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Impact of DOM from composted "alperujo" on soil structure, AM fungi, microbial activity and growth of *Medicago sativa*

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Abstract

Water-soluble extracts from compost may represent an alternative nutrient and organic matter source for crop production under drip irrigation. Dissolved organic matter (DOM), extracted from composted "alperujo", the main by-product from the Spanish olive oil industry, was applied to soil alone or in combination with either *Glomus intraradices* Schenck and Smith or a mixture of *G. intraradices*, *Glomus deserticola* (Trappe, Bloss. and Menge) and *Glomus mosseae* (Nicol and Gerd.) Gerd. and Trappe. Response measurements included mycorrhizal colonisation, nutrient uptake and growth of *Medicago sativa* and microbiological and physical properties in the rhizosphere. Dissolved organic matter was added to soil at concentrations of 0, 50, 100 or 300 mg C kg^{-1} substrate. During the four months of the experiment, the plants were harvested three times. Both mycorrhizal inoculation treatments significantly increased soil aggregate stability. Only the mycorrhizal inoculations increased microbial biomass C and protease and phosphatase activities and decreased water-soluble C, particularly the mixture of arbuscular mycorrhizal fungi. At the third harvest, the greatest increase in growth of *M. sativa* was observed in the inoculated plants with shoot biomass being 38% greater than for plants grown in the soil amended with the highest dose of DOM and 57% greater than for control plants. The addition of DOM was not sufficient to restore soil structure and microbial activity and did not affect the mycorrhizal development of introduced populations of arbuscular mycorrhizal fungi, but, depending on the dose, its fertiliser efficiency for improving plant growth was apparent.

1. Introduction

Biological and physical soil degradation cause a progressive decline in agricultural production. The natural roles of rhizosphere microorganisms have been marginalised due to high inputs of inorganic fertilisers, herbicides and pesticides (Mäder et al., 2002). Such farming practices may affect both arbuscular mycorrhizal (AM) fungal diversity and their effectiveness in the ecosystem containing these endophytes (Jeffries and Barea, 2001). Arbuscular mycorrhizal fungi form mutual associations with more than 80% of plant species, including agronomic plants (Smith and Read, 1997). They are important determinants of plant health and soil fertility because they are known to enhance mineral uptake (particularly P) (Smith and Read, 1997), tolerance to water-stress (Ruíz-Lozano, 2003) and soil aggregation (Caravaca et al., 2002). The identification of efficient AM fungi is a prerequisite for carrying out mycorrhizal technologies, since nutrient uptake and metabolism in mycorrhizal plants depend on the mycorrhizal endophyte (Azcón and Tobar, 1998). Likewise, the host plant response to different AM fungi is highly dependent on environmental conditions (Johnson et al., 1997).

Inadequate management practices have led to a decrease in the levels of organic matter of agricultural soils of Mediterranean regions, causing a loss of their fertility and quality. The addition to soil of liquid organic materials, and especially of organic substances extractable with alkaline solutions, has been proposed as an alternative nutrient and organic matter source for crop production under drip irrigation (Ayuso et al., 1996). Dissolved organic matter

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(DOM) addition may have a positive effect on plant growth, directly and/or through the improvement in soil properties (Chen and Aviad, 1990). The so-called "Alperujo" (AL), a very wet, solid by-product from the extraction of olive oil is a good source of DOM because of its high C and mineral nutrients content (Alburquerque et al., 2004). AL, however, also contains organic acids, phenolic compounds and fats that may have a negative effect on microbial activity, inhibit the establishment of AM symbioses, immobilise available N (Saviozzi et al., 1991) and decrease plant growth (Martín et al., 2002; Linares et al., 2003). Composting with forced aeration and the addition of a bulking agent has been shown to be an effective process with regard to reducing the phytoxicity of AL (Alburquerque et al., 2006). Little is known, however, of the impact of the DOM extracted from properly composted AL on soil native microbial biomass and activity, soil physical properties or the performance of AM-inoculated plants.

The objectives of this study were: (1) to compare the effectiveness of inoculation with an AM fungus or with a mixture of three AM fungi in increasing mycorrhizal colonisation, plant growth and nutrient uptake in *Medicago sativa* and (2) to evaluate the potential application of DOM, obtained by alkaline extraction of composted AL, in improving the establishment of introduced populations of AM fungi, plant performance and physical and chemical properties reflecting soil quality and functioning. The results obtained will determine the usefulness of DOM from AL as a soil amendment and liquid fertiliser for agricultural purposes.

2. Materials and methods

2.1. Materials

An agricultural soil was collected near Murcia (SE Spain). The climate is semi-arid Mediterranean with an average annual rainfall of 300 mm and a mean annual temperature of 19.2 °C. The potential evapotranspiration reaches 1000 mm y^{-1}. The main characteristics of the agricultural soil used were: pH (1:5) = 8.89; electrical conductivity = 0.18 dS m^{-1}; TOC = 1.80%; total $N = 2.01$ g kg^{-1}; available $P = 70$ μg g^{-1}; extractable $K = 440$ μg g^{-1} and cationic exchange capacity = 15 cmol kg^{-1}. The methods used for the soil characterization are described further down.

The amendment used (DOM) was the organic fraction extracted with KOH from composted AL. The raw material was collected from an olive-mill located in Granada, Spain and mixed with fresh cow bedding as bulking agent for composting (Cegarra et al., 2006). The composting process was based on the Rutgers strategy (Finstein et al., 1985) combined with mechanical turning and forced aeration. The extract was obtained by mechanical shaking during 24 h (12 h at 25 °C and 12 h at 70 °C) of the composted AL with 0.1 M KOH (1:20, w/v). The suspension was centrifuged at 14644*g* for 20 min. After centrifugation the supernatant was freed of particulate matter. The analytical characteristics of the DOM are shown in Table 1. The methods used for the DOM characterization are described further down.

2.2. Mycorrhizal inoculation of seedlings

The mycorrhizal fungi used were either *Glomus intraradices* Schenck and Smith (EEZ 1), or a mixture of *G. intraradices*, *Glomus deserticola* (Trappe, Bloss. and Menge) (EEZ 45) and *Glomus mosseae* (Nicol and Gerd.) Gerd. and Trappe (EEZ 43). They were obtained from the collection of the experimental field station of Zaidín, Granada. The acronym EEZ refers to Estación Experimental del Zaidín. The mycorrhizal inoculum consisted of a mixture of rhizosphere soil from trap cultures (*Sorghum* sp.) containing 55 spores of *G. intraradices* g^{-1} or a mixture of 24 spores of *G. intraradices*, 20 spores of *G. deserticola* and 20 spores of *G. mosseae* g^{-1} together with hyphae and mycorrhizal root fragments.

2.3. Experimental design and layout

Five hundred grams of potting substrate, consisting of soil and vermiculite at a ratio of 2:1 (v:v) pasteurised by steaming for 1 h on three consecutive days, were placed in 0.6 l pots. The mesocosm experiment was conducted as a completely randomised factorial design with two factors. The first factor comprised the addition of DOM to soil at a rate of 0, 5.8, 11.7 or 35.1 ml kg^{-1} substrate (0, 50, 100 or 300 mg C kg^{-1} substrate). The second factor had three levels: non-inoculation, inoculation with *G. intraradices* or inoculation with a mixture of *G. intraradices*, *G. deserticola* and *G. mosseae*. Five replicates per treatment were carried out, making a total of 60 pots.

Table 1
Chemical characteristics of the dissolved organic matter (DOM) of composted alperujo (AL)

Dry weight (g l^{-1})	25.0
pH	12.01
EC (mS cm^{-1})	9.78
Organic matter (g l^{-1})	17.4
Extractable C (g l^{-1})	8.6
Humic acid C (g l^{-1})	6.5
Fulvic acid C (g l^{-1})	2.1
Carbohydrates (g l^{-1})	1.03
Phenols (g l^{-1})	1.58
Total N (mg l^{-1})	320
Na (mg l^{-1})	186
K (g l^{-1})	4.44
P (mg l^{-1})	62
Ca (mg l^{-1})	136
Mg (mg l^{-1})	28.9
Fe (mg l^{-1})	3.4
Cu (mg l^{-1})	0.2
Mn (mg l^{-1})	0.3
Zn (mg l^{-1})	1.2

The AM inoculum was mixed with the potting substrate, at a rate of 5% (v/v). In March 2005, *M. sativa* seeds were partly submerged into the growth substrate (ten seeds per pot). Three weeks after seeding, the amended pots received three times 30 ml DOM extracted from AL of each dose of amendment applied for three consecutive days. The electrical conductivity of each dose of amendment was 1.26, 2.48 and 6.34 mS cm^{-1}, respectively. The pH of each dose of amendment was 10.75, 10.84 and 10.87, respectively. The experiment was carried out in the nursery of the University of Murcia, in Murcia, without chemical fertiliser addition. The plants were well watered and kept outdoors under ambient irradiance, temperature and air humidity.

During the four months of the experiment (from March to June) the plants were cut three times. The cuttings were made two, three and four months after seeding. After the third cutting, rhizosphere soil samples were collected from the pots. To collect the rhizosphere soil the root system with rhizosphere soil adhered was introduced into a plastic bag, shook and separated the rhizosphere soil from the root system. Rhizosphere soil samples, air-dried to 20% moisture content and sieved to <2 mm, were divided into two subsamples. One subsample was stored at 2 °C for microbiological analysis and another subsample was allowed to dry at room temperature for physical–chemical analysis.

2.4. Plant analyses

Fresh and dry (105 °C, 5 h) mass of shoots and roots were recorded. Dry plant samples of shoots were ground before chemical analysis. Plant P was determined colorimetrically as molybdovanadate phosphoric acid (Kitson and Mellon, 1944) after digestion in nitric-perchloric acid (2:1) for 1 h at 150 °C and 2 h at 210 °C according to Abrisqueta and Romero (1969). Plant N was determined using an EuroVector Elemental Analyser (EuroEA3000), whereas plant K was estimated by atomic absorption spectrophotometry in the nitric-perchloric extract. The phosphorus, nitrogen and potassium contents have been expressed on dry shoot biomass basis.

The percentage of root length colonized by arbuscular mycorrhizal fungi was calculated by the gridline intersect method (Giovannetti and Mosse, 1980) after staining with trypan blue (Phillips and Hayman, 1970).

2.5. Soil and amendment analyses

Electrical conductivity and pH were measured in a 1:5 (w/v) aqueous solution. Water-soluble C (1:5, w/v), extractable C and fulvic acid C (after precipitation of humic acid at pH 2.0) were determined with an automatic Carbon Analyser for liquid samples (Shimadzu TOC-5050 A). Humic acid C was calculated by subtracting the fulvic acid C from extractable C. Phenolic substances were measured with the Folin method and carbohydrates by the anthrone method (Brink et al., 1960). Total N and C were determined with an automatic elemental microanalyzer after combustion at 1020 °C. Available P was extracted with sodium bicarbonate and determined by colorimetry according to Kitson and Mellon (1944). Cation exchange capacity was determined by Ba^{2+} retention after percolation with a solution of 0.2 N $BaCl_2$-triethanolamine at pH 8.1 (Carpena et al., 1972). After digestion in nitric-perchloric acid, K and Na were determined by flame photometry and Ca, Mg, F, Cu, Mn and Zn by atomic absorption spectrophotometry.

Microbial biomass C was determined by the fumigation-extraction method (Vance et al., 1987). Ten grams of soil at 60% of field capacity were fumigated in a 125 ml Erlenmeyer flask with purified $CHCl_3$ for 24 h. After removal of residual $CHCl_3$, 40 ml of 0.5 M K_2SO_4 solution was added and the sample was shaken for 1 h before filtration of the mixture. The K_2SO_4-extracted C was measured as indicated for water-soluble C. Microbial biomass C was calculated as the difference between the C of fumigated and non-fumigated samples divided for the calibration factor $K_{EC} = 0.38$.

Dehydrogenase activity was determined according to Trevors et al. (1982) using 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride (INT) as oxidizing agent.

Urease and *N*-α-benzoyl-L-argininamide (BAA) hydrolyzing activities were determined in 0.1 M phosphate buffer at pH 7; 1 M urea (Tabatabai and Bremner, 1972) and 0.03 M BAA (Ladd and Butler, 1972) were used as substrates, respectively. Both activities were measured using colorimetric determination of the NH_4^+ released in the hydrolysis reaction (Kandeler and Gerber, 1988).

Alkaline phosphatase and β-glucosidase activities were determined using *p*-nitrophenyl phosphate disodium and *p*-nitrophenyl-β-D-glucopyranoside as substrates, respectively according to Tabatabai and Bremner (1969).

The percentage of stable aggregates was determined by the method described by Lax et al. (1994). Sieved (0.2–4 mm) soil (4 g) was placed on a small 0.250 mm sieve and wetted by spray. After 15 min, the soil was subjected to an artificial rainfall of 150 ml with energy of 270 Jm^{-2}. The remaining soil on the sieve was placed in a previously weighed capsule (T), dried at 105 °C and weighed (P1). Then, the soil was soaked in distilled water and, after 2 h, passed through the same 0.250 mm sieve with the assistance of a small stick to break the remaining aggregates. The residue remaining on the sieve, which was made up of plant debris and sand particles, was dried at 105 °C and weighed (P2). The percentage of stable aggregates with regard to the total aggregates was calculated by the following relationship $(P1 - P2) \times 100/(4 - P2 + T)$.

2.6. Statistical analysis

Data were log transformed to achieve normality. Amendment addition, mycorrhizal inoculation and their interactions effects on measured variables were tested by

a two-way analysis of variance. Means separation for significant ($P = 0.05$) main effects was accomplished by Tukey's mean separation test. Orthogonal polynomial contrasts were used to test for linear, quadratic and cubic trends for amendment application rate. Statistical procedures were carried out with the software package SPSS 10.0 for Windows.

3. Results

3.1. Physical–chemical parameters

Neither the addition of DOM from composted AL nor the mycorrhizal inoculation with AM fungi had any significant effect on soil pH or electrical conductivity (Table 2).

The inoculation with *G. intraradices* and with the mixture of AM fungi significantly reduced the concentration of water-soluble C in the non-amended soil and in the soil amended, particularly with the doses of 50 and 100 mg C/kg substrate (Table 3). The water-soluble carbohydrates concentration was slightly decreased by *G. intraradices*. However, the addition of DOM did not affect the concentrations of water-soluble C or carbohydrates.

The ANOVA indicated that both mycorrhizal inoculation and the addition of DOM had significant effects on soil aggregate stability (Table 2). The percentage of stable aggregates was increased by both mycorrhizal inoculation treatments, which resulted in an average 39% increase with respect to non-inoculated seedlings (Table 3). Except for *G. intraradices*-inoculated plants, there was significant interaction between the effects of mycorrhizal inoculation and amendment. The trend of soil aggregate stability in response to applied amendment was negative linear for the non-inoculated plants ($P < 0.02$) and for the plants inoculated with the mixture of AM fungi ($P < 0.001$). The addition of DOM at the doses of 100 and 300 mg C/kg substrate significantly reduced the aggregate stability of the soil inoculated with the mixture of AM fungi, although the values were higher than those recorded in the non-inoculated counterpart soils.

Table 2
Two factor ANOVA (amendment and mycorrhiza) for all parameters studied of the third harvest of *Medicago sativa*

	Amendment (A)	Mycorrhiza (M)	Interaction (A × M)
pH (H_2O)	NS	NS	NS
Electrical conductivity	NS	NS	NS
Water-soluble C	NS	<0.001	0.010
Water-soluble CH	NS	0.005	NS
Aggregate stability	0.003	<0.001	0.013
Microbial biomass C	NS	<0.001	NS
Dehydrogenase	0.026	<0.001	<0.001
Urease	0.003	<0.001	0.002
Protease-BAA	NS	<0.001	<0.001
β-glucosidase	NS	<0.001	0.004
Phosphatase	NS	<0.001	0.001
Shoot	0.033	<0.001	NS
Root	NS	<0.001	NS
Nitrogen	NS	<0.001	<0.001
Phosphorus	NS	<0.001	NS
Potassium	NS	<0.001	NS
Colonisation	NS	<0.001	NS

P significance values.

3.2. Biochemical parameters

The addition of DOM did not affect soil microbial biomass C, whereas both mycorrhizal inoculation treatments significantly increased this soil biological parameter (Tables 2 and 5). The amendment and mycorrhizal inoculation had a significant effect on dehydrogenase activity. There was a positive interaction between the doses of 100 and 300 mg C/kg substrate and the inoculation with the mixture of AM fungi on dehydrogenase activity. The addition of DOM did not affect the protease, β-glucosidase and phosphatase activities (Table 5). Orthogonal polynomial contrasts yielded significant ($P < 0.001$) linear trends for urease activity in the non-inoculated soil and in the soil inoculated with *G. intraradices*. Analysis of variance indicated highly significant effects of the mycorrhizal inoculation on biochemical parameters (Table 2). The highest protease activitiy was recorded in the soil inoculated with the mixture of AM fungi (Table 5). The dose of DOM and the mycorrhizal inoculation exhibited negative interactions on urease activity in *G. intraradices*-inoculated plants

Table 3
Means for the main effects of mycorrhizal inoculation and dissolved organic matter (DOM) addition on soil physical–chemical properties after the third harvest of *Medicago sativa*

	Amendment				Mycorrhiza		
	0[A]	1	2	3	No mycorrhiza	GI	M
pH (H_2O)	8.93a[*]	8.94a	8.95a	8.96a	8.94a	8.95a	8.94a
Electrical conductivity (μS cm^{-1})	451a	431a	444a	449a	426a	490a	415a
Water-soluble C (μg g^{-1})	105a	111a	108a	117a	129b	102a	100a
Water-soluble CH (μg g^{-1})	11a	12a	11a	13a	13b	10a	12ab
Aggregate stability (%)	28.5ab	29.4b	25.4a	26.1a	21.7a	29.3b	31.1b

GI: *Glomus intraradices*; M: Mixture of AM fungi.
[A] 0, 1, 2 or 3 = 0, 50, 100 or 300 mg C of DOM kg^{-1} substrate, respectively.
[*] Significant differences between the levels of each factor are indicated by different letters (Tukey's HSD test, $P = 0.05$).

Table 4
Means for the interaction effects of mycorrhizal inoculation and dissolved organic matter (DOM) addition on soil physical–chemical properties, enzyme activities and foliar N after third harvest of *Medicago sativa*

	No mycorrhiza				*Glomus intraradices*				Mixture of AM fungi			
	0[a]	1	2	3	0	1	2	3	0	1	2	3
Water-soluble C (μg g^{-1})	119	125	140	133	110	106	91	104	86	104	94	116
Aggregate stability (%)	22.6	25.1	18.4	20.6	27.4	30.4	29.2	30.1	35.3	32.8	28.7	27.6
Dehydrogenase activity (μg INTF g^{-1} soil 20 h^{-1})	107	95	99	106	112	120	101	111	108	101	118	122
Urease activity (μmol NH_3 g^{-1} h^{-1})	1.75	1.51	1.46	1.27	1.74	1.70	1.34	1.29	1.69	1.79	1.73	1.78
Protease-BAA activity (μmol NH_3 g^{-1} h^{-1})	0.83	0.85	0.69	0.70	1.15	1.07	1.58	1.45	1.45	1.59	1.57	1.34
β-glucosidase activity (μmol PNP g^{-1} h^{-1})	0.93	1.20	1.26	1.36	0.83	0.81	0.66	0.71	0.93	0.95	1.04	1.02
Phosphatase activity (μmol PNP g^{-1} h^{-1})	1.00	0.92	0.80	1.08	1.09	1.34	1.28	1.16	1.42	1.29	1.25	1.17
Nitrogen (mg $plant^{-1}$)	34	30	43	38	66	71	50	55	60	65	61	49

Values in rows followed by the same letter are not significant different at the 0.05 level according to the Tukey's HSD test.
[a] 0, 1, 2 or 3 = 0, 50, 100 or 300 mg C of DOM kg^{-1} substrate, respectively.

Table 5
Means for the main effects of mycorrhizal inoculation and dissolved organic matter (DOM) addition on soil microbial biomass C and enzyme activities after the third harvest of *Medicago sativa*

	Amendment				Mycorrhiza		
	0[A]	1	2	3	No mycorrhiza	GI	M
Microbial biomass C (μg g^{-1})	387a[*]	387a	356a	343a	280a	359b	467c
Protease-BAA activity (μmol NH_3 g^{-1} h^{-1})	1.14a	1.17ab	1.28b	1.16a	0.77a	1.31b	1.49c
β-glucosidase activity (μmol PNP g^{-1} h^{-1})	0.90a	0.99a	0.99a	1.03a	1.19c	0.75a	0.99b
Phosphatase activity (μmol PNP g^{-1} h^{-1})	1.17a	1.19a	1.11a	1.14a	0.95a	1.22b	1.28b

GI: *Glomus intraradices*; M: Mixture of AM fungi.
[A] 0, 1, 2 or 3 = 0, 50, 100 or 300 mg C of DOM kg^{-1} substrate, respectively.
[*] Significant differences between the levels of each factor are indicated by different letters (Tukey's HSD test, $P = 0.05$).

and on phosphatase activity in mixture-inoculated plants (Table 4). In contrast, the doses of 100 and 300 mg C/kg substrate and the inoculation with *G. intraradices* increased protease activity.

3.3. Growth and mycorrhizal infection of Medicago sativa

At the first cutting, shoot biomass was increased by the application of amendment (Tables 6 and 7) and was slightly decreased by the inoculation with *G. intraradices*. The content of some nutrients (N and P) in the shoots presented a linear and quadratic response with increasing the dose of DOM. At the second harvest, shoot biomass of *G. intraradices*-inoculated plants was also lower than for the non-inoculated plants and for the plants inoculated with the mixture of AM fungi. From this harvest onwards, both mycorrhizal inoculation treatments generally increased the foliar N and P contents. Only the inoculation with the mixture of AM fungi increased significantly the content of foliar K.

At the third cutting, both the addition of DOM and the mycorrhizal inoculation treatments had significantly stimulated the shoot biomass of *M. sativa* (Tables 2 and 8), the greatest increase being observed in the inoculated plants (biomass about 38% greater than that of plants grown in the soil amended with the highest dose of DOM and about 57% greater than for control plants). There were no significant differences in the growth of plants inoculated with different mycorrhizal inocula. Shoot biomass increased linearly with the amendment dose with high level of significance ($P = 0.008$). No significant interaction between amendment and mycorrhizal inoculation was observed. Plant root biomass was affected only by mycorrhizal inoculation, which provoked a decrease with respect to the non-inoculated plants (Table 8).

Table 6
Two factor ANOVA (amendment and mycorrhiza) for all parameters studied of the first and second harvest of *Medicago sativa*

	Amendment (A)	Mycorrhiza (M)	Interaction (A × M)
First harvest			
Shoot	0.018	<0.001	NS
Nitrogen	<0.001	<0.001	<0.001
Phosphorus	0.009	<0.001	NS
Potassium	0.001	<0.001	NS
Second harvest			
Shoot	NS	<0.001	NS
Nitrogen	0.020	<0.001	<0.001
Phosphorus	NS	<0.001	NS
Potassium	NS	<0.001	NS

P significance values.

Inoculation with the AM fungi increased the foliar N, P and K contents of *M. sativa* plants (Table 6). The plants inoculated with the mixture of AM fungi had nutrient contents of P and K higher than the plants inoculated with *G. intraradices*. The contents of foliar nutrients did not vary

Table 7
Means for main effects of mycorrhizal inoculation and dissolved organic matter (DOM) addition on growth parameters, foliar nutrient content and root infection after the first and second harvest of *Medicago sativa*

	Amendment				Mycorrhiza		
	0[A]	1	2	3	No mycorrhiza	GI	M
First harvest							
Shoot (g dw plant^{-1})	3.08a[*]	3.31ab	3.50b	3.19ab	3.31b	2.97a	3.53b
Phosphorus (mg plant^{-1})	9a	10ab	12b	10ab	10b	9a	12b
Potassium (mg plant^{-1})	237a	243a	288b	244a	308b	231a	221a
Second harvest							
Shoot (g dw plant^{-1})	1.59a	1.63a	1.66a	1.64a	1.67b	1.54a	1.67b
Phosphorus (mg plant^{-1})	6a	7a	7a	7a	4a	7b	9c
Potassium (mg plant^{-1})	107a	114a	121a	108a	111b	95a	131c

GI: *Glomus intraradices*; M: Mixture of AM fungi; dw = dry weight.
[A] 0, 1, 2 or 3 = 0, 50, 100 or 300 mg C of DOM kg^{-1} substrate, respectively.
[*] Significant differences between the levels of each factor are indicated by different letters (Tukey's HSD test, $P = 0.05$).

Table 8
Means for the main effects of mycorrhizal inoculation and dissolved organic matter (DOM) addition on growth parameters, foliar nutrient content and root infection after the third harvest of *Medicago sativa*

	Amendment				Mycorrhiza		
	0[A]	1	2	3	No mycorrhiza	GI	M
Shoot (g dw plant^{-1})	1.85a[*]	1.95a	1.93a	2.04b	1.43a	2.19b	2.20b
Root (g dw plant^{-1})	3.24a	3.37a	3.31a	3.22a	3.83b	3.03a	2.99a
Nitrogen (mg plant^{-1})	53ab	55b	51ab	48a	36a	61b	59b
Phosphorus (mg plant^{-1})	6a	7a	6a	7a	4a	7b	9c
Potassium (mg plant^{-1})	107a	114a	106a	117a	76a	120b	138c
Colonisation (%)	36.5a	37.5a	37.7a	35.9a	0.2a	58.9c	51.8b

GI: *Glomus intraradices*; M: Mixture of AM fungi; dw = dry weight.
[A] 0, 1, 2 or 3 = 0, 50, 100 or 300 mg C of DOM kg^{-1} substrate, respectively.
[*] Significant differences between the levels of each factor are indicated by different letters (Tukey's HSD test, $P = 0.05$).

significantly with the addition of DO. Except for foliar N, the amendment *x* mycorrhizal inoculation interaction had no significant effect on the foliar contents.

At the third cutting, the inoculated seedlings had significantly higher percentages of root colonisation (on average 55% of the root length was infected) than the non-inoculated plants, whose roots showed negligible levels of AM colonisation (Table 6). The addition of DOM had no effect on the mycorrhizal infection of *M. sativa* plants.

4. Discussion

Both the addition of DOM from composted AL and the mycorrhizal inoculation with different mycorrhizal inocula stimulated the growth of *M. sativa*. A clear growth advantage due to DOM and the inoculation with the mixture of AM fungi was produced at the first harvest. In the short-term, *M. sativa* showed a high specificity of response to the inoculation with different *Glomus* species. Inoculation with multiple AM fungi increases the probability of plant-fungus matches that stimulate optimal plant growth, compared with inoculation with a single AM fungus species (Van der Heijden et al., 1998). However, at the third harvest, the plants inoculated with *G. intraradices* had comparable biomass to those inoculated with the mixture of AM fungi, both values being greater than that of plants amended even with the highest dose of DOM. The level of mycorrhizal colonisation of roots of the inoculated plants was high, indicating the high mycorrhizal infectivity of the soil and the persistence of mycorrhizal propagules after three harvests. If the shoot/root ratio reflects the degree of effectiveness of AM (Tobar et al., 1994), then *M. sativa* responded significantly to the inoculation with the different inoculants. The increased growth associated with AM infection in nutrient-deficient soils has been attributed to enhanced nutrient uptake, especially N and P (Smith and Read, 1997; Toro et al., 1998). In our study, mycorrhizal inoculation appeared effective in improving nutrient content, independent of the introduced *Glomus* species. The increased plant N content found in this legume may be due to the ability of AM fungi to enhance N capture from soil and to increase P uptake, which could promote nodule formation by *Rhizobium* and biological N_2 fixation (Azcón and Barea, 1992).

The effectiveness of the amendment with respect to stimulating plant growth depended on the dose added. No negative effects on plant growth were observed with the addition of DOM extracted from composted AL, even with

the highest dose. This result corroborates the suitability of this material for agricultural purposes and demonstrates the effectiveness of the composting process for eliminating and/or reducing the potentially phytotoxic compounds of AL (Alburquerque et al., 2006). For example, phenolic compounds contained in the AL can have toxic effects on plant growth (Leadir et al., 1997). Most phenolic acids began to manifest their phytotoxicity to lettuce and soybean plants at a concentration of 60 mg of phenolic content kg^{-1} soil (Martín et al., 2002). The highest dose of DOM corresponded to an application of 55 mg kg^{-1} of phenolic compounds and this concentration was not enough to inhibit the growth of plants. Arbuscular mycorrhizal fungi can increase the sensitivity of colonised plants to the phytotoxicity of phenolic compounds, facilitating the action or transfer of these toxic substances to plants (Martín et al., 2002). In our particular case, no negative effect of the amendment on the colonisation or on the growth of the AM-inoculated plants was observed. The benefit of the combined treatment (addition of amendment and mycorrhizal inoculation), with respect to the growth of seedlings, was similar to that from each treatment individually.

Humic substances are known to affect enzymatic activities of several microbial species (Visser, 1985a) and to stimulate microbial growth through alteration of membrane permeability (Visser, 1985b). Dehydrogenase activity is usually measured as an index of microbial activity (García et al., 1997). An increase in the soil dehydrogenase activity could be expected due to the input of easily-degradable C and N substrates from the DOM. However, the enzyme activity was independent of the addition of DOM. This could mean that with time (four months after application of DOM) most of the added easily-available organic matter was decomposed, as is supported by the concentrations of labile C fractions. Consequently, global microbial activity returned to its original level in the third harvest. The β-D-glucosidase is an exoenzyme that catalyses the hydrolysis of β-glucosides in soil or in decomposing plant residues. The increase of β-glucosidase activity with the increase of the amendment dose could indicate that the soil achieved the capability to utilise the carbohydrate material added with the DOM. Urease and protease act in the hydrolysis of organic nitrogen to inorganic nitrogen, the former using urea-type substrates and the latter simple peptidic substrates. The urease activity decreased proportionally with the amendment dose, whereas the protease activity was not affected by the addition of DOM. It has been shown that urease activity is reduced considerably after interaction of the enzyme with humic acid fractions of low molecular weight (10–20 kDa) at pH 8.0 (Marzadori et al., 2000). Phosphatases catalyse the hydrolysis of organic phosphorus compounds to inorganic phosphorus. No significant variations occurred with the addition of DOM, which could indicate that the DOM had a scarce content of substrates capable of activating the synthesis of this enzyme.

Several authors have demonstrated that total percentages of mycorrhizal colonisation and sporulation are correlated negatively with the concentration of soluble C fractions (Muthukumar and Udaiyan, 2000) and with the root development, indicating that mycorrhizal fungi act as strong sinks for photosynthates. The rhizosphere soil of the plants inoculated with the mixture of AM fungi showed lower concentration of water-soluble C and root biomass than those of the control soil. Such a change in the rhizodeposition can affect the composition, activity and size of the rhizosphere soil microflora (Wamberg et al., 2003; Alguacil et al., 2005). Both mycorrhizal treatments were effective in increasing microbial biomass C and benzoyl argininamide-hydrolysing and phosphatase activities, related to the N and P cycles, respectively. Increased phosphatase activity in the rhizosphere of plants inoculated with the mixture of AM fungi or *G. intraradices* may have been due to a direct fungal secretion or an induced secretion by the plant roots of extracellular phosphatases, as pointed out by Joner et al. (2000). In contrast, mycorrhizal inoculation treatments led to a decrease in β-D-glucosidase activity, which could be related to a decrease of carbon compounds in these treatments as indicated above.

Roots and associated mycorrhizal hyphae may form a three-dimensional network that aggregates small soil particles (Roldán et al., 1994; Lax et al., 1997). Recent studies have indicated also that arbuscular mycorrhizal fungi produce a glycoprotein, glomalin, that acts as an insoluble glue to stabilise aggregates (Wright and Anderson, 2000). The mixture of AM fungi was more effective than a single species at increasing the percentage of stable aggregates of soil. This effect observed in the soil inoculated with the three AM fungi was notably reduced with increasing the dose of DOM. The presence of higher contents of sales in the amendment applied could explain the negative interaction between the dose of DOM and the inoculation with the mixture of AM fungi. According to such hypothesis, the lack of changes with DOM rate in the aggregate stability of soil inoculated with *G. intraradices* would be related to the formation of aggregates more resistant to dispersive effects of the sales from amendment. As suggested by Bearden and Petersen (2000), the symbiosis between arbuscular mycorrhizal fungi and plants would have increased the stability of the soil aggregates. In our study, plants inoculated with the two different inoculants showed similarly high levels of AM root colonisation. Of particular note was the fact that the addition of DOM at concentrations from 50 to 300 mg C kg^{-1} substrate did not affect mycorrhizal infection due to either *G. intraradices* or the mixture of AM fungi.

In conclusion, the addition of DOM, obtained by alkaline extraction of composted AL, was not sufficient to restore soil structure and microbial activity and did not affect the mycorrhizal development of introduced populations of AM fungi, but, depending on the dose, its fertiliser efficiency for improving plant growth was apparent. The effectiveness of this amendment with regard to improving plant growth depended on its dose. The mycorrhizal

inoculation with an AM fungus or a mixture of three AM fungi was the most effective treatment for improving both the growth of *M. sativa* plants and the soil physical and microbiological quality after the third harvest. The improvement in plant growth could be related to the persistence of infective AM propagules from previous harvests, leading to a higher extent of mycorrhizal colonisation. The combined treatment of mycorrhizal inoculation of seedlings and addition of DOM to soil produced effects on plant growth similar to inoculation with AM fungi.

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