



Arbuscular mycorrhizal colonization and growth of soybean (*Glycine max*) and lettuce (*Lactuca sativa*) and phytotoxic effects of olive mill residues

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Abstract

We studied the influence of olive mill dry residue (DOR) on growth of soybean (*Glycine max*) and lettuce (*Lactuca sativa*) colonized with the arbuscular fungi *Glomus mosseae* or *G. deserticola*. The DOR has 6 mg g^{-1} of soluble phenolic compounds and the application of 2.5 g kg^{-1} of DOR (15 mg kg^{-1} of phenolic content) to soil decreased the growth of plants colonized with the AM fungi. The application to the soil of 10 g kg^{-1} of DOR (60 mg kg^{-1} of phenolic content) decreased the dry weight of nonarbuscular mycorrhizal (AM) plants. The application of DOR decreased the percentage of AM colonization of plants except those were inoculated with *G. mosseae* or *G. deserticola* 4 weeks before the application of DOR. No effect of the residue on the colonization of these previously AM inoculated plants was observed. DOR had no effect neither on the fungal succinate dehydrogenase activity nor on the most probable number of AM propagules in all treated soils. The obtained results showed that AM fungi increased the phytotoxicity of DOR in lettuce and soybean. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Arbuscular mycorrhizas; *Glomus deserticola*; *Glomus mosseae*; Olive mill dry residues; Phenols; Phytotoxicity

1. Introduction

Mediterranean countries produce annually about 10 million tons of olives (Barranco et al., 1997). In recent years, a two-stage centrifugation process to extract olive oil has been increasingly used because it extracts more oil products from the olives, consumes less water and energy, and avoids the production of olive mill wastewater, whose disposal is regarded as a serious environmental issue in the olive-growing regions (Alba et al., 1993). In this extraction process a wet residue (80% of the weight of the olive) is obtained along with the oil. This wet olive residue is dried and extracted with solvents to obtain a dry residue (DOR). Due to its high C and mineral nutrients content (Nogales et al., 1999), DOR might be used as fertilizer. However, phenolic compounds have been detected in DOR (Martín García et al., 1997). Studies carried out with the olive mill wastewater obtained by the former process of extraction of olive oil have showed that the phenols contained in it were

the principal causers of its great antimicrobial activity (Moreno et al., 1987; Perez et al., 1992). Exogenous phenolic compounds can also have phytotoxic effect (Carpasso et al., 1992).

Arbuscular mycorrhizal (AM) fungi can improve plant growth by taking up relatively immobile nutrients such as phosphate (Barea and Jeffries, 1995). There is increasing evidence that phenolics are able to influence plant growth and root colonization (Siqueira et al., 1991). However, the role of phenolics in the AM symbiosis is not well established. Some exogenously applied phenolics may stimulate indigenous populations of AM fungi in the field resulting in increased plant growth and yields (Siqueira et al., 1992). However, phenolic compounds may also inhibit the establishment of AM symbioses and decrease the host growth (Leadir et al., 1997).

Thus addition of DOR to soil might result in increased colonization of root by AM fungi. However, these residues contain phenolics that may have negative effect on plant growth directly and/or through the decrease of AM symbiosis. The aim of this work was to study the effect of

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dry residues from olive extraction on AM symbiosis and plant growth.

2. Materials and methods

The experiments were carried out in 0.3 l pots filled with a gray loam soil obtained from the field of the Estación Experimental del Zaidín (Granada, Spain). The soil had a pH of 8.1 in a 1:1 soil:water ratio. P, N, K, Fe, Mn, Cu and Zn were determined by methods of Lachica et al. (1965). NaHCO_3 -extractable P was 6.2 mg kg^{-1} , N was 2.5 mg kg^{-1} , K was 132 mg kg^{-1} , Fe was 9.6 g kg^{-1} , Mn was 110 mg kg^{-1} , Cu was 5.8 mg kg^{-1} and Zn was 5.7 mg kg^{-1} . The soil texture was 358 g kg^{-1} sand, 436 g kg^{-1} silt, 205 g kg^{-1} clay and 18 g kg^{-1} organic matter. The soil was used either nonsterilized or steam-sterilized and mixed with sterilized quartz sand 2:3 by volume.

Olive mill dry residue was collected from an orujo manufacturer (COLGRA S.L., Granada, Spain). Total C, N, P, K, Fe, Mn, Cu and Zn content of the DOR were determined by Nogales et al. (1999). C was 464 g kg^{-1} , N was 11.2 g kg^{-1} , P was 1 g kg^{-1} and K was 6.5 g kg^{-1} , Fe was 1.5 g kg^{-1} , Mn was 23 mg kg^{-1} , Cu was 14 mg kg^{-1} and Zn was 15 mg kg^{-1} . The phenolic content of the DOR was determined by extraction with distilled water (200 g l^{-1}) after agitation for 18 h. Total phenolic contents of extracts was estimated according to Ribereau-Gayon (1968) using syringic acid as standard.

Glomus mosseae (Nicol. and Gerd.) Gerd. and Trappe (BEG no. 12) from Rothamsted Experimental Station and *G. deserticola* Trappe, Bloss and Menge from the Instituto de Investigaciones Agrobiológicas de Galicia (CSIC) were the AM fungi used in these experiments. The AM fungal inoculum was a root-and-soil inoculum consisting of rhizosphere soil containing spores and colonized root fragments of *Medicago sativa* L. in amounts of 4 or 8 g per pot, which were predetermined to have achieved high levels of root colonization. Uninoculated were given a filtrate (Whatman no. 1 paper) of the inoculum containing the common soil microflora, but free of AM fungal propagules.

Lettuce (*L. sativa* L.) and soybean (*Glycine max*) were used as tests plants. Seeds were pregerminated and selected for uniformity prior to transplanting. Plants were grown in a greenhouse with natural light supplemented by Sylvania incandescent and cool-white lamps giving $400 \text{ nmol m}^{-2} \text{ s}^{-1}$ at 400–700 nm; there was a 16–8 h light–dark cycle at 25–19 °C and 500 ml l^{-1} relative humidity. Plants were watered from below, and fed with a nutrient solution lacking phosphate at 20 ml per pot weekly (Hewitt, 1952).

Three experiments were carried out: (1) Plants were grown in nonsterilized soil. (2) Plants were grown in sterilized soil inoculated with 8 g of inoculum of *G. mosseae* or *G. deserticola*. In both experiments plants were grown in

Table 1

Shoot and root dry weight of lettuce (*L. sativa*) and soybean (*G. max*) grown in nonsterilized soil in presence of different concentrations of mill dry olive residue

Dry olive residue concentration (g kg^{-1} soil)	Shoot dry weight (mg)		Root dry weight (mg)	
	Lettuce	Soybean	Lettuce	Soybean
0	227d	118c	144c	76c
2.5	103c	92b	48b	53b
5	34b	73b	6a	51b
10	30b	74b	5a	41a
20	4a	51a	2a	34a

Column values followed by the same letter are not significantly different as determined by Duncan's multiple range test ($P = 0.05$).

0.3 l pots and harvested after 4 weeks. (3) Plants were grown in 40 ml pots of a sterilized mix of peat and vermiculite in equal volumes. Plants were inoculated with 4 g of inoculum of *G. mosseae* or *G. deserticola*. After 4 weeks growth, plants were transplanted to a 0.3 l pots filled with sterilized soil and harvested after other 4 weeks.

Olive mill dry residue was applied to the 0.3 l soil pots in the three experiments at concentrations of 0, 2.5, 5, 10 and 20 g kg^{-1} soil/sand mixture.

Plants were harvested and dry mass was determined. After the harvest two samples of fresh weight were taken from the entire root system at random. One of the samples were cleared and stained (Phillips and Hayman, 1970), and the percentage of root length colonization was measured (Giovannetti and Mosse, 1980). In the second sample succinate dehydrogenase (EC 1.3.99.1) (SDH) activity was measured in fungal mycelia by the reduction of tetrazolium salts at the expense of added succinate (MacDonald and Lewis, 1978); the percentage of AM fungal mycelia with SDH activity was determined under a compound microscope (Ocampo and Barea, 1985).

Table 2

Percentage of AM root length colonization of lettuce (*L. sativa*) and soybean (*G. max*) grown in nonsterilized soil in presence of different concentrations of mill dry olive residue

Dry olive residue concentration (g kg^{-1} soil)	Percent root length colonization		Percent AM mycelium with SDH activity (%)	
	Lettuce	Soybean	Lettuce	Soybean
0	42.1c	71.2c	52.3a	58.5a
2.5	30.3b	39.4b	51.2a	58.5a
5	34.2bc	39.3b	57.1a	55.3a
10	29.4b	35.7b	50.8a	56.2a
20	5.5a	7.6a	54.6	52.8a

Column values followed by the same letter are not significantly different as determined by Duncan's multiple range test ($P = 0.05$).

Dry olive residue concentration (g kg ⁻¹ soil)	Shoot dry weight (mg)			Root dry weight (mg)		
	Lettuce			Soybean		
	M	G. mosseae	G. deserticola	M	G. mosseae	G. deserticola
0	81b 1	155d 2	174c 2	83b 1	148c 2	149c 2
2.5	81b 1	115c 2	111b 2	72ab 1	103b 2	112b 2
5	85b 1	108c 2	119b 2	77ab 1	104b 2	115b 2
10	63a 1	57b 1	50a 1	62a 1	64b 1	57b 1
20	59a 2	18a 1	21a 1	61a 2	48a 1	40a 1

The number of effective AM fungal propagules of the pots with nonsterilized soil or sterilized soil inoculated with *G. mosseae* or *G. deserticola* was estimated at the beginning and at the end of the experiment by the most probable number (MPN) method (Porter, 1979) using *M. sativa* as the test plant. To determine the MPN, five samples of 10 ml from each of the five pots were used. From each sample, five dilutions (10^{-1} – 10^{-5}) were prepared diluting 10 ml of the sample in 99 ml of sterilized soil. Each dilution was tested in five 100 ml pots. The *M. sativa* plants were harvested after 6 weeks to record the presence or absence of mycorrhizal colonization.

For statistical treatments, the percent values were arcsine transformed. The data obtained for dry weight, percentage of AM colonization, percentage of AM mycelia with SDH activity and the most probable number of propagules of AM fungi were subjected to ANOVA. The mean values of five replicate pots were compared using Duncan's multiple range test ($P = 0.05$).

3. Results

Total phenolic content of the DOR was 6 mg g^{-1} of phenolic compounds after water extraction.

3.1. Experiment 1

Shoot and root dry weights (Table 1) and the percentage of AM root length colonization (Table 2) of lettuce and soybean cultivated in nonsterilized soil were decreased by all doses of DOR used. The dry weight of lettuce was lower in presence of 5 g kg⁻¹ of DOR than in presence of 2.5 g kg⁻¹ of DOR but the dry weight of soybean was similar at both DOR concentrations. The metabolic activity after AM colonization, measured as the percentage of mycelium with SDH activity was not affected by the addition of DOR. (Table 2).

3.2. Experiment 2

The application of DOR at the doses of 10 and 20 g kg⁻¹ decreased the shoot and root dry weights of noninoculated plants. Whereas the shoot and root dry weights of plants inoculated with *G. mosseae* or *G. deserticola* decreased in presence of all doses of DOR (Table 3). The shoot and root dry weights of lettuce and soybean inoculated with *G. mosseae* or *G. deserticola* were higher than uninoculated controls in the absence of DOR. Shoot and root dry weights of mycorrhizal plants were higher than nonmycorrhizal plants in the presence of 2.5 and 5 g kg⁻¹ of DOR. Both plants had similar dry weight in presence of 10 g kg⁻¹ of DOR. However, the dry matter of nonmycorrhizal plants was higher than those of AM plants when 20 g kg⁻¹ of DOR was applied (Table 3). The percentage of AM root length colonization of lettuce and soybean inoculated with

Table 4

Percentage of root length colonization and percentage of AM fungus-mycelium with SDH activity in lettuce (*L. sativa*) and soybean (*G. max*) inoculated with *Glomus mosseae* and *G. deserticola* in presence of different concentrations of mill dry olive residue

Dry olive residue concentration (g kg ⁻¹ soil)	Percent root length colonization				Percent AM mycelium with SDH activity			
	Lettuce		Soybean		Lettuce		Soybean	
	<i>G. mosseae</i>	<i>G. deserticola</i>	<i>G. mosseae</i>	<i>G. deserticola</i>	<i>G. mosseae</i>	<i>G. deserticola</i>	<i>G. mosseae</i>	<i>G. deserticola</i>
0	24.3c	48.4c	32.3bc	53.1b	63.4a	79.2a	67.3a	77.4a
2.5	25.4c	59.1c	35.6bc	41.5b	62.1a	80.2a	67.2a	70.5a
5	22.2bc	50.2c	30.6bc	56.1b	73.3a	82.4a	65.2a	79.3a
10	14.3ab	35.3b	17.6a	21.2a	60.8a	86.4a	66.4a	73.6a
20	11.7a	15.6a	15.3a	11.6a	63.5a	87.4a	62.7a	78.2a

Column values followed by the same letter are not significantly different as determined by Duncan's multiple range test ($P = 0.05$).

G. mosseae or with *G. deserticola* decreased in presence of 10 and 20 g kg⁻¹ of DOR (Table 4). However, the percentage of mycelium with SDH activity was not affected by the presence of DOR (Table 4).

3.3. Experiment 3

The application of all doses of DOR decreased the shoot and root dry weight of lettuce and soybean when these plants were inoculated with *G. mosseae* or *G. deserticola* 4 weeks before the application of the DOR. However, the shoot and root dry weight of the noninoculated controls decreased when 10 and 20 g kg⁻¹ of DOR were used (Table 5). The shoot and root dry weight of plants previously inoculated with *G. mosseae* or *G. deserticola* were higher than noninoculated plants at all doses of DOR used (Table 5). DOR did not decrease either the percentage of AM root length colonization or the percentage of AM mycelium with SDH activity of plants inoculated with AM fungi 4 weeks before the application of DOR (Table 6).

The number of AM fungal propagules in soil pots at the beginning of the experiment was 15 ± 4 in nonsterilized soil, 31 ± 7 in sterilized soil inoculated with *G. mosseae* and 52 ± 12 in sterilized soil inoculated with *G. deserticola*. At the end of the experiment the most probable number of propagules in nonsterilized and in sterilized soil inoculated with *G. mosseae* or *G. deserticola* was not affected by all doses of DOR applied (Table 7).

4. Discussion

A dose of 4 kg m⁻² is recommended for farmyard manure applications (Ocampo et al., 1975). The application of an equivalent dose in this study (20 g kg⁻¹ of DOR), decreased the dry weight of lettuce and soybean plants. Addition of 10 g kg⁻¹ of DOR to soil decreased the dry weight of non AM inoculated plants. Moreover, when plants were colonized with AM fungi the dry weight of plants was

decreased with the application of 2.5 g kg⁻¹ of DOR. These results indicates that DOR have phytotoxic substances and suggest that AM fungi may facilitate the action or transfer of these toxic substances to plant. In some cases an increase of sensitivity of plant caused by AM fungi to toxic substances such as some heavy metals and herbicides has been observed (Leyval et al., 1997; Nelson and Khan, 1990). However, AM fungi can differ in their responses to phenolics compounds (Douds et al., 1996). Lettuce seem to be more sensitive than soybean to the action of DOR when these plants were colonized with the soil indigenous AM fungi but, lettuce and soybean had similar sensitivity to DOR when were colonized with *G. mosseae* or *G. deserticola*.

The dry weight of plants inoculated with *G. mosseae* or with *G. deserticola* at the same time that DOR was applied, was higher than non AM inoculated controls when the residue was applied until the dose of 10 g kg⁻¹. When the dose of DOR was increased to 20 g kg⁻¹ the dry weight of plants inoculated with *G. mosseae* or with *G. deserticola*, decreased drastically as compared to uninoculated plants. These results reinforce the hypothesis that AM fungi increase the sensitivity of plants to the phytotoxicity caused by the application of DOR. However, the dry weight of plants inoculated with *G. mosseae* or with *G. deserticola* 4 weeks before the application of DOR was higher than noninoculated controls at all doses of DOR used. As have been observed with other phytotoxic substances, the best development of AM colonized plants before to the application of DOR may explain their resistance to the phytotoxic action of this residue (Ocampo, 1993).

Phenolic compounds in soil can have toxic effects on the growth of the plants (Fiestas Ros de Ursinos, 1986). Most of phenolic acids began to manifest their phytotoxicity at a concentration of 50 mg kg⁻¹ (Wang et al., 1967). The DOR has 6 mg g⁻¹ of soluble phenolic compounds. Growth reduction of mycorrhizal plants by the soil application of some phenols have been observed (Wacker et al., 1990). Our results show that the application of 2.5 g kg⁻¹ of DOR

Table 5

Shoot and root dry weight of lettuce (*L. sativa*) and soybean (*G. max*) inoculated or not with *Glomus mosseae* and *G. deserticola* 4 weeks before the application of different concentrations of mill dry olive residue

Dry olive residue concentration (g kg ⁻¹ soil)	Shoot dry weight (mg)		Root dry weight (mg)					
			Lettuce			Soybean		
	M	<i>G. mosseae</i>	<i>G. deserticola</i>	<i>G. mosseae</i>	<i>G. deserticola</i>	M	<i>G. mosseae</i>	<i>G. deserticola</i>
0	365b 1	582d 2	650d 2	105b 1	419c 2	180b 1	299c 2	359c 2
2.5	299b 1	459c 2	466c 2	97b 1	243b2	137b 1	220b 2	224b 2
5	276b 1	370b 2	397b 1	87b 1	172a 2	126b 1	195b 2	193b 2
10	93a 1	212b 2	241ab 2	67a 1	141a 2	46a 1	110a 2	132a 2
20	76a 1	175a 2	198a 2	59a 1	129a 2	37a 1	88a 2	109a 2

Column values followed by the same letter or row values followed by the same number are not significantly different as determined by Duncan's multiple range test ($P = 0.05$).

Table 6

Percentage of root length colonization and percentage of AM fungus-mycelium with SDH activity in lettuce (*L. sativa*) and soybean (*G. max*) inoculated with *Glomus mosseae* and *G. deserticola* 4 weeks before the application of different concentrations of mill dry olive residue

Dry olive residue concentration (g kg ⁻¹ soil)	Percent root length colonization		Percent AM mycelium with SDH activity					
			Lettuce			Soybean		
	<i>G. mosseae</i>	<i>G. deserticola</i>	<i>G. mosseae</i>	<i>G. deserticola</i>	<i>G. mosseae</i>	<i>G. deserticola</i>	<i>G. mosseae</i>	<i>G. deserticola</i>
0	35.7a	71.3a	34.4a	82.4a	62.6a	60.8a	66.2a	79.6a
2.5	35.8a	60.2a	31.7a	86.2a	63.5a	75.3a	62.5a	84.2a
5	38.1a	75.4a	34.6a	84.3a	63.5a	72.3a	67.4a	88.3a
10	37.7a	70.2a	36.4a	84.6a	62.1a	68.7a	68.2a	85.5a
20	35.6a	74.4a	30.8a	75.9a	70.3a	64.6a	67.6a	82.6a

Column values followed by the same letter are not significantly different as determined by Duncan's multiple range test ($P = 0.05$).

Table 7

The most probable number of propagules of AM fungi in nonsterilized or sterilized soil inoculated with *Glomus mosseae* or *G. deserticola* in presence of different concentrations of mill dry olive residue after lettuce (*L. sativa*) and soybean (*G. max*) culture

Dry olive residue Concentration (g kg ⁻¹ soil)	Number of propagules × 10 g ⁻¹ soil					
	Lettuce			Soybean		
	Nonsterilized soil	<i>G. mosseae</i>	<i>G. deserticola</i>	Nonsterilized soil	<i>G. mosseae</i>	<i>G. deserticola</i>
0	11.2a	25.3a	38.2a	12.1a	28.2a	40.5a
2.5	12.4a	21.2a	35.2a	13.1a	22.2a	45.2a
5	10.5a	27.5a	37.5a	20.5a	30.3a	50.1a
10	21.3a	32.2a	52.6a	25.2a	30.4a	68.4a
20	22.2a	36.1a	67.2a	30.3a	37.2a	70.6a

Column values followed by the same letter are not significantly different as determined by Duncan's multiple range test ($P = 0.05$).

(15 mg kg⁻¹ of phenolic content) decreased the dry weight of AM colonized plants whereas the addition to the soil of 10 g kg⁻¹ of DOR (60 mg kg⁻¹ of phenolic content) was necessary to decrease the dry weight of non AM inoculated plants.

The establishment of the AM symbiosis also seems to depend in great measure on the concentration of phenolic compounds present in the soil (Leadir et al., 1997). According to our results, 2.5 g kg⁻¹ of DOR was enough to decrease the percentage of root length colonized with indigenous AM fungi. However, the percentage of root length colonized by *G. mosseae* or by *G. deserticola*, inoculated at the same time that DOR was applied, only decreased when the dose of DOR rose to 10 g kg⁻¹. Nevertheless, no effect of DOR on the root length colonization of plants inoculated with *G. mosseae* or with *G. deserticola* 4 weeks before the application of DOR. As happen with other soil contaminants, the effect of DOR on AM root colonization seem to vary with the type of fungi and the time of inoculation (Leyval et al., 1997; Ocampo 1993). The effect of many biotic and abiotic factors on AM symbiosis take place when the fungus is in its extramatrical phase of development (Ocampo 1993). DOR might affect the fungus while the fungus relies on its own energy, but once fed from the root, the fungus is unaffected by the toxins. However, no effect of DOR on the number of propagules of AM fungi in soil, determined by the MPN method, was observed. On the other hand, the absence of effect of this residue on the metabolic activity of the AM mycelium into the root, assessed as SDH activity, suggests that the DOR did not affect directly the development of the fungi inside the root but rather that it acted through the plant.

In conclusion, AM fungi not only were resistant to the action of the DOR but also increase the sensitivity of colonized plants to the phytotoxicity of this residue. The possibility that AM fungi increase plant susceptibility to DOR through the higher uptake of phenols or other phytotoxic substances will be investigated.

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