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Improvement of soil characteristics and growth of *Dorycnium pentaphyllum* by amendment with agrowastes and inoculation with AM fungi and/or the yeast *Yarowia lipolytica*

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Abstract

The effectiveness of two microbiologically treated agrowastes [dry olive cake (DOC) and/or sugar beet (SB)] on plant growth, soil enzymatic activities and other soil characteristics was determined in a natural soil from a desertified area. *Dorycnium pentaphyllum*, a legume plant adapted to stress situations, was the test plant to evaluate the effect of inoculation of native arbuscular mycorrhizal (AM) fungi and/or *Yarowia lipolytica* (a dry soil adapted yeast) on amended and non-amended soils. Plant growth and nutrition, symbiotic developments and soil enzymatic activities were limited in non-amended soil where microbial inoculations did not improve plant development. The lack of nodules formation and AM colonization can explain the limited plant growth in this natural soil. The effectiveness and performance of inocula applied was only evident in amended soils. AM colonization and spores number in natural soil were increased by amendments and the inoculation with *Y. lipolytica* promoted this value. The effect of the inoculations on plant N-acquisition was only important in AM-inoculated plants growing in SB medium. Enzymatic activities as urease and protease activities were particularly increased in DOC amended soil meanwhile dehydrogenase activity was greatest in treatments inoculated with *Y. lipolytica* in SB added soil. The biological activities in rhizosphere of agrowaste amended soil, used as indices of changes in soil properties and fertility, were affected not only by the nature of amendments but also by the inoculant applied. All these results show that the lignocellulosic agrowastes treated with a selected microorganism and its further interaction with beneficial microbial groups (native AM fungi and/or *Y. lipolytica*) is a useful tool to modify soil physico-chemical, biological and fertility properties that enhance the plant performance probably by making nutrients more available to plants.

Keywords: AM fungi; Organic amendment; *Yarowia lipolytica*; Soil enzymatic activities

1. Introduction

Restoration of disturbed ecosystems has become, in recent years, a practice focused on maintenance and regeneration of the original plant communities (Herrera

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et al., 1993a,b). Biotic environmental conditions are major factors that require particular attention in restoration ecology (Jeffries and Barea, 1994). These aspects as well as those related to soil structure, stability and erosion are key factors for successful reclamation strategies and revegetation of desertified areas (Bethlenfalvay and Linderman, 1992; Herrera et al., 1993a,b; Smith and Read, 1997; Caravaca et al., 2002a). In degraded soils the microbial population is too small (Boddington and Dodd, 2000) and under such situations, reintroduction of beneficial rhizosphere microbes is a possible way to restore the original target plant community (Jeffries and Barea, 1994). Nitrogen inputs derived from symbiotic N_2-fixation may be critical for plant development in degraded areas (Barea et al., 1992a) and arbuscular mycorrhizal (AM) symbioses play an essential role in plant phosphorus uptake and in sustaining a vegetation cover in desertification-threatened ecosystems (Requena et al., 1996; Azcón and Barea, 1997; Caravaca et al., 2002b). Thus, management of indigenous populations is currently one promising option (Boddington and Dodd, 2000).

In degraded soils, the activity of microbiota is low because of the lack of suitable organic substrates. The application of organic matter can double soil carbon and nitrogen levels in about 40 years (Jenkinson, 1988). In addition, it has been reported that the accumulation of organic matter might increase the AM spore density and the survival and independent growth of AM fungi, but the information is limited (Harinikumar et al., 1990). In general, AM propagules from low-input systems has a greatly enhanced capacity to initiate AM symbiosis (Mäder et al., 2000a) which facilitate colonization of plants (Ezawa et al., 2000).

In this regard, one alternative, as Atkinson and Watson (2000) suggested, is an organic-based system where organic nutrients are gradually released into mineral forms, synchronizing nutrient availability in soils with plant needs. The organic system includes a high proportion of recalcitrant organic compounds that are slowly biodegradable, to minimize leaching losses.

Large quantities of agrowastes as olive waste materials are produced during the extraction of oil from the olive fruits. This product poses serious environmental problems due to the phenolic part of the waste materials (Pérez et al., 1986; Paredes et al., 1987). Such lignocellulosic materials can be mineralized in composting and biotransformation processes avoiding soil contamination. One attractive approach is to use such organic compounds as fertilizers, following biological degradation, since the organic and inorganic minerals released are beneficial to plants (Rodríguez et al., 1999).

In previous studies, using agricultural soils and crop plants (Vassilev et al., 1996, 1997a,b), microbially mediated processes having chelating reactions have been practiced for solubilizing inorganic P forms [(fluorapatite) rock-phosphate (RP)], using a selected strain of *Aspergillus niger* able to grow on sugar beet (SB) and dry olive cake (DOC) waste materials. In the transformation processes, such materials are broken down into simple sugars, which provide energy sources required for the growth and metabolic activity of soil heterotrophic microorganisms such as N_2-fixing bacteria or those with P-solubilizing abilities.

This biotechnological approach, based on microbial RP solubilization by *A. niger* on agroindustrial wastes, is very attractive for use in sustainable systems and reclamation strategies but no information is available on the use of such materials for these purposes.

Woody legume shrubs as *Dorycnium pentaphyllum*, belonging to the natural succession in plant communities of Mediterranean ecosystems, has been chosen for artificial acceleration of this area in restoration programs.

The aim of this study was to determine the fertilizing effect of these microbially mediated processes applied to a natural degraded soil inoculated or not with AM fungi (obtained from native inoculum) and/or *Yarowia lipolytica* a plant growth-promoting yeast (PGPY) with RP-solubilizing ability (Vassileva et al., 2000; Vassilev et al., 2001), using *D. pentaphyllum* as test plant. Thus, values of plant growth and nitrogen nutrition, amount of root length AM colonized and nodule formation as well as those related to soil enzymatic activities (dehydrogenase, urease and protease), pH and electric conductivity and AM fungal spores number as affected by the treatment applied were determined.

2. Materials and methods

2.1. Fermentation experiments

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

Sugar beet (SB) wastes and dry olive cake (DOC) were used as substrates in the fermentation trials. Their characteristics were: cellulose [29% (SB) and 18% (DOC)], hemicellulose [23% (SB) and 16% (DOC)] and lignin [5% (SB) and 26% (DOC)].

The solid residues were dried in a 60 °C oven and then ground to pass a 2-mm-pore screen. Portions of 15 g of each solid substrate and a mixture, 1:1 (w/w), were placed in 250-ml Erlenmeyer flasks. Czapek-Dox mineral salt solution, 40 ml, was added to all flasks. Rock phosphate (Morocco fluorapatite, 12.8% soluble P, 1 mm mesh), was added to all treatments at a rate of 0.75 g/50 ml.

Media were sterilized by autoclaving at 120 °C for 30 min. 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 days.

2.2. Soil–plant experiment

The treatments used in this experiment were as follows: unamended soil: control (i); soil amended with treated SB + RP (ii); DOC + RP (iii); SB + DOC + RP (iv). The fermentation products from treatments (ii)–(iv), prepared as described before, were mixed with a soil–sand mixture (1:1, v/v) and left for equilibration for 4 weeks at room temperature. The topsoil (0–20 cm) from a field in Murcia province (Spain) was used. The main soil characteristics were pH 8.90, 1.36 μg/g (Olsen test), organic carbon 0.94%, total N 0.22%, and on electric conductivity of 1.55. Solid lignocellulosic (SB, DOC, SB + DOC) and RP mixtures were added to the soil at a rate supplying 3/100 g soil, with a corresponding amount of RP (0.15/100 g soil). One seedling of *Dorycnium pentaphylum* was transplanted in each pot ($d = 12.2$ cm; 500 g capacity; 5 pots per treatment) inoculated or not with a mixture of 7 autochthonous arbuscular mycorrhizal fungi [*Glomus mosseae* (EEZ-43), *G. constrictum* (EEZ-42), *G. coronatum* (EEZ-44), *G. microagregatum* (EEZ-40), *Glomus* sp. (EEZ-41), *G. albidum* (EEZ-39), *G. claroideum* (EEZ-47)]. These mycorrhizal fungi were isolated from the desertified soil of a Mediterranean area used in this study (Murcia province, Spain) and they were morphologically identified. They were separately bulked in an open-pot culture of red clover, and used as a stock culture. From this stock culture, the mycorrhizal inoculum was obtained. It consisted of spores, mycelia and mycorrhizal root fragments. Five grams of inocula mixture per pot, having similar characteristics (an average of 30 spores per g and roots with 75% of AM colonization) was applied to each one of the corresponding pots, (M treatments) in the bottom of a 5-cm deep hole. Similarly, *Y. lipolytica* was introduced, when appropriate, into the soil–plant systems at a rate of 1 ml per pot (1.7×10^6 cfu ml^{-1}). Cell suspension of *Y. lipolytica*, was prepared as described earlier (Vassileva et al., 2000). The plants were grown in a greenhouse under a day/night cycle of 16/8 h, 21/15 °C, and 50% relative humidity. Photosynthetic photon flux density (PPFD) was 503 μmol/m^2/s, as measured with a lighmeter (LICOR, model LI-188B). Water loss was compensated for by watering every day, after weighing pots.

2.3. Analytical methods

The plants were harvested after two months. Shoot dry weight was recorded after drying at 70 °C. The concentration of N was measured colorimetrically with an autoanalyzer according to the manufacturer's instructions (Technicon, 1974). The percentage of mycorrhizal root length was determined by microscopic examination of stained root samples (Phillips and Hayman, 1970), using the gridline intersect method of Giovannetti and Mosse (1980) where the root sample was spread out evenly in dishes that had gridlines marked on the bottom to form 1.27 cm^2. Vertical and horizontal gridlines were scanned under a dissecting microscope at 40–100× magnification. The absence or presence of AM colonization was recorded at each point where a root intersected a line and at least 100 gridline intersects were tallied as the authors recommended.

pH and electrical conductivity were measured in a 1:5 (w/v) aqueous extract. Activity of the following enzymes was determined as follows: Dehydrogenase activity was determined following Skujins' method (1976), as modified by García et al. (1997). For this, 1 g of soil at 60% of its field capacity was exposed to 0.2 ml of 0.4% INT (2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride) in distilled water for 20 h, at 22 °C in darkness. The INTF (iodo-nitrotetrazolium formazan) formed was extracted with 10 ml of methanol, by shaking vigorously for 1 min and filtering through a Whatman No. 5 filter paper. INTF was measured spectrophotometrically at 490 nm.

Urease and *N*-α-benzoyl-L-argininamide (BAA)-hydrolyzing protease activities were determined in 0.1 M phosphate buffer at pH 7; 1 M urea and 0.03 M BAA were used as substrates, respectively. Two milliliters of buffer and 0.5 ml of substrate were added to 0.5 g of sample, which was incubated at 30 °C (for urease) or 39 °C (for protease) for 90 min. Both activities were determined as the NH_4^+ released in the hydrolysis reaction (Nannipieri et al., 1980).

AM fungal spores were extracted from soil by wet sieving and decanting, followed by sucrose centrifugation (del Val et al., 1999). After centrifugation, the supernatant was poured through a 50-μm mesh and quickly rinsed with tap water. Spores were counted using a Doncaster dish, under a dissecting microscope.

2.4. Statistical analysis

Data were subjected to an analysis of variance (ANOVA), followed by Duncan's multiple range test (Duncan, 1995). Percentage values were ARCsine transformed before statistical analysis.

3. Results

DOC amendment increased shoot biomass, particularly in association with *Y. lipolytica*, but the effectiveness of SB application, in terms of plant growth, was greater than that of DOC. The mixture of both residues

did not exert any significant effect compared to the single application of DOC (the amendment with less plant growth effect). In non-amended soil, plant growth was very limited and microbial inoculations were not effective in improving plant biomass (Fig. 1(a)).

Nevertheless, root growth was stimulated in non-amended soil by AM colonization. The greatest root development was in SB-amended non-inoculated plants. AM colonization or *Y. lipolytica* inoculation increased root fresh biomass, as compared with non-inoculated in DOC-added plants, by 192% (*Y. lipolytica*) and by 184% (mycorrhizal inoculum). In contrast, both these treatments decreased root biomass in SB- and SB + DOC-treated plants (Fig. 1(b)).

In this soil, mycorrhizal inoculation was a critical factor for obtaining mycorrhizal plants. Significant differences were found between the lengths of AM-colonized root in amended and non-amended plants (Fig. 2(a)).

In AM-inoculated plants, the percentage of AM-colonization was increased by DOC, SB and DOC + SB amendments. The highest percentage of AM colonization was reached in dually (*Y. lipolytica* plus AM fungi) inoculated plants in amended soil. The application of *Y. lipolytica* significantly promoted AM infection in DOC-, SB- or DOC + SB-amended plants. These results indicate that in this natural arid soil the AM potential depended not only on the inoculation but also on the organic amendments. Without such amendments, the free-living microbial inoculum of *Y. lipolytica* did not affect the percentage of AM colonization, and inoculated mycorrhizal fungi colonized only a maximum of 10% of the total root length (Fig. 2(a)).

Plant nitrogen uptake was increased by the amendments applied. The effect of inoculations on plant N acquisition was important only for AM-inoculated plants growing in SB medium (Fig. 1(c)).

Nodules were not formed in non-inoculated plants. DOC increased nodulation, particularly in dually inoculated plants, and SB only increased nodule formation in single-inoculated plants. DOC + SB application reduced nodulation, which fell to zero in all the plants (Fig. 2(b)).

Dehydrogenase activity increased in treated agro-waste residue-amended soil, particularly when SB was added, the lowest value was obtained when AM fungi was inoculated. But in DOC-amended soil, non-significant responses to microbial treatments or dehydrogenase activity were found (Table 1).

Urease activity was highly increased in the rhizosphere of DOC- or SB-amended plants. The highest value was in the rhizosphere of mycorrhizal plants growing in DOC treatments (Table 1). The application of treated residues enhanced protease activity and *Y. lipolytica* significantly increased such activity in DOC-amended soil (Table 1). Values of pH in rhizosphere soil after plant growth show that the application of SB lowered pH, but the double inoculation with AM fungi plus *Y. lipolytica* increased pH values in non-treated and

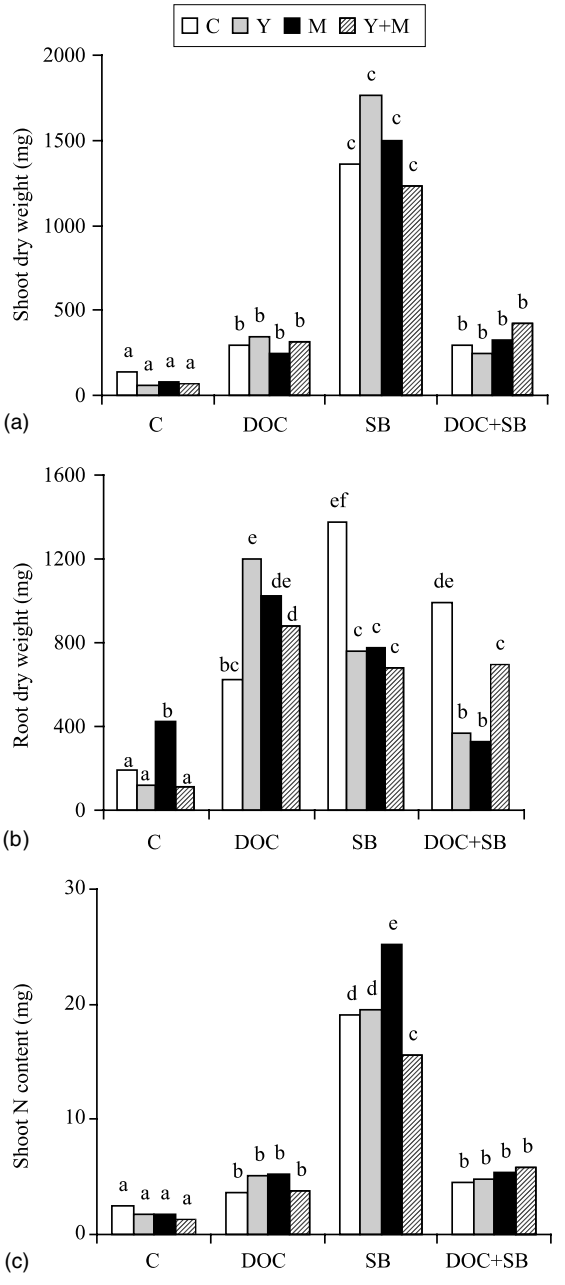

Fig. 1. Shoot dry weight (mg) (a), root dry weight (mg) (b) and shoot N content (mg) (c) of *Dorycnium* sp. plants treated or not with fermented organic residues from dry olive cake (DOC), sugar beet (SB) or both (DOC + SB) and inoculated or not with *Y. lipolytica* (Y), AM fungi (M) or both inocula (Y + M). Values not sharing a letter in common differ significantly ($P < 0.05$ from each other) as determined by Duncan's multiple range test.

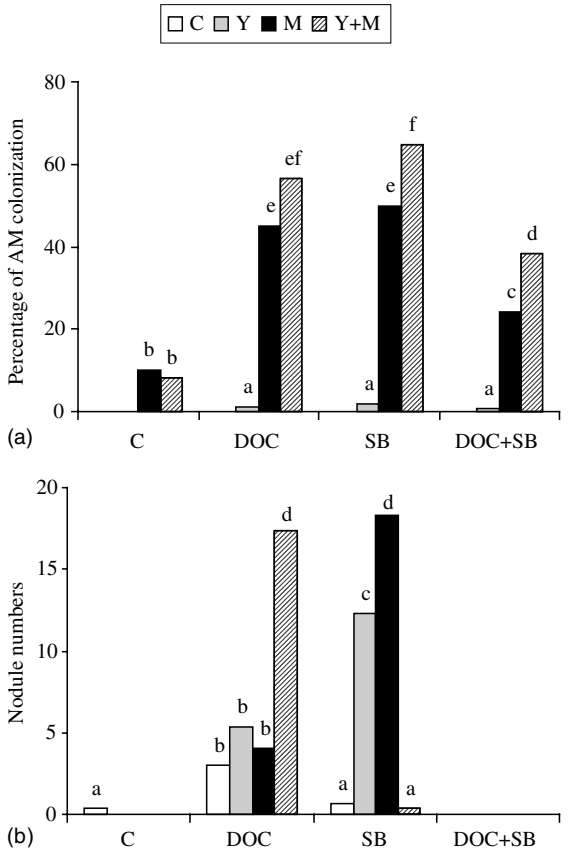

Fig. 2. Percentage of AM colonization (a) and number of nodules (b) in *Dorycnium* sp. plants treated or not with fermented organic residues from dry olive cake (DOC), sugar beet (SB) or both (DOC + SB) and inoculated or not with *Y. lipolytica* (Y), AM fungi (M) or both inocula (Y + M). Values not sharing a letter in common differ significantly ($P < 0.05$ from each other) as determined by Duncan's multiple range test.

in all treated soils (Table 2). Conversely, the electrical conductivity was the highest when organic residue was added. *Y. lipolytica* reduced the electric conductivity in amended soil particularly when AM inoculum was added (Table 2).

Natural mycorrhizal spore populations in rhizosphere soil were increased in all treatments inoculated with *Y. lipolytica* and this effect was more significant in single DOC-treated soil. Nevertheless, the combination of *Y. lipolytica* plus AM inoculum did not affect or decreased (compared with single DOC or SB treatments) the number of AM spores in rhizosphere soil (Table 2).

4. Discussion

The aim of this study was to investigate how management practices involving microbial inoculations and organic amendments could affect soil biological properties and fertility. The results show that plant growth and nutrition as well as soil biological values were greatly increased by the management practices used here.

Inputs are critical for plant development under such conditions as was already reported by Barea et al., 1992a. As expected, *A. niger* exhibited cellulosic activity and RP-solubilizing ability, using these substrates as hydrocarbon sources (Vassilev et al., 1986). In addition, the effectiveness and performance of inoculant applied as plant growth-promoting microorganisms was only evident in organic-amended soil.

The results from the present study demonstrate that indigenous AM fungi or *Rhizobium* were not able to colonize *Dorycnium* plants. These findings indicate that natural symbiotic colonization in desertified soils is extremely low (Herrera et al., 1993a,b; Azcón and Barea, 1997). Nevertheless, the organic DOC or SB amendments increased the AM spore population along the three months after application. These results agree well with those reported by Douds and Schenck (1990). But along the experimental period the amendments did not affect the AM colonization, which continued being zero. AM inoculation was effective in increasing this value, particularly in amended soils. As Joner and Jakobsen (1994) reported we also found in this study a great increase in AM spore population provoked by each one of the organic amendments used. Values ranged from 319%, in DOC, to 761%, in SB.

Microbial processes are required for plant development in degraded areas and particular interest show N derived from N_2-fixation and arbuscular-mycorrhizal colonization, responsible for P uptake from soil deficient in available P.

AM colonization achieved by the mycorrhizal inoculation in SB-treated plants greatly increased the N uptake. In fact, nodule number was also increased in this treatment. The ability of AM fungi to increase N_2-fixation is a well-documented subject (Barea et al., 1992b, 2002). Moreover, AM fungi also improve N uptake from soil, as was assessed using ^{15}N under field and greenhouse conditions (Tobar et al., 1994; Azcón and El-Atrash, 1997; Mäder et al., 2000b).

Urease and protease activities have been directly implicated in the increased acquisition of N by plants. But regarding the present results, these activities were maximal in DOC-amended soil. Thus, such enzymatic activities seem not to be involved in the high effectiveness of AM inocula in the N nutrition of plants grown on SB added soil. Concerning revegetation studies, the most important step in plant establishment is to have a good root system development and treatments applied here were, in general, very effective for increasing root growth.

Table 1
Dehydrogenase activity (μg INF g^{-1}) urease (μg NH_3 g^{-1} h^{-1}) and protease (μmol NH_3 g^{-1} h^{-1}) activities in rhizosphere soil *Dorycnium* sp. plants treated or not with fermented organic residues from dry olive cake (DOC), sugar beet (SB) or both (DOC + SB) and inoculated or not with *Y. lipolytica* (Y), AM fungi (M) or both inocula (Y + M)

Microbial treatments	C	Organic residue		
		DOC	SB	DOC + SB
Dehydrogenase				
Control	39 a	75 bcd	86 de	66 bc
Y	40 a	70 bc	92 e	65 b
M	42 a	76 bcd	75 bcd	78 cd
Y + M	47 a	73 bc	90 e	71 bc
Urease				
Control	910 a	2160 ef	2100 def	1550 b
Y	1000 a	2150 ef	1980 cde	1720 bcd
M	790 a	2520 f	1710 bcd	1620 bc
Y + M	1140 a	2290 ef	2140 def	2030 cde
Protease				
Control	530 a	1460 c	1440 c	1180 bc
Y	540 a	1860 d	1380 bc	1170 bc
M	570 a	1610 c	1250 bc	1010 b
Y + M	610 a	1390 bc	1500 c	1160 bc

Values not sharing a letter in common differ significantly ($P \leqslant 0.05$ from each other) as determined by Duncan's multiple range test.

Table 2
Values of pH, electric conductivity (μS cm^{-1}) and mycorrhizal spore number in rhizosphere soil of *Dorycnium* sp. plants treated or not with fermented organic residues from dry olive cake (DOC), sugar beet (SB) or both (DOC + SB) and inoculated or not with *Y. lipolytica* (Y), AM fungi (M) or both inocula (Y + M)

Microbial treatments	C	Organic residue		
		DOC	SB	DOC + SB
pH				
Control	8.65 f	8.55 de	8.37 a	8.42 ab
Y	8.75 g	8.52 cd	8.39 ab	8.42 ab
M	8.61 ef	8.58 def	8.45 bc	8.45 bc
Y + M	8.88 h	8.73 g	8.58 def	8.55 de
Electric conductivity				
Control	358 ab	644 efg	712 fgh	759 h
Y	259 a	554 de	546 cd	601 ef
M	370 abc	699 fgh	750 gh	802 h
Y + M	299 a	454 bcd	486 d	531 de
Mycorrhizal spore number				
Control	36 a	223 bcd	274 de	86 abc
Y	114 abcd	553 f	434 ef	148 abcd
M	113 abcd	255 cd	296 de	121 abcd
Y + M	66 ab	115 abcd	54 ab	84 abc

Values not sharing a letter in common differ significantly ($P \leqslant 0.05$ from each other) as determined by Duncan's multiple range test.

The rhizosphere effect on enzymatic activities has been studied (Reddy et al., 1987), but no information is available about the effect of treated agrowastes residues on these activities. In the degraded soil used, the activity of microbiota is low because of the lack of suitable organic substrates. This is not a limiting step if agrowaste amendments are incorporated into the unfertile soil.

Thus, the higher values for dehydrogenase activity in the rhizosphere of amended soils reflect higher oxidative activities of the soil microflora (Skujins, 1976) which is indicative of a better functioning and fertility in the treated soil.

Parameters related with rhizosphere microbial activities are used as biomarkers of soil rehabilitation pro-

cesses (Naseby and Lynch, 1997; Caravaca et al., 2002c), we clearly see higher microbial activities in agrowaste-treated soils than in the natural degraded soil used here.

The decrease in the rhizosphere pH caused by treated amendments may be the result of RP solubilization by *A. niger* in the fermentation process previous to the bioassay. This effect was previously reported by Vassilev et al. (1996) and Rodríguez et al. (1999).

Also values of electrical conductivity were highest in amended soil and the inoculation with *Y. lipolytica* decreased soil electrical conductivity, which is an indication of the positive effect of this yeast in reducing this factor that may be detrimental at a high level. Here, *Y. lipolytica* was able to increase the number of autochthonous AM spores by 317% (unamended soil), 244% (in DOC-amended medium), or 158% (in SB-amended medium). This effectiveness of *Y. lipolytica* on AM spore number was not related to an improvement in the AM colonization but it could affect subsequent mycorrhizal development since the spores, as fungal propagules, have a dormancy period and are not viable as inocula until the next period of root growth. The yeast effect on extraradical fungal mycelium development was not here evaluated.

The increased AM spore population caused by *Y. lipolytica* underlines the importance of this yeast as a factor contributing to the mycorrhizal potential in this stressed soil. The ability of this yeast for solubilizing rock phosphate (Vassilev et al., 2001) may be involved in the found effects.

In conclusion, the application of agrowastes to degraded lands may be an useful practice because of the high content of organic and inorganic plant nutrients in these residues. According to these results, the application of treated agrowastes as DOC or SB is able to improve soil quality and fertility and thus can be considered a practice of great interest, in particular if such residues, when not previously microbially treated, have environmental problems, as in the case of DOC (Pérez et al., 1986; Paredes et al., 1987).

In degraded land, this practice can be recommended, since agrowaste decomposition by *A. niger* increased the content of organic and inorganic plant nutrients available to improve plant growth, particularly in interaction with selected beneficial microorganisms (AM fungi, *Y. lipolytica*, *Rhizobium* sp.), as well as improving soil biochemical and biological characteristics.

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