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The interactive effect of an AM fungus and an organic amendment with regard to improving inoculum potential and the growth and nutrition of *Trifolium repens* in Cd-contaminated soils

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ABSTRACT

Aspergillus niger-treated dry olive cake (DryOC) can be used as a soil organic amendment and the aim of this work was to study the effectiveness of this amendment and a Cd-adapted arbuscular mycorrhizal (AM) fungus in improving *Trifolium repens* growth and nutrition in Cd-contaminated soil. In a compartmentalized growth system, consisting of a root compartment (RC) and two hyphal compartments (HCs), we investigated the influence of the amendment on intraradical and extraradical AM fungi development. In addition, we studied the viability and infectivity of the detached extraradical mycelium in plants, designated as receptor plants, grown in the HC after removal of the RC. Both the amendment and the AM fungus increased shoot and root biomass and nodulation in both the non-contaminated and Cd-contaminated soils. The positive interaction between the microbiologically treated DryOC 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 the immobilisation of Cd in amended soils. However, *A. niger*-treated DryOC 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.

The combination of the AM fungus and *A. niger*-treated DryOC increased plant tolerance to Cd in terms of plant growth and nutrition and can be regarded as an important strategy for reclaiming Cd-contaminated soils.

1. Introduction

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

However, as a result of HM contamination, plant productivity is seriously limited in these areas (Puppi et al., 1994). In this respect, microbial inoculations can help plants to cope with the adverse conditions (Barea and Jeffries, 1995; Barea et al., 2002). Arbuscular mycorrhizal (AM) fungi have an extraordinary importance since they increase nutrient acquisition by the plant as well as resistance to biotic and abiotic stresses (Bethlenfalvay and Linderman, 1992). In fact, the symbiosis with AM fungi has been proposed as one of the mechanisms of plant HM-tolerance (Hildebrandt et al., 2007). AM fungi from contaminated soils have been reported to cope better with HM-toxicity than those not exposed to such long-term selection pressure (Weissenhorn et al., 1993). In this study on Cd-contaminated soils, we used a Cd-adapted *Glomus mosseae* strain which has been shown to reduce Cd plant toxicity and promote plant establishment (Vivas et al., 2003b; Medina et al., 2005).

However, HM-polluted soils are generally characterised by poor soil structure, low water-holding capacity, lack of organic matter and nutrient deficiency. Thus, in order to establish the plant cover successfully in a Cd-contaminated soil, physical and biological soil characteristics need to be improved. To reach this objective, the application of microbially treated organic agrowaste has been proposed (Medina et al., 2004a,b, 2005). Large quantities of waste materials are produced during the extraction of olive oil from the olive fruits. These pose serious environmental problems due to their content of phenolics (Pérez et al., 1986; Paredes et al., 1987). Yet, such lignocellulosic material can be used, after biotransformation processes, as an organic amendment. Dry olive cake

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(DryOC), treated with *Aspergillus niger* and supplemented with rock phosphate (RP), has been applied to soil together with AM fungi for revegetation purposes (Medina et al., 2004a,b); nevertheless, no information exists on the use of such materials in a programme for reclamation of contaminated areas.

The main characteristics that an organic material should display for its use as a substrate in soil remediation are to be locally abundant, inexpensive and to have sufficient carbon bioavailability. During the fermentation of DryOC by *A. niger*, RP is solubilised and DryOC is transformed into more-simple compounds. These simple compounds can be used as energy sources by heterotrophic microorganisms, which require them for growth and metabolic activities (Bowen and Rovira, 1999). In this way, soil fertility is improved. Moreover, immobilisation of HMs in soil could be accomplished using amendments, since organic matter forms strong complexes with HMs (Bolan and Duraisamy, 2003; Hartley et al., 2004).

It has been shown that there is a positive interaction between *A. niger*-treated DryOC and AM fungi in terms of plant growth and nutrition, and increased AM intraradical colonisation has been observed (Medina et al., 2004a,b). However, there is no information so far about the influence of *A. niger*-treated DryOC on the AM extraradical mycelium, either in HM-contaminated soils or in non-contaminated soils. The mycelial network has been considered as the main source of AM propagules in a stressed ecosystem (Requena et al., 1996). Changes in the length of intraradical and/or extraradical mycorrhizal hyphae may be induced by HMs, reducing their infective capacity; thus, the study of the influence of amendments on the AM extraradical mycelium, especially in Cd-contaminated soils, is important regarding AM root colonisation and function.

This study consisted of two experiments. The first was designed to study the influence of *A. niger*-treated DryOC on Cd availability in soil. The second was carried out in two successive parts. The aim of the first part was to determine the effectiveness of a Cd-tolerant *G. mosseae* strain in an interaction with *A. niger*-treated DryOC, either in non-contaminated soil or in Cd-contaminated soil. The effectiveness of the inocula and the amendment was evaluated not only in terms of plant growth and nutrition but also in the intraradical and extraradical AM development, as affected by Cd contamination and the amendment application.

In the second part, the viability, infectivity and effectiveness of detached extraradical mycelium from amended or non-amended donor plants, as well as the effect of Cd on the AM inocula, were determined in plants, designated as receptor plants, grown in the HC after the removal of the donor plant.

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 citric acid on complex substrates (Vassilev et al., 1986) and to mineralise lignocellulosic materials (Vassilev et al., 1998). For inoculum preparation, *A. niger* was grown on a slant at 30 °C for 7 d and spores were scraped in sterile distilled water.

Dry olive cake (DryOC) was dried in an oven at 60 °C and then ground to pass a 2-mm pore screen. Portions of 15 g were placed in 250-mL Erlenmeyer flasks. Czapek-Dox mineral salt solution, 40 mL, was added to all flasks. Rock phosphate (Morocco fluorapatite) was added to all treatments at a rate of 0.75 g/50 mL. The media were sterilised by autoclaving at 120 °C for 30 min. A spore suspension of *A. niger* (1.2×10^7) was spread carefully over the surface of the respective media.

The fermentation was performed at 30 °C for 20 d.

2.2. Soil and DryOC characteristics and cadmium applications

The soil used for the experiment was from Granada province (Spain), with pH 7.2 (water), 1.6% organic matter and nutrient concentrations (mg kg^{-1}) of N (total) 2.1, P (Olsen) 1.7 and K (NH_4^+-extractable) 0.8. The soil texture was 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 soil:sand ratio of 2:1 (v/v) and sterilised by steaming for three sterilisation cycles (100 °C for 1 h, on each of 3 d).

The DryOC characteristics were cellulose content—18%, hemicellulose content—16%, lignin content—26%; C_{total} (g kg^{-1} dw)—464, N_{total} (g kg^{-1} dw)—11, and P_{total} (g kg^{-1} dw)—0.6. DryOC was added to the soil at a rate of 5 g solid waste in 100 g of soil.

After sterilisation, the soil was supplemented with Cd by adding an adequate amount of an aqueous solution of $CdSO_4$. After 2 weeks of incubation (for metal stabilisation), the available Cd was 50 μg g^{-1}—determined using EDTA as extractant (Lakanen and Erviö, 1971).

2.3. Experimental design

2.3.1. Experiment 1

Soil contaminated with Cd was incubated or not with *A. niger*-treated DryOC at a rate of 5%, for 30 d. After this, the total and extractable Cd from non-contaminated and contaminated (with and without *A. niger*-treated DryOC) soils were analysed.

2.3.2. Experiment 2

Plants were cultivated in containers of 21 cm length, 5 cm width and 10 cm depth, which had three compartments of similar size: the root compartment (RC), in the centre of the container, and two hyphal compartments (HCs) located at both sides of the RC. The RC was separated from the HCs by a 45-μm nylon mesh, which prevented root penetration but allowed AM hyphae to pass to the HCs. The containers were filled with the experimental soil, either contaminated or not with Cd.

2.3.2.1. Part I. A greenhouse pot experiment was carried out with three main factors in a fully factorial design: (1) organic amendment in terms of *A. niger*-treated dry olive cake (two levels, without and with); (2) AM (two levels, without and with inoculation with the AM fungus *G. mosseae*); and (3) Cd contamination (two levels, without and with), giving a total of eight treatments each with five replicates and thus a total of 40 containers.

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 RC) was applied to the RC that had not received the RP-supplemented amendment.

Ten seeds of *T. repens* were sown in the RC and, after emergence, were thinned to four seedlings per RC.

2.3.2.2. Part II. The second part of the experiment had the same design as part I, and was planned to evaluate the infectivity of mycelia generated by the donor root grown in RC, as affected by Cd contamination and *A. niger*-treated DryOC amendment. This part was carried out after completion of part I. Hence, the only source of inoculum was the detached mycelia (and eventually spores) developed in the HC. Eight seeds of *T. repens* were sown in both HCs when the plants grown in the RC were harvested and, after emergence, they were thinned to four seedlings per HC.

2.4. Microbial inoculation of soil

Mycorrhizal inoculum was only applied in part I of the experiment. It was bulked in an open-pot culture of red clover

and consisted of spores, mycelium and mycorrhizal root fragments, having a colonisation efficiency of 75%. Inoculum (10 g) was applied to each of the corresponding RCs, in the bottom of a 5-cm deep hole. The mycorrhizal strain was isolated from a Cd-treated, long-term field experiment (10 years old) in Nagyhörcsök (Hungary) (Vöros et al., 1998). The non-mycorrhizal treatment received the same amount of autoclaved inoculum together with a 2-mL aliquot of a filtrate (<20 μm) of the AM inoculum in order to provide a general microbial population free of AM propagules.

A suspension of *Rhizobium leguminosarum* bv *trifolii* was added to each pot (1 mL, 10^8 CFU per pot).

2.5. Plant growth conditions

T. repens was grown for 180 d (part I) and 45 d (part II) under greenhouse conditions (18–24 °C, with an 18/6 h light/dark period and 70/80% relative humidity). The photoperiod was provided by fluorescent (24-F96T12VHO/CM) and incandescent (45–40 W) Sylvania and Phillips lamps, respectively. The photosynthetic photon flux (PPF) was ca. 500 μmol m^{-2} s^{-1}. Water was applied daily to maintain accurate humidity in the soil.

2.6. Measurements

2.6.1. Experiment I

The Cd–EDTA was determined by metal extraction using EDTA (0.5 M) at pH 7 (López-Sánchez et al., 2002).

The total Cd was determined by digesting 0.1 g of sample with 3 mL of aqua regia in pressurised PFA digestion vessels in a microwave digester, under a working programme consisting of 5 min at 300 W, 0 min at 250 W and 15 min at 600 W.

2.6.2. Experiment II

2.6.2.1. Part I. Shoots were harvested sequentially 60, 90, 120, 150 and 180 d after sowing. After each harvest, the shoot fresh and dry (after drying at 70 °C) weights were recorded. At the last (sixth) harvest, the shoot and root parts were harvested and analysed further. The concentrations (mg g^{-1}) of N (micro-Kjeldahl), K and P (Olsen and Dean, 1965), as well as Cd (μg g^{-1}), were determined in the shoot (from pooled material of the six harvests per replicate), after 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). Nodule numbers were estimated by direct observation using a binocular microscope.

The roots were washed carefully and stained by classical, non-vital trypan blue (TB) staining (Phillips and Hayman, 1970). Mycorrhizal development was evaluated according to the method of Trouvelot et al. (1986) and expressed as the frequency of AM colonisation ("*F*", root fragments showing fungal colonisation) and the intensity of AM colonisation ["*M*" gives an estimation of the amount of root cortex that has become mycorrhized and refers to the whole root system, while "*m*" refers only to the mycorrhizal root fraction. "*A*" is the arbuscule abundance and gives an estimation of the arbuscule richness in the whole root system, while "*a*" refers to the mycorrhizal root fraction only].

Hyphal length densities were measured by a grid-line-intersect method (Jakobsen et al., 1992).

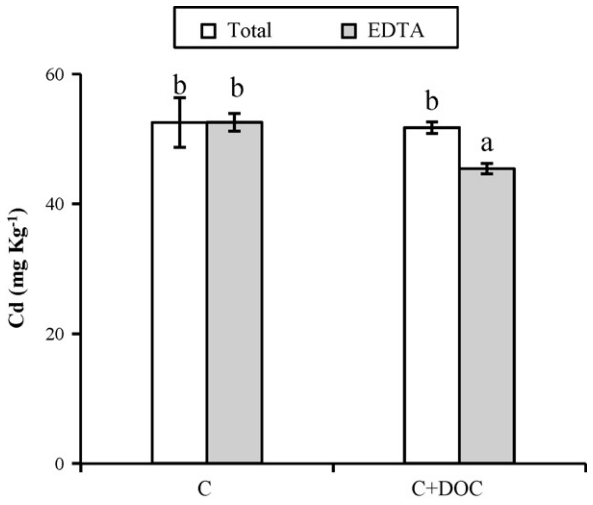

Fig. 1. Influence of the addition or not (control, C) of *A. niger*-treated dry olive cake (DOC) on the total and extractable (EDTA) Cd concentration of an artificially Cd-contaminated soil. Vertical bars represent standard errors. Values of all the treatments not sharing a letter in common differ significantly ($P < 0.05$) from each other as determined by LSD multiple range test.

2.6.2.2. Part II. Plants grown in the HC were harvested 45 d after sowing. The only source of inoculum was the detached mycelia (and eventually spores) developed in the HC. Shoot and root fresh weight, shoot dry weight, nodule number and mycorrhizal development were determined following the methods described above.

2.7. Statistical analysis

The results were evaluated statistically by factorial analysis of variance with AM treatment, *A. niger*-treated dry olive cake treatment, Cd treatment and their interaction as sources of variation. Time was included as a treatment in the analysis of variance of shoot dry weight along the six harvests. Means were compared by the $LSD_{0.05}$ multiple range test. Statgraphics Plus for Windows was used to perform the statistical tests.

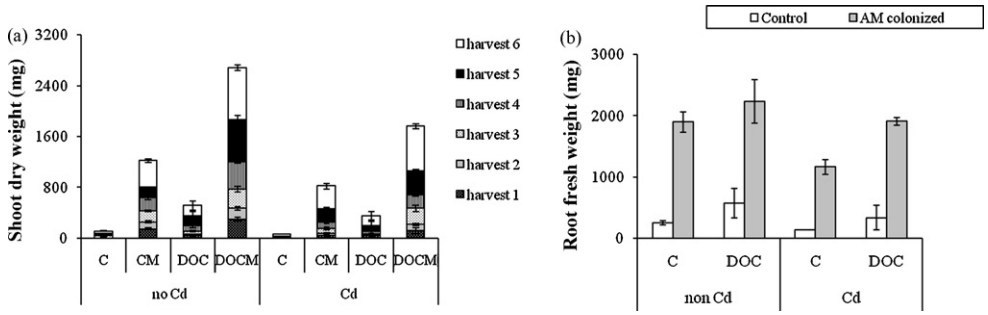

Fig. 2. Effect of *A. niger*-treated dry olive cake (DOC) amendment and AM inoculation on shoot biomass (a) and root biomass (b) after successive harvests of *Trifolium repens* grown in root compartment with non-contaminated and Cd-contaminated (50 g g^{-1}) soil (part I). Vertical bars represent standard errors.

3. Results

3.1. Experiment I

The addition of *A. niger*-treated DryOC to the soil decreased the extractable Cd concentration by 11.76% (Fig. 1).

3.2. Experiment II

3.2.1. Part I

The *T. repens* plants failed to establish when Cd-contaminated soil was not amended by treated agrowaste and was lacking AM inoculum (Fig. 2).

In both the Cd-contaminated and non-contaminated soils, the application of the amendment and AM inoculation significantly increased shoot growth at all harvest times (Fig. 2a, Table 1). The positive interaction between *A. niger*-treated DryOC and the AM fungus resulted in an increasing shoot biomass with time (Table 1, Fig. 2a). The highest shoot biomass (the sum of the six harvests) was reached in mycorrhizal plants grown in *A. niger*-treated DryOC-amended soils, with increases of 2933% (in non-contaminated soils) and 2376% (in contaminated soils) compared to the control treatments (without mycorrhiza and *A. niger*-treated DryOC) (Fig. 2a). Similarly, root development was increased significantly by AM inoculation and *A. niger*-treated DryOC (Table 2, Fig. 2b).

The shoot and root biomass of clover plants were reduced in Cd-contaminated soils; nevertheless, inoculation with the AM fungus and amendment with *A. niger*-treated DryOC diminished this decrease (Fig. 2a and b). In fact, mycorrhizal plants grown in Cd-polluted soils amended with *A. niger*-treated DryOC had similar root biomass to that of plants grown in non-contaminated soils (Fig. 2b). These results show that both AM colonisation and the *A. niger*-treated DryOC amendment were critical for plant growth, especially in Cd-polluted soil.

Table 1
F-values and probabilities of significance based on factorial ANOVA from shoot dry weight along the six harvests, with *A. niger*-treated dry olive cake (*A*), mycorrhiza (*M*), Cd contamination (Cd) and time (*t*) as the main factors and their interactions.

	F-values
M	676.69 ***
A	204.45 ***
Cd	54.08 ***
t	79.85 ***
M × *A*	67.55 ***
M × Cd	25.16 ***
M × *t*	47.07 ***
A × Cd	7.81 **
A × *t*	14.06 ***
Cd × *t*	1.06 NS
M × *A* × Cd	4.58 *
M × *A* × *t*	4.05 **
M × Cd × *t*	0.81 NS
A × Cd × *t*	3.07 *
M × *A* × Cd × *t*	1.93 NS

NS: not significant.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

Table 2
F-values and probabilities of significance based on factorial ANOVA from the measured parameters with *A. niger*-treated dry olive cake (*A*), mycorrhiza (*M*) and Cd contamination (Cd) as the main factors and their interactions.

	F-values						
	M	*A*	Cd	*M* × *A*	*M* × Cd	*A* × Cd	*M* × *A* × Cd
Shoot dry weight	273.14 ***	82.58 ***	21.88 ***	27.16 ***	10.10 **	3.12 NS	1.87 NS
Root fresh weight	131.55 ***	10.57 **	8.24 **	0.74 NS	1.31 NS	0.55 NS	0.69 NS
Nodules number	119.20 ***	16.42 ***	11.57 **	0.05 NS	2.73 NS	3.08 NS	1.00 NS
Shoot K content	245.21 ***	73.32 ***	13.97 ***	28.25 ***	1.44 NS	3.58 NS	0.12 NS
Shoot N content	268.31 ***	68.35 ***	22.41 ***	35.17 ***	8.15 **	6.00 *	2.46 NS
Shoot P content	97.45 ***	96.19 ***	18.99 ***	42.71 ***	2.28 NS	18.67 ***	2.72 NS
F[a]		3.73 NS	5.29 *			0.68 NS	
M[a]		1.69 NS	1.56 NS			0.38 NS	
m[a]		1.96 NS	1.05 NS			0.63 NS	
a[a]		5.59 *	0.00 NS			0.08 NS	
A[a]		2.09 NS	0.09 NS			0.07 NS	
Hyphal length density in RC		0.01 NS	2.00 NS			0.48 NS	
Hyphal length density in HC		30.43 ***	8.70 **			10.94 **	

NS: not significant.
[a] Mycorrhiza values were—*F*: amount of root fragments with fungal colonisation, *M*: fractional colonisation extent in the root system, *m*: length of colonisation in the root fraction of 1 cm, *a*: arbuscule abundance in the root fraction of 1 cm, and *A*: length of root cortex with arbuscules.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

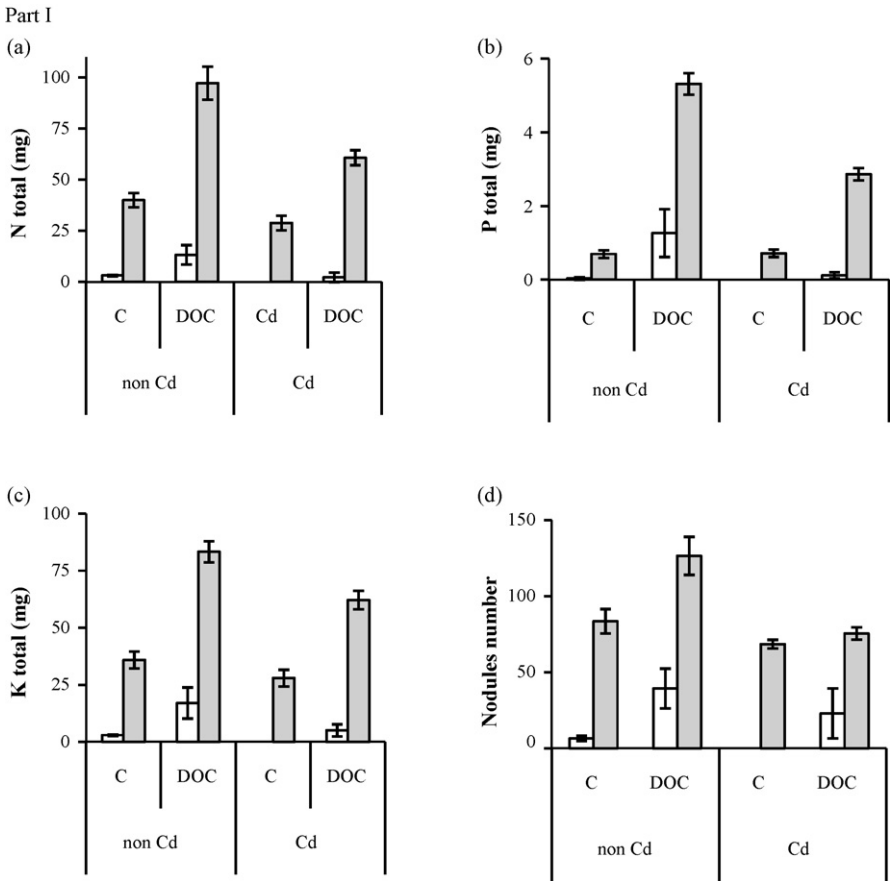

Fig. 3. Effect of *A. niger*-treated dry olive cake (DOC) amendment and AM inoculation on N (a), P (b), K (c) shoot content and nodules number (d) of *Trifolium repens* grown in root compartment with non-contaminated and Cd-contaminated (50 g g^{-1}) soil (part I). Vertical bars represent standard errors.

In Cd-contaminated soils, there was no nodulation in non-amended plants lacking AM inoculum. However, both the mycorrhiza and microbially treated DryOC significantly increased the number of nodules formed (Table 2, Fig. 3d).

Regarding nutrient acquisition, the shoot N, P and K contents displayed trends similar to that described for plant growth. Both AM inoculum and *A. niger*-treated DryOC, particularly when associated, increased the shoot contents of all the nutrients measured, either in contaminated or non-contaminated soils (Fig. 3a–c). Mycorrhizal plants grown in Cd-polluted soils reached shoot nutrient contents similar to those of plants grown in non-polluted soil. The highest N, P and K values were observed in mycorrhizal plants amended with *A. niger*-treated DryOC, in both contaminated and non-contaminated soils (Table 2, Fig. 3a–c).

Non-AM-inoculated plants remained uncolonised. Arbuscular mycorrhization in terms of *F*, *M* and *m* decreased in mycorrhizal plants grown in Cd-polluted soil compared to the non-polluted soil. The addition of the amendment to the soil influenced these values. In mycorrhizal plants grown in Cd-polluted soils, the amount of arbuscules in the mycorrhizal root fractions (*a*) was significantly greater in amended plants than in non-amended plants and similar to that of non-polluted soils (Table 2). In general, Cd in the medium decreased the *F*, *M* and *m* values, while a large increase was observed in *A. niger*-treated DryOC-amended plants (Fig. 4c).

The hyphal length density was similar in all treatments from soil of the root compartment; nevertheless, in the hyphal compartment, both the amendment and Cd significantly decreased extraradical mycelium development (Table 2, Fig. 4a and b).

3.2.2. Part II

In this part, we evaluated the effect of Cd and *A. niger*-treated DryOC on the viability and infectivity of mycelia generated by the AM fungi during the first plant growth period. To do so, we determined the growth and symbiotic parameters of receptor host plants as an estimate of the proportion of active extraradical mycelium.

No roots were detected outside the mesh (HC). Thus, the AM inoculum in the HC was exclusively AM mycelium and spores detached from AM-colonised plants in the RC.

There was no statistically significant difference in shoot dry weight or root fresh weight of the receptor plants in spite of the Cd contamination of the soil. Similarly, *A. niger*-treated DryOC amendment did not influence these values (Table 3, Fig. 5 a and b).

In Cd-contaminated soil, the number of nodules in receptor plants was not significantly different; however, in non-contaminated soil, mycelial inoculum from donor plants grown in *A. niger*-treated DryOC-amended soil increased nodule formation compared with unamended donor plants (Table 3, Fig. 5c).

Colonisation frequency (*F*), absolute intensity and arbuscule abundance in receptor plants were lower when the AM inoculum consisted of mycelia from plants grown in Cd-contaminated soils. The *A. niger*-treated DryOC amendment did not affect significantly most of these morphological parameters related to the colonisation quality (Table 3, Fig. 6).

4. Discussion

Environmental conditions in HM-contaminated soils (having low levels of nutrients and organic matter and high levels of toxic

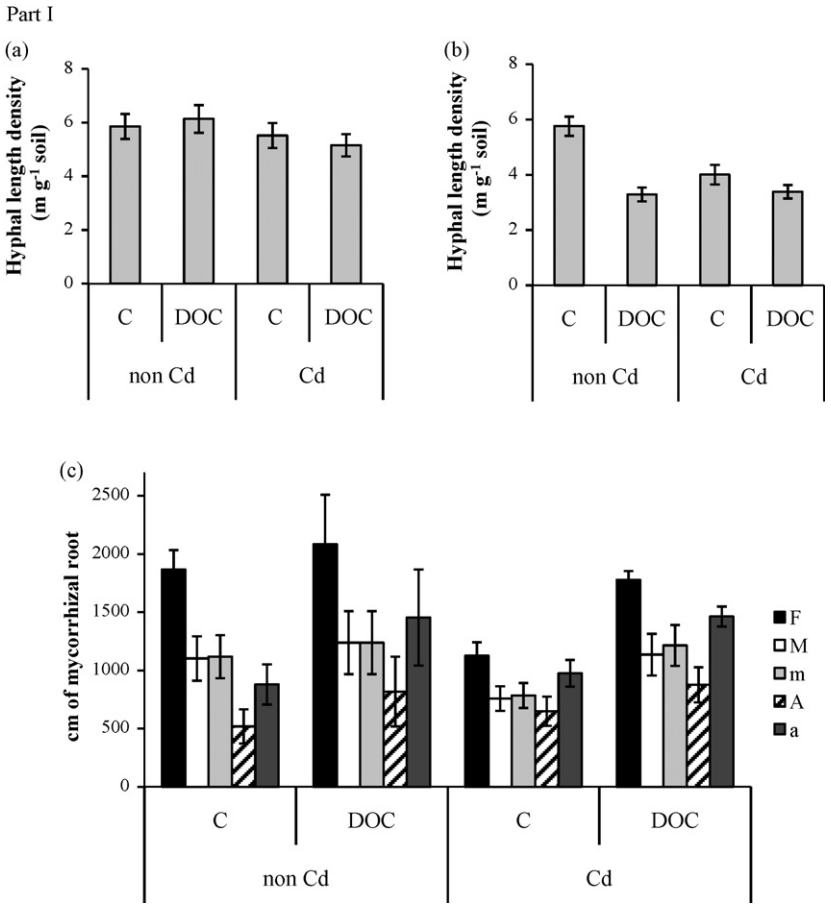

Fig. 4. Effect of *A. niger*-treated dry olive cake (DOC) amendment on AM hyphal length density in RC (a) and HC (b) and on the total arbuscular mycorrhization (c) of *Trifolium repens* grown in non-contaminated and Cd-contaminated (50 g g^{-1}) soil (part I). Vertical bars represent standard errors. Mycorrhizal values were *F*: amount of root fragments with fungal colonisation, *M*: fractional colonisation extent in the root system, *m*: length of colonisation in the root fraction of 1 cm, *a*: arbuscule abundance in the root fraction of 1 cm, and *A*: length of root cortex with arbuscules.

Table 3
F-values and probabilities of significance based on factorial ANOVA from the parameters measured in the receptor plants grown in HC (part II), with *A. niger*-treated dry olive cake (*A*) and Cd contamination (Cd) as the main factors and their interactions.

	F-values		
	A	Cd	*A* × Cd
Shoot dry weight	0.41	3.65	0.79
	NS	NS	NS
Root fresh weight	0.06	5.06	0.06
	NS	*	NS
Nodules number	4.06	15.12	3.34
	NS	**	NS
F	0.09	6.46	0.20
	NS	*	NS
M	0.21	6.62	0.13
	NS	*	NS
m	0.19	5.54	0.06
	NS	*	NS
a	0.05	3.62	0.06
	NS	NS	NS
A	0.00	4.63	0.20
	NS	NS	NS

Mycorrhiza values were—*F*: amount of root fragments with fungal colonisation, *M*: fractional colonisation extent in the root system, *m*: length of colonisation in the root fraction of 1 cm, *a*: arbuscule abundance in the root fraction of 1 cm, and *A*: length of root cortex with arbuscules. NS: not significant.
* $P < 0.05$.
** $P < 0.01$.

contaminant) are unfavourable for the growth and activity of the indigenous microorganisms. *A. niger*-treated DryOC contains high levels of available carbon and nutrients and the microbial fermentation by *A. niger* decreases the toxicity of phenolic compounds, as Vassilev et al. (2006) reported. However, its use as an amendment for environmental reclamation has not been explored so far.

The effectiveness of the *A. niger*-treated DryOC amendment with regard to plant development, particularly when associated with AM fungi, in Cd-contaminated soils is demonstrated in this study. The use of agrowastes as soil amendments is a common practice, widely studied in recent years. They represent an input of organic matter that can improve the structure of the soil and its fertility (Roldán et al., 2006). Previous studies by our research group have shown the positive effect of the application of *A. niger*-treated DryOC to a desertified Mediterranean soil, in terms of improving both soil characteristics and plant growth (Medina et al., 2004a,b). As a following step, we wanted to test the effectiveness of this amendment in an interaction with AM fungi for reclamation of Cd-contaminated soils.

The *A. niger*-treated DryOC increased the shoot and root biomass of plants grown either in Cd-contaminated or non-contaminated soil, compared to non-amended soil. In non-contaminated soils, these effects can be explained by an increase in nutrient availability. In fact, nutrient (N, P, K) content in plants grown in the amended soil was 2-fold (N, K) and 3-fold (P) higher

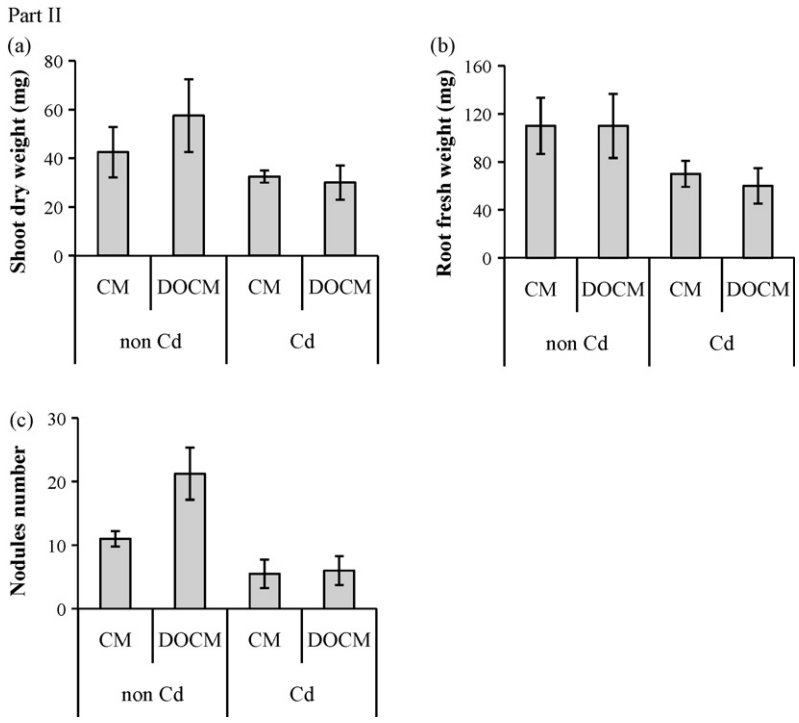

Fig. 5. Shoot (a) and root (b) biomass and nodules number (c) of *Trifolium repens* receptor plants grown in HC grown in non-contaminated and Cd-contaminated (50 g g^{-1}) soil (part II). Vertical bars represent standard errors.

than for those grown in non-amended soil. These results agree with those reported by Tisdall and Oades (1982), who stated that slowly decomposable material like cellulose results in a stable increase, which persists for months, in comparison with labile material like glucose, for which the effect is temporary. This is also in agreement with the studies of Medina et al. (2004a,b), where an increase in plant growth and nutrition was observed in *A. niger*-treated DryOC-amended soils. In Cd-contaminated soils, in addition to these increases in nutrient uptake by the plants, the enhancement of plant growth could be explained also by the immobilisation of Cd by the amendment. In our first experiment, a decrease of Cd availability in soil was achieved when *A. niger*-treated DryOC was added to the soil, confirming the importance of soil organic matter for Cd immobilisation reported by Prokob et al. (2003).

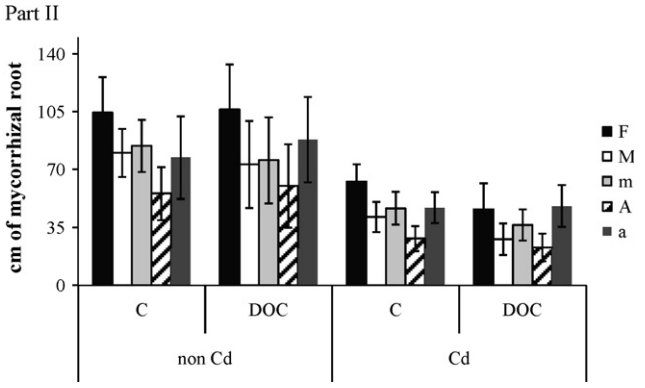

Fig. 6. Total arbuscular mycorrhization of *Trifolium repens* receptor plants grown in HC (part II). Vertical bars represent standard errors. Mycorrhizal values were *F*: amount of root fragments with fungal colonisation, *M*: fractional colonisation extent in the root system, *m*: length of colonisation in the root fraction of 1 cm, *a*: arbuscule abundance in the root fraction of 1 cm, and *A*: length of root cortex with arbuscules.

Previous studies have shown that the Cd-adapted *G. mosseae* strain used as inoculum in our experiment is able to tolerate high metal concentrations (Vivas et al., 2003a, b). Our results show 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 previous work (Vivas et al., 2006). Furthermore, the arbuscular richness (*a*, *A*) was increased in plants grown in *A. niger*-treated DryOC-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 DryOC-amended soil seemed to be more Cd-tolerant than in the absence of *A. niger*-treated DryOC. Probably, the *A. niger*-treated DryOC effect with respect to increasing the arbuscule content was an important part of the tolerance mechanism. For indigenous microorganisms to be effective, they must be capable of resisting chemical pollutants. Plant growth was inhibited by Cd; the inhibition was, however, significantly lower in AM-plants compared to the non-inoculated control plants. Therefore, mycorrhizal plants seemed to be more Cd-tolerant (in terms of growth and nutrition) than non-mycorrhizal plants. Likewise, although the shoot and root biomass of non-amended AM-plants were diminished in Cd-contaminated soils, nutrient uptake remained unaffected. Joner and Leyval (1997) demonstrated that hyphae are less sensitive than roots with respect to heavy metal toxicity. Similarly, Janouškova and Vosátka (2005) observed that carrot roots grown in monoxenic cultures were more sensitive to Cd than extraradical *Glomus intraradices* and *Gigaspora margarita* mycelium. Therefore, hyphal growth and nutrient uptake may be maintained when roots are impaired because of heavy metal toxicity (Joner and Leyval, 2001). AM symbiosis may consequently protect plants against Cd toxicity by improving their nutrient uptake if root functionality is decreased by Cd damage.

The AM hyphal length density decreased in the HC in Cd-contaminated soils compared to the non-contaminated soil. It has been shown that spores and pre-symbiotic hyphae are generally

sensitive to HMs in the absence of plants (Shalaby, 2003). Nevertheless, in spite of the decrease of hyphal length, symbiotic infectivity was similar in receptor plants grown in both contaminated and non-contaminated soils. In the HC, the sole source of AM inoculum was the mycelium and spores developed from the roots grown in the RC. Unfortunately, we did not check spore abundance in the HC. Another possible explanation could be that the estimation of hyphal length density in our study included a fraction of dead or inactive AM biomass which did not correlate well with the infective hyphal biomass.

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 effect of Cd on nodule formation was compensated by microbially treated DryOC or AM colonisation.

The results show that combining the addition of the *A. niger*-treated DryOC amendment and AM inoculation resulted in the highest plant yields, especially on Cd-contaminated soil.

This effect was not transitory since it was observed after successive harvests. These results support the findings of Medina et al. (2004a,b, 2005), where a positive interaction between an organic amendment and AM fungi was observed in *T. repens* growing in heavy metals-contaminated soils.

Organic matter can have beneficial effects on the growth of AM fungi. Giovanetti et al. (2002) suggested that this might be related to increased pore volume in soil, which has a beneficial effect on AM colonisation, the mycorrhizal growth response and AM spore numbers. In our study, we evaluated the influence of *A. niger*-treated DryOC on AM hyphal intraradical and extraradical development. Our results show a positive influence of the amendment on the intraradical parameters measured. This is in accordance with the previous studies of Medina et al. (2004a,b, 2006) that found an increase in M, m and, especially, arbuscule richness (*A*, *a*) in Cd- and Zn-contaminated soils when *A. niger*-treated sugar beet waste was added as an amendment to the soil. Nevertheless, no influence of *A. niger*-treated DryOC on extraradical hyphal length density in soil was observed in our experiment. Several authors have shown enhanced hyphal growth in the presence of organic matter (Labidi et al., 2006; Vaidya et al., 2008), but inhibitory effects of organic matter have been observed as well (Avio and Giovanetti, 1988; Calvet et al., 1992). Ravnskov et al. (1999) concluded that the effects of organic compounds on the growth of AM fungi in soil vary according to the chemical composition of the substrate. Medina et al. (2007) observed that whereas *A. niger*-treated sugar beet waste had a positive effect on AM hyphal growth the untreated sugar beet waste had a negative effect. The authors attributed these results to the mineralisation of the lignocellulosic material during the fermentation by *A. niger*. Lignin and phenolics have been shown to decrease AM hyphal growth (Douds et al., 1996; Nagahashi et al., 1996). The contents of lignin and phenolics in DryOC are higher than in sugar beet waste; therefore, after its mineralisation there are still phenolic compounds in the substrate (Vassilev et al., 2006).

Our results show that the combination of an AM fungus and an organic amendment is a successful biotechnological tool for improving plant growth in Cd-contaminated soils. The AM fungus improved plant nutrition, conferring Cd tolerance to the plants. Also, *A. niger*-treated DryOC seemed to have a direct effect on plant growth by improving plant nutrition and decreasing Cd bioavailability in the soil. Another positive effect may be the recovery of soil properties, as has been reported before (Caravaca et al., 2002; Medina et al., 2004a,b). Tolerance of Cd, as observed in *A. niger*-treated DryOC-amended and AM-colonised plants, may be produced by integrated strategies with nutritional and physicochemical features. These effects resulted in the improvement of the establishment of mycorrhizal plants. Therefore, we conclude that this system can be regarded as an important strategy for reclamation purposes.

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References

Avio, L., Giovanetti, M., 1988. Vesicular–arbuscular mycorrhizal colonization of lucerne roots in a cellulose amended soil. Plant Soil 112, 99–104.
Barea, J.M., Jeffries, P., 1995. Arbuscular mycorrhizas in sustainable soil plant systems. In: Varma, A., Hock, B. (Eds.), Mycorrhiza: Structure, Function, Molecular Biology and Biotechnology. Springer-Verlag, Heidelberg, pp. 521–559.
Barea, J.M., Azcón, R., Azcón-Aguilar, C., 2002. Mycorrhizosphere interactions to improve plant fitness and soil quality. Anton. Van Leeuw. 81, 343–351.
Bethlenfalvay, G.J., Linderman, R.G., 1992. Mycorrhizae and crop productivity. In: Bethlenfalvay, G.J., Linderman, R.G. (Eds.), Mycorrhizae in Sustainable Agriculture. Special Publication No. 54. American Society of Agronomy, Madison, WI, pp. 1–27.
Biró, B., Bayoumi, H.E.A.F., Balázsy, S., Kecskés, M., 1995. Metal sensitivity of some symbiotic N_2-fixing bacteria and *Pseudomonas* strains. Acta Biol. Hung. 46, 9–16.
Bolan, N.S., Duraisamy, V.P., 2003. Role of inorganic and organic soil amendments on immobilisation and phytoavailability of heavy metals: a review involving specific case studies. Aust. J. Soil Res. 41, 533–555.
Bowen, G.D., Rovira, A.D., 1999. The rhizosphere and its management to improve plant growth. Adv. Agron. 66, 1–102.
Calvet, C., Estaun, V., Camprubi, A., 1992. Germination, early mycelial growth and infectivity of vesicular–arbuscular mycorrhizal fungus in organic substrates. Symbiosis 14, 405–411.
Caravaca, F., Barea, J.M., Figueroa, D., Roldán, A., 2002. Assessing the effectiveness of mycorrhizal inoculation and soil compost addition for enhancing reafforestation with *Olea europaea* subsp. *sylvestris* through changes in soil biological and physical parameters. Appl. Soil Ecol. 20, 107–118.
Douds, D.D., Nagahashi, G., Abney, G.D., 1996. The differential effects of cell wall associated phenolics, cell walls, and cytosolic phenolics of host and non-host roots on the growth of two species of AM fungi. New Phytol. 133, 289–294.
Giovanetti, M., Sbrana, C., Avio, L., 2002. Arbuscular mycorrhizal fungal mycelium: from germlings to hyphal networks. In: Gianinazzi, S., Schüepp, H., Barea, J.M., Haselwandter, K. (Eds.), Mycorrhiza Technology in Agriculture: From Genes to Bioproducts. Birkhauser Verlag, Basel, Switzerland, pp. 49–58.
Hartley, W., Eduards, R., Lepp, W.N., 2004. Arsenic and heavy metal mobility in iron oxide-amended contaminated soils as evaluated by short and long term leaching tests. Environ. Pollut. 131, 495–504.
Hildebrandt, U., Regvar, M., Bothe, H., 2007. Arbuscular mycorrhiza and heavy metal tolerance. Phytochemistry 68, 139–146.
Janouškova, M., Vosátka, M., 2005. Response to cadmium of *Daucus carota* hairy roots dual cultures with *Glomus intraradices* or *Gigaspora margarita*. Mycorrhiza 15, 217–224.
Jakobsen, I., Abbott, L.K., Robson, A.D., 1992. External hyphae of vesicular–arbuscular mycorrhizal fungi associated with *Trifolium subterraneaum* L. 1. Spread of hyphae and phosphorus inflow into roots. New Phytol. 120, 371–380.
Jeffries, P., Gianinazzi, S., Perotto, S., Turnau, K., Barea, J.M., 2003. The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. Biol. Fert. Soils 37, 1–16.
Joner, E.J., Leyval, C., 1997. Uptake of 109Cd by roots and hyphae of a *Glomus mosseae*/*Trifolium subterraneum* mycorrhiza from soil amended with high and low concentrations of cadmium. New Phytol. 134, 353–360.
Joner, E.J., Leyval, C., 2001. Time-course of heavy metal uptake in maize and clover as affected by root density and different mycorrhizal inoculation regimes. Biol. Fert. Soils 33, 351–357.
Labidi, S., Nars, H., Zouaghi, M., Wallander, H., 2006. Effects of compost addition on extra-radical growth of arbuscular mycorrhizal fungi in *Acacia tortilis* ssp. *raddiana* savanna in a pre-Saharan area. Appl. Soil Ecol. 35, 184–192.
Lakanen, E., Erviö, R., 1971. A comparison of eight extractants for the determination of plant available micronutrients on soil. Acta Agr. Fenn. 123, 223–232.
López-Sánchez, J.F., Sahuquillo, A., Rauret, G., Lachica, M., Barahona, E., Gómez, A., Ure, A.M., Muntau, H., Quevauviller, Ph., 2002. Extraction procedures for soil analysis. In: Quevauviller, Ph. (Ed.), Methodology in Soil and Sediment Fraction Studies. The Royal Society of Chemistry, Cambridge, UK, pp. 61–63.
Medina, A., Vassilev, N., Alguacil, M.M., Roldán, A., Azcón, R., 2004a. Increased plant growth, nutrient uptake, and soil enzymatic activities in a desertified Mediterranean soil amended with treated residues and inoculated with native mycorrhizal fungi and a plant growth-promoting yeast. Soil Sci. 169, 260–270.

Medina, A., Vassilev, M., Caravaca, F., Roldán, A., Azcón, R., 2004b. Improvement of soil characteristics and growth of *Dorycnium pentaphylum* by amendment with agrowastes and inoculation with AM fungi and/or the yeast *Yarowia lipolytica*. Chemosphere 56, 449–456.

Medina, A., Vassileva, M., Barea, J.M., Azcón, R., 2006. The growth-enhancement of clover by *Aspergillus*-treated sugar beet waste and *Glomus mosseae* inoculation in Zn contaminated soil. Appl. Soil Ecol. 33, 87–98.

Medina, A., Vassilev, N., Barea, J.M., Azcón, R., 2005. Application of *Aspergillus niger*-treated agrowaste residue and *Glomus mosseae* for improving growth and nutrition of *Trifolium repens* in a Cd-contaminated soil. J. Biotechnol. 116, 369–378.

Medina, A., Jakobsen, I., Vassilev, N., Azcón, R., Larsen, J., 2007. Fermentation of sugar beet waste by *Aspergillus niger* facilitates growth and P uptake of external mycelium of mixed populations of arbuscular mycorrhizal fungi. Soil Biol. Biochem. 39, 485–492.

Nagahashi, G., Abney, G.D., Doner, L.W., 1996. A comparative study of phenolics acids associated with cell walls and cytoplasmatic extracts of host and non-host roots for AM fungi. New Phytol. 133, 281–288.

Olsen, S.R., Dean, L.A., 1965. Phosphorus. In: Black, C.A., Evans, D.D., White, J.L., Ensminger, L.E., Clarck, F.E., Dinauer, R.C. (Eds.), Methods of Soil Chemical Analysis. Part 2. American Society of Agronomy, Madison, WI, pp. 1035–1049.

Paredes, M.J., Monteoliva-Sánchez, M., Moreno, E., Pérez, J., Ramos-Cormenzana, A., Martinez, J., 1987. Effect of waste waters from olive oil extraction plants on the bacteria population in soil. Chemosphere 15, 659–664.

Pérez, J.D., Esteban, E., Gómez, M., Gallardo-Lara, F., 1986. Effects of wastewater from olive processing on seed germination and early plant growth of different vegetable species. J. Environ. Sci. Health 4, 349–357.

Phillips, J.M., Hayman, D.S., 1970. Improved procedure of clearing roots and staining parasitic and vesicular–arbuscular mycorrhizal fungi for rapid assessment of infection. Trans. Br. Mycol. Soc. 55, 159–161.

Puppi, G., Azcón, R., Höflich, G., 1994. Management of positive interactions of arbuscular mycorrhizal fungi with essential groups of soil microorganisms. In: Gianinazzi, S., Schüepp, H. (Eds.), Impact of Arbuscular Mycorrhizas on Sustainable Agriculture and Natural Ecosystems. Birkhäuser Verlag, Basel, Switzerland, pp. 201–215.

Prokob, Z., Cupr, P., Zlevorova-Zlamalikova, V., Komarek, J., Dusek, l., Holoubek, I., 2003. Mobility, bioavailability, and toxic effects of cadmium in soil samples. Environ. Res. 91, 119–126.

Ravnskov, S., Larsen, J., Olsson, P.A., Jakobsen, I., 1999. Effects of various compounds on growth and phosphorus uptake of an arbuscular mycorrhizal fungus. New Phytol. 141, 517–524.

Requena, N., Jeffries, P., Barea, J.M., 1996. Assesment of natural mycorrhizal potential in a desertified semi-arid ecosystem. Appl. Environ. Microbiol. 62, 842–847.

Roldán, A., Carrasco, L., Caravaca, F., 2006. Stability of desiccated rhizosphere soil aggregates of mycorrhizal *Juniperus oxycedrus* grown in a desertified soil amended with a composted organic residue. Soil Biol. Biochem. 38, 2722–2730.

Seregin, I.V., Ivanov, V.B., 2001. Physiological aspects of cadmium and lead toxic effects on higher plants. Russ. J. Plant Physiol. 48, 523–544.

Shalaby, M., 2003. Responses of arbuscular mycorrhizal fungal spores isolated from heavy metals polluted and unpolluted soil to Zn, Cd, Pb and their interactions in vitro. Pakistan J. Biol. Sci. 6, 1416–1422.

Takács, T., Biró, B., Vörös, I., 2001. Arbuscular mycorrhizal effect on heavy metal uptake of ryegrass (*Lolium perenne* L.) in pot culture with polluted soil. In: Horst, W.W.J., Scheck, M.K., Bürkert, A., Claassen, N., Flessa, H., Frommer, W.B., Goldback, H.W., Olfs, H.W., Römheld, V., Sattelmacher, B., Schmidhalter, U., Schubert, S., von Wirén, N., Wittenmayer, L. (Eds.), Plant Nutrition: Food Security and Sustainability of Agro-Ecosystems Through Basic and Applied Research (Developments in Plant and Soil Sciences). Kluwer Academic Publishers, The Netherlands, pp. 480–481.

Tisdall, J.M., Oades, J.M., 1982. Organic-matter and water-stable aggregates in soils. J. Soil Sci. 33, 141–163.

Trouvelot, A., Fardeau, J.C., Plenchett, C., Gianinazzi, S., 1986. Nutritional balance and symbiotic expression in mycorrhizal wheat. Physiol. Veg. 24, 300.

Vaidya, G.S., Shrestha, K., Khadge, B.R., Johnson, N.C., Wallander, H., 2008. Organic matter stimulates bacteria and arbuscular mycorrhizal fungi in *Bauhinia purpurea* and *Leucaena diversifolia* plantations on eroded slopes in Nepal. Restor. Ecol. 16, 79–87.

Vassilev, N., Vassileva, M., Ganchev, I., 1986. Citric acid production by *Aspergillus niger* on starch hydrolysate media. Acta Microbiol. Bulg. 18, 62–67.

Vassilev, N., Baca, M.T., Vassileva, M., Franco, I., De Nobili, M., 1998. Mineralization of three agro-industrial wastes by and acid-producing strain of *Aspergillus niger*. In: de Bertoldi, M., Sequi, P., Lenunes, B., Papi, T. (Eds.), The Science of Composting. Chapman and Hall, London, pp. 1375–1379.

Vassilev, N., Medina, A., Azcón, R., Vassileva, M., 2006. Microbial solubilization of rock phosphate on media containing agro-industrial wastes and effect of the resulting products on plant growth and P uptake. Plant Soil 287, 77–84.

Vivas, A., Biró, B., Ruíz-Lozano, J.M., Barea, J.M., Azcón, R., 2006. Two bacterial strains isolated from a Zn-polluted soil enhance plant growth and mycorrhizal efficiency under Zn-toxicity. Chemosphere 62, 1523–1533.

Vivas, A., Vörös, I., Biró, B., Campos, E., Barea, J.M., Azcón, R., 2003a. Symbiotic efficiency of autochtonous arbuscular Mycorrhizal fungus (*G. mosseae*) and *Brevibacillus brevis* isolated from cadmium polluted soil under increasing cadmium levels. Environ. Pollut. 126, 179–189.

Vivas, A., Vörös, I., Biró, B., Barea, J.M., Ruiz-Lozano, J.M., Azcón, R., 2003b. Beneficial effects of indigenous Cd-tolerant and Cd-sensitive *Glomus mosseae* associated with a Cd-adapted strain of *Brevibacillus brevis* in improving plant tolerance to Cd contamination. Appl. Soil Ecol. 24, 177–186.

Vöros, I., Biró, B., Takács, T., Köves-Péchy, K., Bujtás, K., 1998. Effect of arbuscular mycorrhizal fungi on heavy metal toxicity to *Trifolium pratense* in soils contaminated with Cd, Zn and Ni salts. Agrokém. Talajtan 47, 227–288.

Weissenhorn, I., Leyval, C., Berthelin, J., 1993. Cd-tolerant arbuscular mycorrhizal (AM) fungi from heavy-metal polluted soils. Plant Soil 157, 247–256.