent ecological key factors governing the cycles of major plant nutrients and have significant

influence on plant health and productivity (Jeffries *et al.*, 2003; Requena *et al.*, 2001). On the other hand, the quality and productivity of degraded soils can be improved by organic amendments (Roldán *et al.*, 1996a, b, c).

Influence of the application of organic amendments and AMF on plant growth

The viability of applying microbially treated amendments (DOC or SB) it has been demonstrated in order to improve the growth and (NPK) nutrient status of the different target shrub species, *Dorycnium pentaphyllum* (Medina *et al.*, 2004a, b), *Cistus albidus* L. (Alguacil *et al.*, 2003b), *Juniperus oxycedrus* (Caravaca 2006c) and *Quercus coccifera* (Caravaca *et al.*, 2005b). All these plant species belong to the natural succession in certain plant communities of semiarid Mediterranean ecosystems in the southeast of Spain. They are well adapted to drought conditions and form symbiosis with mycorrhizal fungi. Thus, they represent target species selected for revegetation of the semiarid Mediterranean area. The effectiveness of these amendments relies on the improvement of soil fertility. Mineralization of lignocellulosic agrowastes by microbial processes and simultaneous solubilisation of inorganic insoluble phosphates provides the plants with an organic amendment rich in mineral nutrients, especially available P (when RP is applied in the fermentation process). It has been shown that *D. pentaphyllum* plants grown in *A. niger*-treated SB or DOC amended soils had higher (N, P, K) nutrient contents in their tissues than non-amended control plants (Medina *et al.*, 2004a, b). Caravaca *et al.* (2004a) found a correlation between the increase in N, P, K content in shoots of *D. pentaphyllum* grown in *A. niger*-treated SB amended soil and the increase in total N, available P and extractable K content in

this amended soil. The greatest increase was found in available P, confirming the solubilisation of RP by *A. niger* during the fermentation of the agrowaste. Similar to these results, Alguacil *et al.* (2008a, b) found an improvement in the available nutrient supply for the plant in soil amended with *A. niger*-treated DOC.

The amendment added to the soil can be used as C and energy source for soil microorganisms. This could lead to an enhancement of soil enzyme activities and, as a result of this, to an increase in the availability of nutrients to plants (see section 2.4.2). Alguacil *et al.* (2008a, 2003b) reported an increase in enzyme activities in soil amended with *A. niger*-treated SB waste and *A. niger*-treated DOC, respectively. This increase was associated with a rise in macronutrients (NPK) in this amended soil.

Moreover, these amendments have been shown to be highly efficient in association with arbuscular mycorrhiza. Alguacil *et al.* (2008a), showed a positive interaction between the amendment *A. niger*-treated DOC and *G. mosseae* in terms of plant growth. Similarly, Caravaca *et al.* (2005b, c; 2006c), reported that the combined treatments, involving mycorrhizal inoculation with *G. intraradices* and the addition of fermented DOC increased the growth of *J. oxycedrus* to a higher extent than each treatment applied separately. Medina *et al.* (2004a, b) found that in *D. pentaphyllum* N and P acquisition was higher in mycorrhizal plants grown in *A. niger*-treated SB or DOC amended soil. While organic amendments can improve nutrient supply, inoculation with mycorrhizal fungi can enhance plant nutrient uptake. Furthermore, it has been found that the amendments increased nodule number Medina *et al.* (2004b), percentage of micorrhizal root colonization (Medina *et al.*, 2004a), and hyphal length in soil

(Medina *et al.*, 2007) which can be related to an increase in nutrient uptake and plant growth.

One important factor in revegetation programmes is the improvement in the development of the root system. Roots contribute to the establishment of plants, and consequently, to the protection of the soil against erosion. Medina *et al.* (2004a, b; 2010b) found that *D. pentaphyllum* root biomass was increased by both inoculation with AM fungi and emendation of the soil with the fermented DOC, and the highest values were reached in plants receiving the dual treatment. Moreover, it has been found that shoot and root biomass were higher in mycorrhizal plants grown in *A. niger*-treated SB amended soil, and no statistical significant difference was recorded in these plants grown under water stress or non-stress conditions (unpublished data).

Influence of the application of organic amendments and AMF on plant biochemical parameters related to drought tolerance

Desertified areas are characterized by a severe climate with little and irregular precipitation and frequent drought. Therefore, the main objective in restoration purposes is the improvement of plant drought tolerance. The osmolytes proline and total sugars are considered as indexes of drought avoidance and antioxidative plant defence response (Ruiz-Lozano *et al.*, 1995). Medina *et al.* (2010a) compared the response of P fertilized *Trifolium repens* to that of mycorrhizal *A. niger*-treated DOC amended ones under water stress conditions. Interestingly mycorrhizal plants grown in the amended soil reached the highest proline and sugar contents and were the least-damaged (in terms of plant growth) by drought.

Water stress was less compensated in P-fertilized than in *A. niger*-treated DOC amended plants. The mechanisms underlining these results may be related to the coordination effects achieved by the improved physical characteristics of composted soil and the ability of these fungi to acquire water (Porcel *et al.*, 2006; Ruíz-Lozano and Azcón, 1996).

Nitrate reductase (NR) has been proposed as an index for assaying the effectiveness of AM fungi-host plant combinations for mitigation of water-deficit stress (Caravaca *et al.*, 2003a, 2005c). This enzyme, responsible for nitrate assimilation, is highly sensitive to metabolic and physiological plant status and it is induced by high nitrate supply (Kandlbinder *et al.*, 2000). A synergistic effect between the fermented DOC amendment and AM fungi in increasing NR activity in roots of *J. Oxycedrus* and *D. pentaphyllum* has been reported (Caravaca *et al.*, 2006a, c, Caravaca *et al.*, 2004b). The higher nutrient supply arising from the fermented amendment can explain the increase in NR activity recorder in those experiments. Moreover, nitrate is the preferential N source of AM fungi associated with plants grown in neutral to alkaline soils (Azcón *et al.*, 2001). The reduction process is highly energy-demanding and hence frequently limited by P availability. Some authors have indicated that the increase in NR of mycorrhizal plants with respect to non-mycorrhizal ones can be related to the phosphate requirements of this enzyme (Ruíz-Lozano and Azcón, 1996). In these experiments, the combining effect from the NR activation through nitrate addition via organic amendment with improved P uptake through mycorrhizal fungi enhance the N assimilation of the plant.

Moreover, the benefits of organic amendments are also due to the improvement of the physical characteristics of the soil, which in turn

favours the establishment or viability of a stable plant cover (Alguacil *et al.*, 2008a, b). Soil structure has a prevailing role in soil infiltration and biogeochemical processes. Therefore, improved soil structure means increased water retention, nutrient uptake, drainage, aeration and root growth.

Influence of the application of organic amendments and AMF on symbiotic parameters

Medina *et al.* (2004a, b; 2010b) found that in the degraded soil from semiarid areas, natural rhizobial and arbuscular mycorrhizal populations were very low and inefficient in promoting the growth of legume *D. pentaphyllum*. The inoculation with autochthonous AM fungi was required to get colonized plants. Moreover, the addition of *A.niger*-treated SB and or *A.niger*-treated DOC to the soil enhanced the formation of such symbiotic structures (Medina *et al.*, 2004b).

Similarly, Caravaca *et al.* (2005a), reported no natural ectomycorrhizal colonization in non-inoculated *C.albidus* and *Q. coccifera* one year after being planted in the field.

Caravaca *et al.* (2005a) found the highest levels of *Scleroderma verrucosum* root colonization in inoculated seedlings of *C.albidus* and *Q. coccifera* grown in soil amended with SB waste, rock phosphate and *A. niger* added directly into the planting hole. On the contrary, Alguacil *et al.* (2003b) found no significant difference in *Pisolithus tinctorius* root colonization between *C.albidus* grown in *A.niger*-treated amended or in non amended soil. These contrasting results could be due to the different ectomycorrhizal strains used for the experiments.

D. pentaphyllum is a woody legume very useful for revegetation of semiarid sites because of its ability to develop

symbiotic associations with both rhizobial bacteria and arbuscular mycorrhizal fungi. Medina *et al.* (2004a, b) reported that the percentage of AM root colonization in AM-inoculated *D. pentaphyllum* was increased by the amendments *A. niger*-treated DOC and SB. Moreover, these plants reached the highest nodule formation. The important role of AM fungi in increasing nodule number is a well-documented subject (Barea *et al.*, 2002c). Besides, the addition of the mineralized amendments represent a source of C and nutrients that provide energy sources for the growth and metabolic activity of soil heterotrophic bacteria such as *Rhizobium*.

Organic matter has been shown to increase mycorrhizal fungal growth (Joner and Jakobsen, 1995a, b). Medina *et al.* (2004a, b) reported an increase on the number and biodiversity of AM fungi and spores populations in soils amended with *A. niger*-treated DOC and SB. Moreover, *A. niger*-treated SB has been shown to increase hyphal length density and activity of AM fungi grown in the hyphal compartment (HC) of compartmentalized pots (Medina *et al.*, 2007). Changes in soil microbial community structure induced by the amendments has been suggested to be involved in those findings (Medina *et al.*, 2007). In addition, the chemical composition of the amendments has been shown to have a strong influence on AM hyphal growth (Medina *et al.*, unpublished data; Ravnskov *et al.*, 1999). On the contrary Medina *et al.*, 2007 reported no AM fungal growth in the HC soil amended with the non-fermented SB.

Influence of the application of organic amendments and AMF on soil properties

During the course of the studies with *A. niger*-treated agrowastes, the multiple beneficial effects of the biotechnological

products on a number of soil properties such as soil structure, soil enzyme activity and soil microbial community have been demonstrated.

Physical and physico-chemical soil parameters

As we explained in section 2.1, the effectiveness of fermented agrowaste with respect to stimulation of plant growth could be due to an improvement in the available nutrient supply in soil. During the process of *A. niger* fermentation, the RP solubilizes increasing the level of bioavailable P in the amendment. Thus, the use of the fermented improves potentially limiting soil nutrients, such as N and P. However, the benefits of organic amendments are also due to the improvement of the physical soil characteristics. Soil structure largely determines soil quality and fertility, which, in turn, favours the establishment and viability of a stable plant cover (Caravaca *et al.*, 2002). Structural soil stability of a semiarid soil has been shown to be significantly improved by the addition of *A. niger*-SB waste, on average, by about 79% compared with non-amended soils (Alguacil *et al.*, 2003a, b). Moreover, these authors found a reduction in bulk density of that soil when the amendment was added. The organic materials are less dense than the mineral fraction of soils. In consequence, their application reduces soil bulk density and leads to an increase in soil porosity improving its structural stability (Pagliari *et al.*, 1981).

Aggregate stability is an important criterion of a healthy, managed ecosystem (Miller *et al.*, 1992). The mechanisms involved in aggregate stabilization are based on the enmeshment of soil particles by hyphae and roots, and on the exudation of polysaccharides (Bearden and Petersen, 2000). According to Roldán *et al.* (1994)

the binding effect of roots and hyphae is long-lived, while that of polysaccharides is transient because they are decomposed rapidly by microbes.

Medina *et al.*, 2004b found a significant increase of aggregate stability in the rhizosphere of *D. pentaphyllum* by the addition of *A. niger*-treated SB and DOC. This increase was correlated with an increase in water-soluble C and water-soluble carbohydrates. The water-soluble organic matter fraction consists of a heterogeneous mixture of components of varying molecular weight, such as mono- and polysaccharides, polyphenols, proteins and low molecular weight organic acids. Many authors suggest that the production of polysaccharides is responsible for improvement aggregates stability since they act as cementing agents (Jastrow *et al.*, 1998).

Moreover, this fraction can be used as carbon and energy sources by soil microorganisms, such as bacteria and fungi, which are mainly responsible for the formation of aggregates larger than 0.2 mm (Roldán *et al.*, 1994). In fact, an increase in microbial activity has been reported by the addition of these amendments (Alguacil *et al.*, 2003a, b; Medina *et al.*, 2004a, b). Microorganisms participate mechanically (union by hyphae) or by the excretion of polysaccharides into the medium. On the other hand, *A. niger*-treated SB has been shown to increase root development and stimulate hyphal growth (Medina *et al.*, 2007, 2010b). Both roots and associated hyphae may form a three-dimensional network that enmeshes fine particles of soil into aggregates. In addition, the organic C released by roots promotes a dense microbial community in the immediate environment of the root. Moreover, it is known that glomalin, produced by AM fungi, acts as an insoluble glue to stabilise aggregates (Wright and Anderson, 2000). An

important mechanism affecting available water in soil is aggregate stabilization. This mechanism could explain the enhanced development of *A. niger*-treated DOC amended mycorrhizal plants under drought-stress conditions (Medina *et al.*, 2010b).

Soil biological and biochemical parameters

It is widely accepted that soil enzyme activities are highly sensitive biochemical parameters indicating perturbations caused by soil treatments (Naseby and Lynch, 1997). In the degraded soil belonging to semiarid sites, the activity of microbiota is low because of the lack of suitable organic substrates (Medina *et al.*, 2004b). Nevertheless, the addition to the soil of the organic amendments, *A. niger*-treated SB and/or DOC, significantly increased soil microbial activity as reflected by an increase in dehydrogenase activity (Medina *et al.*, 2004b).

Measurement of soil hydrolases provides an early indication of changes in soil fertility, since they are involved in the mineralization of important nutrient elements such as N, P, and C. Thus, the increase of enzymatic activities in soil is involved in an increase in the nutrient availability to plants, which, in turn, have a positive influence on soil fertility (García *et al.*, 1997). Many researchers have found that soil hydrolase activities are enhanced by the addition of organic materials (García *et al.*, 1999) and can remain active in an extracellular soil environment. Alguacil *et al.* (2003b) found that urease, protease-BAA, acid phosphatase and glucosidase activities were higher in soil amended with the fermented SB residue than in the non-amended soil. Similarly, Medina *et al.* (2004a, b), reported an increase in all these enzymatic activities when soil was amended with *A. niger*-treated DOC. The

increases observed in enzyme activities may be mainly related to reactivation of the rhizosphere microbial population as a consequence of the addition of the fermented product in combination or not with mycorrhiza, which indicates a rehabilitation of the degraded soil (Medina *et al.*, 2004a; Naseby and Lynch, 1997)

BIOTRANSFORMED AGROWASTE AND AM USE FOR REMEDIATION OF HEAVY METALS CONTAMINATED SOILS

Heavy metals (HM) have caused serious environmental problems and may enter the ecosystem through mining, atmosphere deposition and agrochemicals as pesticides, fertilizer and anthropogenic activities (Liu *et al.*, 1997). Soil contamination by heavy metals is an important problem in industrialized areas, and HMs can cause detrimental effects on ecosystems in soil. High concentrations of metals, such as zinc, nickel, mercury, cadmium, or copper, cause environmental pollution because they have a strong persistence.

The use of plants for bioremediation purpose is based on their ability to tolerate HMs.

Nevertheless, plants are more dependent on microbial activity and microorganisms are able to enhance their metabolic activity to combat stress in polluted areas (Killham and Firestone, 1983; Moreno-Ortego *et al.*, 1999). Depending on the severity of the environmental damage, the diversity and activity of the soil biota are increased to a certain threshold value preceding the final function loss.

There is a duality in plant tolerance to pollutants and its response to pollutant stress, but the establishment of plant species is considered as an effective

strategic for reclaiming contaminated areas. In polluted zones, the decreases in the characteristic biodiversity and the degradation of the natural ecosystem have been proved.

In general, vegetation cover decreases the danger of HM dispersal by water and wind erosion and, in this context, the establishment of plant species is recommended for reclamation of HM-contaminated soils (Jeffries *et al.*, 2003).

The application of AM fungus and *A. niger*-treated SB amendment can be regarded as a successful biotechnological tool for decontaminating a polluted soil, for the recovery of polluted soils and as an important strategy 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.

Measurements of this microbial activity in a treated and/or inoculated soil provide information about the effect of AM symbiosis, treated agrowaste or both, on some biochemical values in the contaminated rhizosphere soil (Medina *et al.*, 2006; 2010b)

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.

Phytoremediation, the use of plants in order to remove toxic metals from soil, is emerging as a potential strategy for cost-effective and environmentally friendly remediation of contaminated soils (Glass, 2000).

The deterioration of biological properties of metal contaminated soils is in part due to their progressive decrease in organic matter content (Turnau *et al.*, 2002). Thus, the application of appropriate levels and kinds of organic amendments may be a valid choice to

improve soil characteristics (Martens and Frankenberger, 1992a, b).

For phytoremediation purpose we need a better understanding of the interactions between plant and soil microorganisms, particularly beneficial microbes as arbuscular mycorrhizal (AM) fungi and plant growth promoting rhizobacteria (PGPR) (Vivas *et al.*, 2003a, b, c, d; Whitfield *et al.*, 2004a, b).

Many rhizosphere colonizing bacteria produce typical metabolites, such as siderophores, biosurfactants or organic acids that stimulate plant growth (Glick, 1995) and also may reduce metal availability in the medium. Until now, mycorrhizal fungi and bacteria have been found in HM contaminated soils which are an indication on AM fungal and bacterial tolerance.

HMs exert a negative influence on soil's microorganisms (Biró *et al.*, 2007; Khan, 2005) which are considered as an index to test soil pollution, because HMs have a deleterious influence on cell functioning and consequently on soil microbial community (Azcón *et al.*, 2009b).

Root exudates have a variety of roles (Marschner, 1995) including that of metal chelators that may reduce 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 the rhizosphere of plants with a more developed root system as occurred with *A. niger*-treated SB and AM inoculated plants (Rodríguez *et al.*, 1999). Thus, the role of such treatments alleviating HM 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 contaminated soil.

Influence of the application of organic amendments and AMF on Cu polluted soils

Copper is an essential element controlling diverse biochemical and regulatory events for plant and fungal metabolism, but it can be toxic to plant and AM fungi at high concentration by interfering respiratory processes and protein synthesis. The extraradical mycelium of AM colonized plants may operate binding processes and have an important role in plant tolerance to Cu. The extraradical mycelium may exclude selectively toxic elements by intracellular precipitation y extracellular glycoprotein or chitin-containing cell walls (Zhou, 1999).

Metal tolerant plant species (metalrophytes) can grow on metal polluted soils (Whiting *et al.*, 2002), and microorganisms as AMF resistant to copper would form the dominant populations (Bååth, 1989; Ferrol *et al.*, 2009).

In a Cu contaminated soil with and without treated agro-wastes, the alleviating effect of the inoculation of an autochthonous Cu-adapted arbuscular mycorrhizal inoculum (GA) compared with a strain from collection of *Glomus claroideum* (GC) on a metalrophyte plant was evaluated. In this study, the negative effect of increasing Cu concentrations on mycorrhizal development was only observed in GC-colonized plants but not in those colonized by the autochthonous Cu adapted GA fungi. The main characteristics of GA inoculum may be ascribed to the ability to maintain high colonizing capacity under the highest Cu levels (500 ppm) assayed. Cu translocated from root to shoot under the highest Cu level was highly reduced in GA colonized plants (by 2.7 times) versus control. In addition, GA colonized plants accumulated less Cu (80% in shoot and 29% in roots) than non-AM plants.

Concomitantly, as an index of Cu tolerance, the antioxidant plant activity was measured. The lowest values of glutathione reductase (GR), superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) were found in GA colonized plants. These results (under revision) suggest that to successfully remediate Cu polluted sites it is essential to colonize metallophyte plants with efficient and adapted AM fungi. The *A. niger*-treated agro-waste application resulted to be a very important component for the less Cu tolerant non AM or GC plant to survive under the highest Cu concentration.

There is a distinct efficiency of AM fungi for maintaining metal homeostasis and buffering metal stress in plant itself through evolution of various physiological and molecular mechanisms. In fact, chelation of toxic metals with exudates, intracellular peptides (glutathiones, metallothioneins, HSPs) and other non-proteinaceous compounds (polyphosphate granules), production of antioxidant enzymes to reduced oxidative stress and transport proteins for metal influx and efflux may be the mechanisms involved. They may be recommended as potential biotechnological tools for remediation and reclamation of metal contaminated habitats.

Influence of the application of organic amendments and AMF on Zn polluted soils

The detrimental effect of Zn excess in the ecosystem is due to its long persistence. Soil application of amendments has resulted in a practical method because of its advantages in increasing soil fertility in heavy metal-degraded soil. Metabolizable C compounds from this SB amendment must be applied to the microbes to ensure their growth and activity.

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 using a Zn contaminated soil (Medina *et al.*, 2006). 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 SB + 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 SB) to 1192% (with SB). 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. Nevertheless Whitfield *et al.*, (2004a, b) 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 reported here, 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 it was observed in Medina *et al.* (2006) study, reported that mycorrhizal effect cannot be explained simply by tissue dilution. In fact, Burleigh *et al.* (2002) recently 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 Zn level within the host plant tissues.

González-Guerrero *et al.* (2005) observed increased transcript levels of a putative Zn transporter gene (*GintZnT1*) of the CDF family in the mycelium of *Glomus intraradices* under Zn exposure,

indicating a possible role of this gene product in Zn homeostasis and protection against Zn stress.

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 Zn polluted soils.

Influence of organic amendment application and AMF on Cd polluted soils

Cadmium (Cd) is a heavy metal (HM) dispersed in natural and agricultural environments mainly through human activities such as mining, refining, municipal waste incinerators and fossil fuel combustion sources. In comparison with other HMs, Cd solubility in soils and its toxicity to plants and animals are high (Medina *et al.*, 2005; Seregin and Ivanov, 2001).

Medina *et al.* (2005) reported a positive interaction between the amendment *A. niger*-treated SB and *G. mosseae* for improving *Trifolium repens* growth and nutrition in a Cd contaminated soil.

Studies done by our research group have shown the positive effect of the application of *A. niger*-treated DOC to a desertified Mediterranean soil, in terms of improving both soil characteristics and plant growth (Medina *et al.*, 2004a, b). Medina *et al.* (2010b) also tested the effectiveness of this amendment in interaction with AM fungi for reclamation of Cd-contaminated soils since in non-contaminated soils, the tested effects can be explained by an increase in nutrient availability. For this study in Cd-contaminated soil was used a

compartmentalized growth system, consisting of a root compartment (RC) and two hyphal compartments (HCs). The influence of *Aspergillus niger*-treated DOC on intraradical and extraradical AM fungi development was investigated in this experimental system. In addition, the viability and infectivity of the detached extraradical mycelium in plants, designated as receptor plants, grown in the HC after removal of the RC was studied. Results showed that AM intraradical and extraradical development in the RC was not eliminated by Cd contamination, which suggests a certain level of Cd tolerance as reported in a previous work (Vivas *et al.*, 2006a). Furthermore, the arbuscular richness (a, A) was increased in plants grown in *A. niger*-treated DOC-amended soils. This parameter shows the functioning of the AM symbiosis, as it is considered to be the interactive structural link between the plant and the fungus. Plants growing in *A. niger*-treated DOC-amended soils seem to be more Cd-tolerant than in the absence of *A. niger*-treated DOC. Probably, the *A. niger*-treated DOC effect with respect to increasing the arbuscule content was an important part of the tolerance mechanism.

Both the amendment and the AM fungus increased shoot and root biomass and nodulation in both the non-contaminated and Cd contaminated soils.

The applied treatments also improved nodule formation by an inoculated *Rhizobium*, that was highly depressed in this Cd-contaminated soil.

The absence of nodules in non-mycorrhizal and non-amended plants grown in Cd-contaminated soil indicates that the inoculated *Rhizobium* was highly sensitive to the high concentration of available Cd in the soil (Biró *et al.*, 1995) and that it was not able to colonise roots in non-amended soil. However, the

detrimental Cd effect on nodule formation was compensated by microbially-treated DOC or AM colonisation.

The positive interaction between the microbiologically treated DOC and the AM fungus resulted in the highest plant yield, which can be explained by enhanced nutrient acquisition and arbuscular richness as well as by Cd immobilisation in amended soils.

However, *A. niger*-treated DOC had no effect on the extraradical mycorrhizal mycelium development. Although Cd decreased, AM hyphal length density, symbiotic infectivity was similar in receptor plants grown in non-contaminated and contaminated soil, thus confirming the AM fungal inoculum potential.

Joner and Leyval (1997) demonstrated that hyphae are less sensitive than roots with respect to heavy metal toxicity. Similarly, Janouskova and Vosatka (2005) observed that carrot roots grown in monoxenic cultures were more sensitive to Cd than extraradical *Glomus intraradices* and *Gigaspora margarita* mycelium.

Environmental conditions in Cd contaminated soils (having low levels of nutrients and organic matter and high toxic contaminant levels) are unfavourable for the growth and activity of the indigenous microorganisms. *A. niger*-treated DOC contains high levels of available carbon and nutrients and the microbial fermentation by *A. niger* decreases the phenolic compound toxicity, as Vassilev *et al.* (2006) reported.

Influence of organic amendment application and AMF on multi-contaminated soil: plant and biological soil properties

The exploitation of natural resources causes important ecological problems. In

fact, in polluted zones decrease the typical biodiversity of such area resulting in the degradation of the natural ecosystem. Such unfavourable conditions for plant growth require applying methods for improving nutrient balance, microbial activity and soil quality (García *et al.*, 1999). In polluted multicontaminated areas, plants are more dependent on microbial activity (Moreno-Ortego *et al.*, 1999) since plant establishment and further development is seriously limited in there. (Baker *et al.*, 1995; Puppi *et al.*, 1994)

However, no clear information is available about the effect on soil biological characteristics of the application of treated agrowastes which include alternative carbohydrate sources affecting the environmental structure and function of microbial community compared to conventional PO_4^{3-} fertilization used in parallel.

The microbial inoculants and amendment application favoured plant growth and the phytoextraction process in the multicontaminated soil. The inoculation with AM fungi in *A. niger* treated SB amended soil increased plant growth similarly to PO_4^{3-} ($100 \mu\text{g kg}^{-1}$) addition, and both treatments matched in P acquisition. However, bacterial biodiversity (estimated by denaturing gradient gel electrophoresis of amplified 16S rDNA sequences) was more stimulated by the presence of the AM fungus than by PO_4^{3-} fertilization. The treated SB amendment plus AM inoculation increased the microbial diversity by 233% and also changed (by 215%) the structure of the bacterial community (Azcón *et al.*, 2009a, b).

In general, the highest enzymatic activities were related to the stimulating effect of root exudates. Nevertheless, the quality of such exudates seems more

important than their quantity, according to some results.

Soil quality is related to the rhizosphere microbial groups. but few studies have considered the dual effect of the mycorrhizal fungi colonization and treated SB amendment on the structure of the bacterial communities and diversity (Maliszewska-Kordybach and Smreczak, 2003; Zhang *et al.*, 2006). 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).

Organic matter from the mineralization of *A. niger*-treated SB can be used as C and energy sources for activities of soil microorganisms (Bowen and Rovira, 1999). This beneficial 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).

Values of β -glucosidase activity that indicate carbohydrates transformation, showed that SBW + AM fungus increased this hydrolytic activity which is important as energy source for rhizospheric microorganisms.

In these polluted zones hydrolase activities were increased by the application of organic products. Soil enzymatic activities, which are indicative of biological performance in the rhizosphere, were also improved by applied treatments. We tested that phosphatase activity in the rhizosphere was 1257% higher by the application of *A. niger*-treated SB plus AM fungus than in control non-treated rhizosphere soil.

Following a similar trend, dehydrogenase and β -glucosidase activities reached the highest values in *A. niger*- treated SB + AM treated soils.

An interesting result is that PO_4^{3-} fertilization and single AM inoculation (used in parallel) similarly promoted plant biomass, but only AM inoculation increased microbial diversity in the presence of SB amendment (Azcón *et al.*, 2009b).

A. niger-treated SB amendment may be a suitable tool for increasing and changing the bacterial community in rhizosphere of the multicontaminated soil. Results show that the application of the treated SB agrowaste not only favored plant development, but also the microbial properties of this multicontaminated soil, such as biodiversity and increased dominance index.

CONCLUSION

Results reported here are indicative of the positive effect of the amendments and beneficial microorganisms, such as AM fungi, on the microbial soil status and the relevance of such biotechnological management on bioremediation in single or multi-contaminated soils as well as on revegetation of semi-arid sites. Mechanisms that govern the interaction in the microbial processes in these semi-arid and/or contaminated soils may be considered in future proposals for reclamation strategies of degraded soils. It is interesting to understand the main microbial activities and mechanisms involved in these positive effects.

According to these results, management practices involving organic amendments and microbial (AM) inoculation seem a promising option for re-afforestation of threatened sites, since

they positively affect soil quality and fertility, the formation of symbiotic structures (nodulation and AM colonization) which improve plant nutrition and growth as well as plant tolerance to stress conditions caused by HM and/or drought.

Application of genetic engineering could prove an asset for efficiency increment and better adaptability of fungal symbionts through alterations in molecular pathways leading to high metal tolerance and/or detoxification, besides incorporating the promising genes responsible for overproduction of metal chelating agents for enhanced metal binding at target sites.

Therefore, it is of great importance to inoculate metal hyper-accumulator plants with efficient and effective mycorrhizal fungal strains better adapted to a particular set of conditions and/or host plant to expedite the process of metal remediation and successful restoration of degraded ecosystems.

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