

Dry matter and root colonization of plants by indigenous arbuscular mycorrhizal fungi with physical fractions of dry olive mill residue inoculated with saprophytic fungi

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Abstract

We studied the influence of indigenous arbuscular mycorrhizal (AM) and saprobe fungi on the phytotoxicity of the physical fractions of dry olive mill residue (DOR). The physical extractions of DOR gave an aqueous (ADOR) and an exhausted (SDOR) fraction with less phytotoxicity for tomato than the original samples. The indigenous AM were able to decrease the phytotoxicity of SDOR inoculated with *Trametes versicolor* and *Pycnoporus cinnabarinus* on tomato. However, incubation of ADOR with both saprophytic fungi did not decrease its phytotoxicity in presence of the indigenous AM fungi. The percentage of root length colonized by indigenous AM strongly decreased in presence of DOR, around 80% of decrease at dose of 25 g kg⁻¹ of DOR, but the level of mycorrhization was higher in presence of ADOR or SDOR (38% and 44% of decrease respectively at the same dose). There were no relationships between the effects of the physical fractions of DOR incubated with the saprobe fungi on AM colonization and on plant dry weight of tomato. Our results suggest that the phytotoxicity of the olive residues can be eliminated by the combination of physical extraction and by saprobe fungal inoculation and the use of this agrowaste as organic amendment in agricultural soil may be possible.

Additional key words: biofertilizer, bioremediation, olive waste, phytotoxicity, saprobe fungi, *Solanum lycopersicum* L.

Resumen

Materia seca y colonización de raíces de plantas por hongos micorrízico arbusculares autóctonos en presencia de fracciones físicas de alpeorujo seco y extractado inoculado con hongos saprobios

En este trabajo se ha estudiado la influencia de los hongos micorrízicos arbusculares autóctonos y hongos saprobios en la fitotoxicidad de las fracciones físicas del alpeorujo. La extracción física del alpeorujo con agua produjo un extracto acuoso (ADOR) y un sólido extractado (SDOR) con menor fitotoxicidad para las plantas de tomate que los controles con alpeorujo. Los hongos arbusculares autóctonos son capaces de disminuir la fitotoxicidad de SDOR inoculado con *Trametes versicolor* y *Pycnoporus cinnabarinus* en plantas de tomate. Sin embargo, la incubación del ADOR con dichos hongos no modificó la fitotoxicidad del residuo en plantas de tomate cultivadas en suelo no estéril. El porcentaje de longitud de raíz colonizada por los endófitos arbusculares autóctonos disminuyó drásticamente en presencia de alpeorujo (alrededor de un 80% de disminución a la dosis de 25 g kg⁻¹ de DOR) aunque el nivel de micorrización fue superior en presencia de ADOR o SDOR (38 y 44% de disminución a la misma dosis). No se ha encontrado relación entre los efectos de las fracciones físicas del alpeorujo incubado con hongos saprobios en la colonización arbuscular y el peso seco de las plantas de tomate. Nuestros resultados indican que la fitotoxicidad de los residuos del olivar puede ser eliminada por el uso combinado de una extracción física y una incubación biológica con hongos saprobios; por tanto podemos considerar el uso de estos residuos como enmendantes orgánicos.

Palabras clave adicionales: biofertilización, biorremediación, fitotoxicidad, hongos saprobios, residuos de aceituna, *Solanum lycopersicum* L.

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Abbreviations used: ADOR (aqueous dry olive mill residue), AM (arbuscular mycorrhizal), DOR (dry olive mill residue), SDOR (exhausted dry olive mill residue).

Introduction

The olive oil industry generates large amounts of byproducts that are harmful to the environment (Morillo *et al.*, 2009). The two-phase extraction process generates a liquid phase (olive oil) and a water-rich solid organic waste (alpeorujo), which is dried and extracted with solvents to obtain an extra yield of oil and the dry olive-mill residue (DOR). It has been estimated that the annual production of DOR in Spain approaches 4 million tons (Alburquerque *et al.*, 2004).

The agronomic utilisation of DOR has increased steadily in recent years as an alternative nutrient and organic matter source, and as an acceptable method for its disposal. However, the use of DOR has been seen to have a detrimental effect on seed germination, plant growth and microbial activity (Martín *et al.*, 2002). In fact, several studies have reported phytotoxic and antimicrobial effects of this residue due to the phenol (Aranda *et al.*, 2007; Sampedro *et al.*, 2007a). Efforts to decrease the environmental impact of olive-mill wastes include biological fermentation with saprophytic fungi (Linares *et al.*, 2003; Giannoutsou *et al.*, 2004; Sampedro *et al.*, 2007b). It has been shown that phytotoxicity of DOR can be reduced by incubating it with some immobilized saprophytic fungi for 4 weeks (Sampedro *et al.*, 2009). Also short period of incubation can reduce the toxic effect of water soluble substances in DOR on plants growth under hydroponic conditions (Aranda *et al.*, 2006).

Most agricultural soils in the Mediterranean regions contain small amounts of organic matter (Caravaca *et al.*, 2002), which is a further limiting factor for plant growth and production. The recycling of agro-industrial residues to replenish soils with nutrients, otherwise provided by chemical fertilizers, is an important part of sustainable agriculture. This practice can also help solve environmental hazards and economic costs related to the disposal of olive wastes (Brunetti *et al.*, 2005). In some soil the lack of suitable organic substrates made very low the development and activity of the microbiota. Nevertheless, this is not limiting step if agrowaste amendments are incorporated into the unfertile soil (Medina *et al.*, 2005).

In order to assess the fertilizer value of the transformed DOR by saprophytic fungi, it is important to determine the role of arbuscular mycorrhizal (AM) fungi in modifying the toxic effects of the olive mill residues. There is evidence that AM fungi play a key role in soil fertility and in plant nutrient acquisition (Requena *et al.*, 1996) and may also help the plant to

thrive under polluted conditions (Shetty *et al.*, 1994). The ability to decrease phytotoxicity of toxic residues seems to depend on which AM endophytes are present (Volante *et al.*, 2005). Some of the AM fungi increased the phytotoxicity of DOR in agronomic plant (Martín *et al.*, 2002). However, *G. deserticola* decrease the sensitivity of plant to the phytotoxicity of the olive residues and the aqueous and exhausted fraction of this residue (ADOR and SDOR respectively) treated with saprophytic fungi. Plant growth promotion in response to alpeorujo treated with saprobe fungi was accompanied by increased AM root colonization with introduced AM fungi (Sampedro *et al.*, 2008; Aranda *et al.*, 2009). However, it is unknown how treated alpeorujo affects the indigenous AM fungi.

The aim of this study was to determine the role of indigenous AM fungi in modifying the toxic effect of the water soluble and exhausted fractions from olive oil residue transformed by saprophytic fungi, on the dry weight of tomato.

Material and methods

Materials

DOR was collected from an olive oil manufacturer (Sierra Sur S.A., Granada, Spain). The main characteristics of DOR determined by Sampedro *et al.* (2007b) were as follows: total organic carbon (TOC), 58.5%; total nitrogen (TN), 1.87%; total phosphorus, 0.21%; lignin, 24.7%; cellulose, 18%; hemicellulose, 11.4%; total phenols, 2.65%; total lipids, 0.2%; ashes, 9.2%. The most abundant elements, the concentration of which is reported in g kg⁻¹ DOR were: K 30.5, Mg 3.8, Ca 13.6, Na 0.17, Fe 1.1, Cu 0.07, Zn 0.06, Mn 0.04.

An aqueous extract from dry DOR was obtained by orbital-shaking this residue with distilled water in a proportion of 1:2 (w/v) for 8 h. The suspension obtained was filtered through several layers of cheesecloth. The filtered (ADOR) and the solid residue obtained after the aqueous extraction (SDOR) that has been incubated or not with the saprobe fungi were tested to determine their phytotoxicity.

Trametes versicolor (A-1369) and *Pycnoporus cinnabarinus* (CECT 20448) were obtained from the Culture Collection of Centro de Investigaciones Biológicas (CIB) of Madrid. Stock cultures were kept at 4°C on 2% malt extract agar. The inoculum for ADOR was produced by growing the fungus under orbital shaking

at 125 rpm and 28°C on Czapek in the presence of 50% ADOR extracts for 15 days. The mycelium was collected by filtration, extensively washed with distilled water and homogenized. The fungi were grown in Erlenmeyer flask (250 mL) containing 70 mL of ADOR extract for 15 days at 28°C with orbital shaking at 125 rpm. Each flask was inoculated with 0.45 g L⁻¹ of each inoculum. For the incubation process of SDOR barley seeds were inoculated with a thin slice of PDA (1 × 1 cm²) with mycelia of a 14 days old culture of saprobe fungi grown at 28°C. Erlenmeyer flask containing 125 g of SDOR steam-sterilized were inoculated with 8 barley seeds grown with the saprobe fungi under static incubation at 28°C for 20 weeks. Appropriate controls were prepared by incubating non-inoculated sterilized ADOR and SDOR under the same conditions used for fungal cultures.

Phytotoxicity experiment

The phytotoxicity experiments were carried out in 0.3 L pots containing non sterilized soil mixed with sterilized quartz sand 1:1 by volume. The soil used was a grey loam 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. The P, N, K, Fe, Mn, Cu and Zn contents were determined by the methods of Lachica *et al.* (1965). These were NaHCO₃-extractable P 6.2 mg kg⁻¹, N 2.5 mg kg⁻¹, K 132 mg kg⁻¹, Fe 9.6 g kg⁻¹, Mn 110 mg g⁻¹, Cu 5.8 mg g⁻¹, Zn 5.7 mg kg⁻¹.

Tomato (*Solanum lycopersicum* L.) was used as the test plant. Seeds were sterilised, pregerminated and

selected for uniformity prior to transplanting. Plants were grown in a greenhouse with supplementary light provided by Sylvania incandescent and cold-white lamps, 400 nmol m⁻² s⁻¹ at 400-700 nm, with a 16/8 h day night cycle at 25/19°C and 50% relative humidity. Plants were watered from below, and were fed with 10 ml nutrient solution per week (Hewitt, 1966).

DOR, ADOR and SDOR were applied to the 0.3 L soil pots at concentrations of 0, 12.5 and 25 g kg⁻¹ soil:sand mixture.

Plants were harvested after 4 weeks and their dry mass was determined. After the harvest fresh samples of roots were taken from the entire root system at random. The samples were cleared and stained using trypan blue in lactic acid (Phillips and Hayman, 1970), and the percentage of root length colonization was measured by the line-intersect method (Giovannetti and Mosse, 1980).

Statistical treatment of data

The data were subjected to one-way ANOVA. The mean values of four replicate pots were compared using the LSD test ($P=0.05$). Percentage data were subjected to arcsine transformation before analysis.

Results

The application of olive mill residue and all the physical fractions at the dose of 25 g kg⁻¹ reduce the shoot dry weight related to that of plants grown in absence of DOR in non sterilized soil (Fig. 1a). The phytotoxic

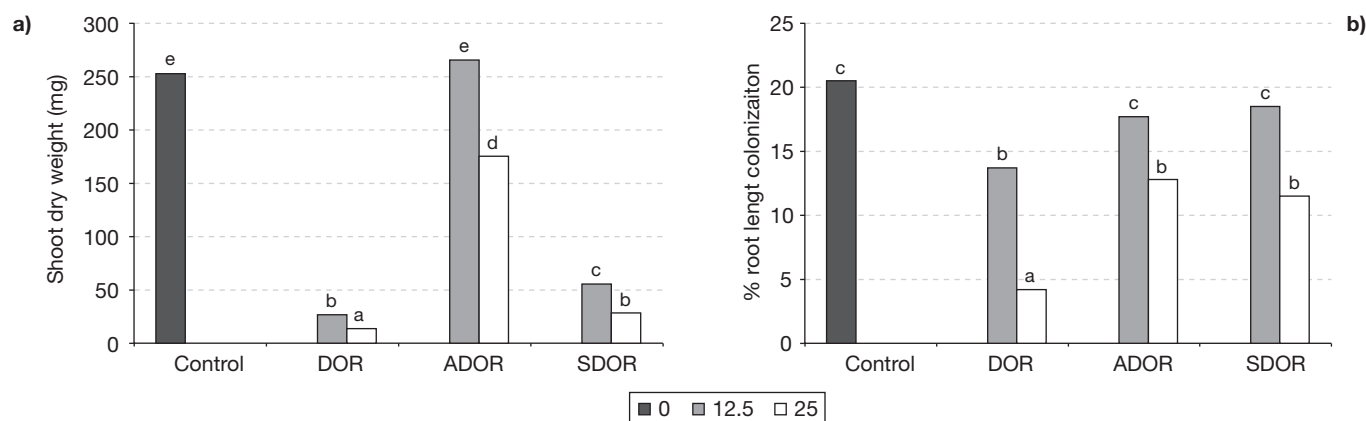


Figure 1. Shoot dry weight (a) and root length (b) in tomato (*Solanum lycopersicum* L.) grown in non sterilized soil in presence of different concentrations (0, 12.5 and 25 g kg⁻¹) of dry olive mill residue (DOR), aqueous extract of dry olive mill residue (ADOR) and solid residue obtained after the aqueous extraction (SDOR). Same lowercase letters above bars indicate lack of statistical significance ($P \leq 0.05$) between different treatments.

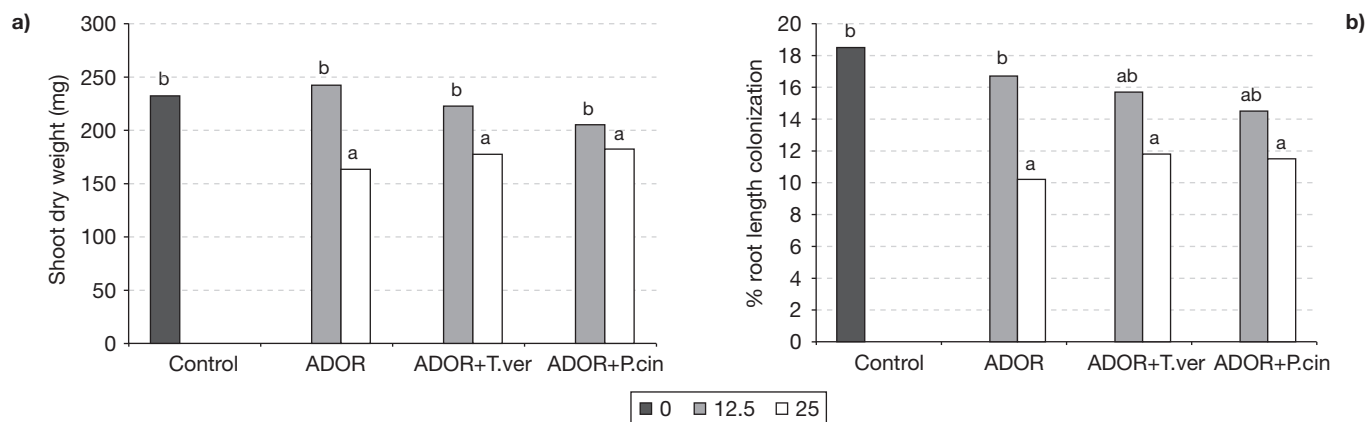


Figure 2. Shoot dry weight (a) and root length (b) in tomato (*Solanum lycopersicum* L.) grown in non sterilized soil in presence of different concentrations (0, 12.5 and 25 g kg⁻¹) of aqueous extract of dry olive mill residue (ADOR) incubated with *Trametes versicolor* (T. ver) and *Pycnoporus cinnabarinus* (P. cin). Same lowercase letters above bars indicate lack of statistical significance ($P \leq 0.05$) between different treatments.

effect of DOR was higher than that of ADOR or SDOR. The shoot dry weight was lower in presence of SDOR than in presence of ADOR. No phytotoxic effect was found in the presence of 12.5 g kg⁻¹ of ADOR (Fig. 1a).

The percentage of AM root length colonization of plants grown in non-sterilized soil decreased in presence of 25 g kg⁻¹ of DOR, ADOR or SDOR. However, the level of mycorrhization reached by tomato was only diminished in presence of 12.5 g kg⁻¹ of DOR (Fig. 1b).

There were no significant differences between the shoot dry weights of plants grown in non-sterilized soil with 12.5 g kg⁻¹ of ADOR. However doses of 25 g kg⁻¹ of the residue decrease the shoot dry weight of tomato plants relative to that of plants grown in absence of this residue. Incubation of ADOR with *T. versicolor* and *P. cinnabarinus* did not decrease its toxicity to tomato plants (Fig. 2a).

A similar percentage of AM tomato root length was colonized by the indigenous endophytes when plants were grown in presence of low doses of ADOR and when they were grown in absence of ADOR (Fig. 2b). Only a reduction of the level of mycorrhization was found in the tomato plant when they were added with 25 g kg⁻¹ of olive residue either alone or incubated with the saprophytic fungi. The incubation of ADOR with the saprophytic fungi did not change the percentage of tomato root colonized by the indigenous AM fungi (Fig. 2b).

The application of SDOR on tomatoes grown in non sterilized soil, without incubation with saprophytic fungi, reduced the shoot dry weight of tomato plants (Fig. 3a). As Figure 3 shows the dry weight of tomatoes

grown in presence of SDOR incubated with the saprophytic fungi increased compared with that of plants grown in presence of SDOR not incubated with saprophytic fungi.

The application of low doses of SDOR had no effect on the level of mycorrhization reached by the AM indigenous fungi (Fig. 3b). The application of SDOR inoculated with *P. cinnabarinus* at both doses and *T. versicolor* at the highest one decreased the percentage of AM root length colonization of tomato to a lower level than when this residue was not incubated with the saprobe fungus (Fig. 3b).

Discussion

DOR is strongly phytotoxic and hinders tomato growth to a great extent. In fact, several authors have indicated that olive mill residue has phytotoxic effects that are particularly evident during the germination and seedling development of higher plants (Casa *et al.*, 2003; Sampedro *et al.*, 2004). It is generally accepted that olive residue phytotoxicity is largely due to the presence of water soluble substances, although other DOR constituents such as volatile fatty acids, aldehydes and alcohols have also negative effect on plant growth (Komilis *et al.*, 2005). Ginos *et al.* (2006) reported that the phytotoxicity of olive mill wastewater decreased considerably after various physical pre-treatments. This was attributed to the removal of phytotoxic substances from the liquid phase. The physical extraction methods of DOR tested in our experiments gave an

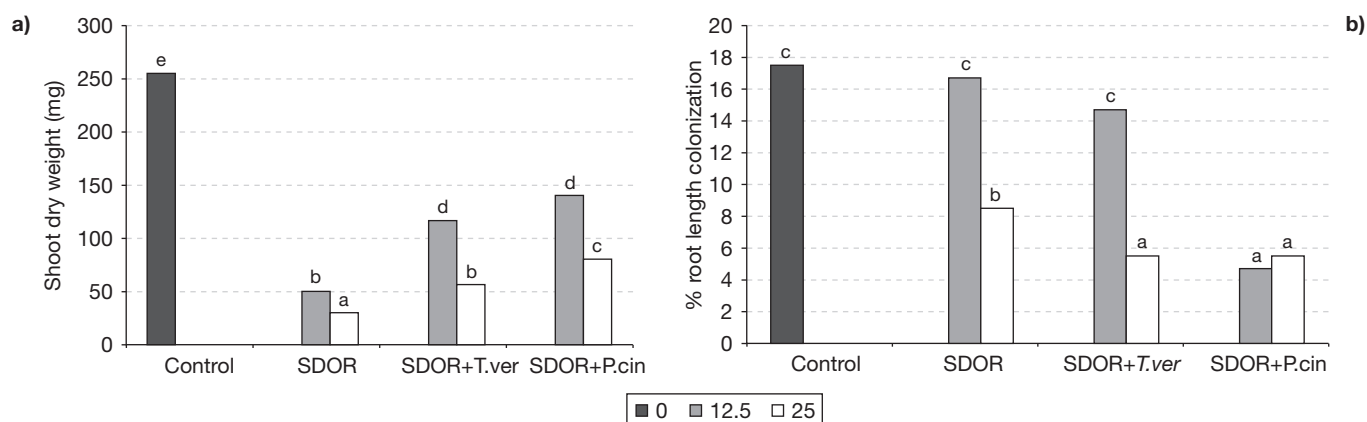


Figure 3. Shoot dry weight (a) and root length (b) in tomato (*Solanum lycopersicum* L.) grown in non sterilized soil in presence of different concentrations (0, 12.5 and 25 g kg⁻¹) of solid residue obtained after the aqueous extract of dry olive mill residue (SDOR) incubated with *Trametes versicolor* (*T. ver*) and *Pycnoporus cinnabarinus* (*P. cin*). Same lowercase letters above bars indicate lack of statistical significance ($P \leq 0.05$) between different treatments.

aqueous (ADOR) and an exhausted (SDOR) fraction with less phytotoxicity than the original DOR. However this physical treatment was not able to eliminate the toxicity.

Saprobe fungi were able to decrease the phytotoxicity of ADOR and SDOR on tomato but their effect was variable. In fact *T. versicolor* and *P. cinnabarinus* reduced the phytotoxic effect of SDOR on shoot dry weight of tomato grown in non sterilized soil, whereas any of the saprophytic fungi were able to eliminate the phytotoxicity of ADOR. The phytotoxicity of dry olive mill residues has been attributed mainly to phenols and the decrease of phenols in olive residues by saprobe fungi has been observed (Sampedro *et al.*, 2004; Aranda *et al.*, 2006). However, they differed in their ability to decrease the toxicity of both residues on tomato grown in soil. These results indicated that saprobe fungi degrade different phenol compounds in both residues, which may have different effects on plant growth.

AM fungi may facilitate the action or transfer of toxic substances to the plants, increasing the sensitivity of plant to the toxicity of DOR and this sensitivity seems to be dependent on AM fungal species (Martin *et al.*, 2002; Bonanomi *et al.*, 2006). In fact, the sensitivity of tomato plant to the toxicity of DOR was different when they were colonized with *G. deserticola* or with the indigenous AM fungi (Sampedro *et al.*, 2009). The effect of AM fungi on plant sensitivity to the toxins in the different olive mill residue fractions is not known. However, the level of phytotoxic substances in soil, such as heavy metals and pesticides (Ocampo, 1993; Khan

et al., 2000), may be implicated in the increase of sensitivity and/or resistance of plants to olive residues conferred by the AM fungi. In our experiments we observed a beneficial effect of SDOR incubated with *T. versicolor* and *P. cinnabarinus* on the shoot dry weight of tomato grown in non sterilized soil that was eliminated in presence of ADOR. Therefore these effects can be attributed to the level of phenols because their decrease was higher in ADOR than SDOR.

The establishment of AM symbiosis seems to be greatly affected by the concentration of toxic compounds in the soil (Leadir *et al.*, 1997; Medina *et al.*, 2005). As was previously observed, the percentage of root length colonized by *G. deserticola* or by the indigenous AM fungi strongly decreased in presence of DOR (Martín *et al.*, 2002). It has been suggested that DOR does not affect AM fungi directly and its negative effect are mediated by the plant (Martín *et al.*, 2002). The effect of ADOR on AM colonization also seems to occur through the plant. In fact the decrease of AM root length colonization of tomato caused by ADOR fractions paralleled the decrease of the plant dry weight. However no relation between the effect of DOR on plant dry weight and AM colonization. The physical fraction of DOR incubated with the saprobe fungi *T. versicolor* and *P. cinnabarinus* had different effects on the percentage of AM colonization of tomato depending more on the physical fraction than on the effect through the plant because no relationship between the effect on AM root colonization and plant dry weight was observed. Neither of the saprobe fungi contributed to the improvement of AM colonization of plants when

they were grown in presence of ADOR and SDOR either incubated with *T. versicolor* and *P. cinnabarinus*. The fact that in general some of the fungus decreased or not increase the percentage of root length colonization may be due to the very complex mechanisms of the saprobe fungi exudates on the AM fungal colonization of roots (Fracchia *et al.*, 2004).

Close relationships between the percentage of AM colonization and its effect on plant growth are not commonly found (García-Garrido *et al.*, 2000; Adriano-Anaya *et al.*, 2006). In our experiments no relationship between the percentage of AM colonization and plant dry weight of tomato affected by the physical fractions of DOR incubated with the saprobe fungi was found.

In general the application of ADOR and SDOR-cubated with the saprobe fungi maintain or increased the shoot dry weight of tomato grown in non sterilized soil which indicates that with the combination of physical and biological treatments the use of this agrowaste as organic amendment in agricultural soil may be possible. However, as the AM fungi is essential to the beneficial effect on plant dry weight caused by the residues incubated with saprobe fungi, the role of soil microorganisms in the effect of agrowaste application to soil must be taken into account.

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