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In vitro Assessment of the Antifungal Activity of Several Compost Extracts Obtained from Composted Animal Manure Mixtures

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Abstract: *In vitro* experiments by direct confrontation, were conducted to assess the inhibitory effect of nine compost extracts, made with different mixtures of animal manure proportions, on some phytopathogenic fungi (*Fusarium oxysporum* f. sp. *radicis-lycopersici*, *F. solani*, *F. graminearum*, *Fusicoccum amygdalis*, *Alternaria* sp., *Colletotrichum coccodes*, *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Aspergillus niger*, *Rhizoctonia solani*, *R. bataticola*, *Pythium* sp. and *Verticillium dahliae*). Compost extracts decreased the radial growth of all fungi tested, except for *Aspergillus niger*. In fact, fungal radial growth inhibition ranged from 0 for *A. niger* to 63.17% for *V. dahliae*. Higher antifungal activity was also noted against *F. oxysporum* f. sp. *radicis-lycopersici* and *F. solani* with 38.12 and 31.87%, respectively. However, lesser inhibition, of about 10.33%, was obtained against *R. bataticola*. Compost extract C2, based on 60% cattle manure, 30% sheep manure and 10% ground straw and compost extract C7, based on 40% cattle manure, 40% sheep manure and 20% vegetable wastes, were found to be the most effective against the fungi tested. These extracts contain an important microflora which seem to be involved in this antifungal activity.

Key words: Compost microflora, inhibition ratio, mycelial growth, phytopathogenic fungi

INTRODUCTION

Several reports showed that composts prepared from heterogeneous organic wastes (animal manures, bark biosolids, grape marcs, vegetable wastes) have the potential to provide biological control of plant diseases caused by soilborne pathogens (Hoitink *et al*., 1997). These composts contain an important microflora (bacteria, fungi and actinomycetes) that can improve soil microbial population and reduce severity of plant diseases. Nelson and Boehm (2002) reported that chicken manure compost enhanced the soil population by 68.4% of bacteria having suppressive effects on *Pythium aphanidermatum*. Aryantha *et al.* (2000) showed that the application of 25% of animal manures compost increased the organic matter content of substrates, the total biological activity and the population of actinomycetes, fungi and bacteria. This type of compost reduced significantly the development of *Phytophthora cinnamomi* on *Lupinus albus* seedlings.

Compost extracts (or compost teas) are shown to be efficacious in the biocontrol of several plant diseases caused by fungi (Ingham, 2002). Elad and Shtienberg (1994) showed that compost water extracts prepared from animal manures reduced the incidence of leaf grey mould caused by *Botrytis cinerea* by more than 56%. Tratch and Bettiol (1997) showed that sprays with compost extracts, diluted at 10%, inhibited the mycelial growth of *Rhizoctonia solani*, *Fusarium oxysporum*, *Botrytis*

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cinerea, *Alternaria solani* and *Septoria lycopersici* on several plants. Zhang *et al.* (1998) reported that compost water extracts sprays reduced severity of *Pseudomonas syringae* populations in *Arabidopsis thaliana*. In *in vitro* and *in vivo* experiments, Znaidi (2002) found that compost extracts reduced the development of *Phytophthora* spp. and *Fusarium solani* on potato tubers by 50%. However, compost extracts differing in their initial composition in ability to suppress diseases (Scheuerell and Mahaffee, 2002). In fact, Nelson and Boehm (2002) reported that extracts prepared from 50:50 mixture of chicken manure and cow manure composts were more efficient in reducing root rot caused by *Pythium* sp. than others prepared from leaves.

In this report, we present a preliminary assay of the *in vitro* antifungal activity of some compost extracts, prepared from various animal manures and differing in initial composition, against some phytopathogenic fungi for their eventual use *in vivo* experiments.

MATERIALS AND METHODS

Initial Composts

Tested extracts were prepared from nine mature composts (>12 months), based on animal manures: Cattle manure, sheep manure, chicken manure and horse manure. Some vegetable wastes and ground straw were also added to some of them (Table 1). They were produced by the composting-unit of the Technical Center of the Organic Agriculture of Chott Mariem (Tunisia) according to an aerobic process described by Znaidi (2002).

Compost Extracts Preparation

The compost extracts were prepared according to the procedure of Weltzien (1992) and Brinton *et al.* (1996). In fact, composts were suspended in tap water (1:5, vol/vol) in large containers. The mixture was stirred for about 10 minutes every day during an extraction period of 5 days. Subsequently, the extracts were filtered through cheesecloth (250 µm) and stored at 4°C. They were taken out 30 min before use (Znaidi, 2002).

Tested Phytopathogenic Fungi

Some phytopathogenic fungi such as *Fusarium oxysporum* f. sp. *radici-lycopersici*, *F. solani*, *F. graminearum*, *Fusicoccum amygdalis*, *Alternaria* sp., *Colletotrichum coccodes*, *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Aspergillus niger*, *Rhizoctonia solani*, *R. bataticola*, *Pythium* sp. and *Verticillium dahliae*, causing some plant diseases in Tunisia, were tested in this study. Fungal isolates were grown on Potato-Dextrose Agar (PDA) at 25°C and stored at 4°C for long term preservation.

In Vitro Test of the Antifungal Activity of Compost Extracts

Extract's antifungal activity was tested via a direct confrontation on agar plates (PDA). In fact, an active mycelial disk (6 mm in diameter) of the pathogen was plated, 1 cm from the edge of a 9 cm Petri plate containing freshly prepared PDA medium. In a diametrically opposed position, 1 cm away from the other set of the plate, a well was occasioned using a sterile 6 mm cork borer, where 50 µL of raw compost extract was poured (El-Masry *et al.*, 2002).

Table 1: Initial composition of composts used for extracts preparation

Composts	Composition
C1	50% CM+ 25% SM+25% PM
C2	60% CM + 30% SM + 10% ground straw
C3	50% CM + 25% SM + 25%HM
C4	50% CM + 20% SM + 20%PM+10% ground straw
C5	25% CM + 25% SM + 25%PM+25%HM
C6	30% CM + 30% SM + 30% PM+10% ground straw
C7	40% CM + 40% SM + 20 % vegetable wastes
C8	25% CM + 25% SM + 25%PM+15%HM+10% ground straw
C9	25% CM + 25% SM + 25%PM+25%HM

C1-C9: compost1-compost 9; CM: Cattle Manure; SM: Sheep Manure; PM: Poultry Manure; HM: Horse Manure

Fifty microliter of distilled water were used for the control plates. The inhibition of the pathogen growth was determined after incubation at 25°C for 4 to 24 days according to the tested fungi. Ten plates were used per elementary treatment.

For each fungus, the inhibition ratio of each compost extract was determined when the radial growth in the control colonies reached the edge of the plates. This ratio was calculated according to the following formula used by Hibar *et al.* (2005) where:

Inhibition Ratio (%) = (1-(average treated colony diameter/average control colony diameter))*100. This study was conducted on March 2006.

Microbial Population Density and Content of Initial Composts

Initial solid composts, source of the tested extracts, were also analyzed for their microbial density and content using three different media. Nutrient Agar (NA; Oxoid) medium (36 g L^{-1}) and Potato Dextrose Agar (PDA; Sigma) supplemented with 5 mg L^{-1} Penicillium-G were used for bacterial and fungal isolations, respectively. Actinomycetes colonies were isolated and counted on Water Yeast Agar (WAY) medium based on yeast extract (Oxoid) (0.25 g L^{-1}), K_2HPO_4 (0.5 g L^{-1}), cycloheximide (Sigma) (0.05 g L^{-1}) and agar (Oxoid No. 3) (18 g L^{-1}).

Ten gram of each solid compost were suspended in 90 mL of autoclaved distilled water in 250 mL Erlenmeyer. The samples were stirred for 1 h at 200 rpm. A six successive dilutions (10^{-6}) were carried out.One hundred microlitre aliquots of different concentrations (10^{-2}, 10^{-4} and 10^{-6}) were spread on the surface of three replicate plates of NA, PDA and WAY media. Colonies were counted after incubation at 20°C for 3 (bacteria), 3-5 (fungi) and 10 days (actinomycetes) (El-Masry *et al.*, 1994; Diab *et al.*, 2003; McQuillen *et al.*, 1994). Populations were determined as the number of colonies per gram of compost. Identification was based on biochemical tests for bacteria and microscopic tests for fungi.

Experimental Design and Statistical Analyses

Petri plates used in this experiment were distributed in a completely randomized design with ten replications per elementary treatment. Phytopathogenic fungi were divided into 8 groups (Table 2) according to the incubation period required for their maximal growth in the control plates (90 mm).

An analysis of variance (ANOVA) was performed for data using the SPSS statistical program version 11.0. Means were compared by the Duncan test and if p values indicated a significant difference ($p \leq 0.05$). Means were separated by Fisher's Least Significant Difference (LSD).

Table 2: Different assays of direct confrontation following the incubation time of the fungi tested

Essay No.	Tested phytopathogenic fungi	Incubation period (in days)
1	*Pythium* sp. *Rhizoctonia solani*	4 days (Daami-Remadi, 2001; McQuilken *et al.*, 1994)
2	*Rhizoctonia bataticola* *Sclerotinia sclerotiorum* *Aspergillus niger*	5 days (McQuillen *et al.*, 1994; Nelson and Boehm, 2002; Sta Baba *et al.*, 2006)
3	*Botrytis cinerea* *Fusarium graminearum*	6 days (Ben Ahmed, 2001; Zhang *et al.*, 2002)
4	*Fusarium oxysporum* f. sp. *radicis lycopersici*	7 days (Hibar *et al.*, 2005)
5	*Alternaria* sp. *Colletotrichum coccodes*	8 days (Daami-Remadi and El Mahjoub, 2004)
6	*Fusarium solani*	9 days (Daami-Remadi, 1996)
7	*Fusicoccum amygdalis*	10 days (Bladgett *et al.*, 2002)
8	*Verticillium dahliae*	24 days (Jabnoun-Khiareddine *et al.*, 2006)

RESULTS

In vitro Antifungal Activity of Compost Extracts

Results showed that the tested compost extracts showed a variable interaction with the tested phytopathogenic fungi. In fact, the mycelial growth of *Rhizoctonia solani* and *Pythium* sp. were significantly reduced by 10.8 to 20% and by 9 to 17%, respectively and this after 4 days of incubation at 25°C, in comparison to the untreated controls. However, all extracts did not show an inhibitory effect on *Aspergillus niger* (Table 3).

The compost extract C2 was the most effective against *Rhizoctonia bataticola* with 23.5%. A similar antifungal activity was also obtained by C6 against *Sclerotinia sclerotiorum* with 28.2% (Table 4).

The radial growth of *Botrytis cinerea* and *Fusarium graminearum*, noted after 6 days of incubation, was significantly reduced in comparison to the untreated controls and the most effective extracts were C7 and C1 showing inhibition ratios of 23.75 and 14.65%, respectively (Table 5).

Fusarium oxysporum f. sp. *radicis-lycopersici*, *Alternaria* sp. and *Colletotrichum coccodes* were significantly inhibited by the various compost extracts and mainly C7 where inhibition ratios were 42.6, 42.9 and 44.4%, respectively (Table 6 and 7).

Table 3: Inhibition ratio of the mycelial growth of *Rhizoctonia solani* and *Pythium* sp., due to the different compost extracts tested, after 4 days of incubation at 25°C on PDA

	Inhibition ratio (%)									
Fungi	C1	C2	C3	C4	C5	C6	C7	C8	C9	Mean
Rhizoctonia solani	16.5±4.5	20.1±4.0	11.6±3.1	12.6±3.3	10.8±3.4	13.1±3.9	14.3±3.9	18.6±4.5	13.5±3.9	14.6
Pythium sp.	11.0±2.6	9.1±2.7	12.2±2.7	14.7±2.6	15.9±2.5	15.3±2.4	14.5±2.7	17.1±2.4	9.1±2.2	13.21

LSD (Compost extracts x Fungi) = 0.52 at p≤0.05, Each value represents the mean of 10 values±standard error

Table 4: Inhibition ratio of the mycelial growth of *Rhizoctonia bataticola* and *Sclerotinia sclerotiorum*, due to the different compost extracts tested, after 5 days of incubation at 25°C on PDA

	Inhibition ratio (%)									
Fungi	C1	C2	C3	C4	C5	C6	C7	C8	C9	Mean
Rhizoctonia bataticola	8.2±3.9	23.5±6.0	17.7±5.3	5.7±3.6	3.4±2.8	6.0±3.8	15.9±5.3	5.1±3.6	7.4±3.8	10.33
Sclerotinia sclerotiorum	19.2±6.8	26.8±7.7	26.0±6.2	27.3±6.1	22.8±6.6	27.4±6.6	28.6±7.4	25.1±6.9	20.4±6.1	24.84

LDS (Compost extractsxFungi) = 0.56 at p≤0.05, Each value represents the mean of 10 values±standard error

Table 5: Inhibition ratio of the mycelial growth of *Botrytis cinerea* and *Fusarium graminearum* due to the different compost extracts tested, after 6 days of incubation at 25°C on PDA

	Inhibition ratio (%)-----									
Fungi	C1	C2	C3	C4	C5	C6	C7	C8	C9	Mean
Botrytis cinerea	14.1±5.2	22.8±6.3	22.0±6.4	15.2±5.2	22.1±6.7	19.3±5.6	24.9±5.2	16.8±6.0	20.2±6.4	19.71
Fusarium graminearum	15.2±5.4	15.2±5.4	15.9±5.5	19.1±6.5	23.2±6.4	19.0±5.8	22.6±5.0	23.2±4.7	22.5±5.1	19.54

LDS (Compost extractsxFungi) = 0.41 at p≤0.05, Each value represents the mean of 10 values±standard error

Table 6: Inhibition ratio of the mycelial growth of *Fusarium oxysporum*, due to the different compost extracts tested, after 7 days of incubation at 25°C on PDA

	Inhibition ratio (%)									
Fungi	C1	C2	C3	C4	C5	C6	C7	C8	C9	Mean
Fusarium oxysporum	34.7± 4.8ab	41.9± 3.7a	41.8± 5.5a	38.0± 4.9ab	32.6± 4.5b	26.4± 4.7b	42.6± 4.0a	40.5± 4.8ab	34.5± 5.2ab	38.12

Each value represents the mean of 10 values±standard error, Different letter (s) within columns represent values that are significantly different at p = 0.05 based on ANOVA and Duncan test

A significant inhibitory effect of the *Fusarium solani* growth, by 36.5%, was obtained after 9 days of incubation, after its dual culture with C2 compost extract. Moreover, all tested extracts inhibited *Fusicoccum amygdalis* and *Verticillium dahliae* (Table 8). The most important inhibition ratio, of about 76.2%, was reached by the extract C9 against *V. dahliae*.

The two extracts C2 and C7 were shown to be the most effective in reducing the mycelial growth of the tested phytopathogenic fungi. The highest inhibition ratios were 72.5 and 48.5%, respectively. The extracts C4 and C9 showed the most important suppressive effect against *Fusicoccum amygdalis* (42.6%) and *Verticillium dahliae* (76.2%).

Furthermore, the direct confrontation of compost extracts with the tested phytopathogenic fungi revealed some antagonistic reactions. In fact, an overlapping of the pathogen's colonies by some microorganisms present in the compost extracts was observed (Fig. 1). *Rhizopus* sp. and *Trichoderma* spp. were detected and they totally covered the plates showing a great competitive ability (Fig. 2). Compost extracts also induced, when confronted with some of the tested fungi, antibiosis zones (Fig. 3) between pathogen's and compost microorganisms colonies. However, the intensity of these phenomena was variable according to the compost extract and the tested phytopathogenic fungus.

Table 7: Inhibition ratio of the mycelial growth of *Alternaria* sp. and *Colletotrichum coccodes* , due to the different compost extracts tested, after 8 days of incubation at 25°C on PDA

	Inhibition ratio (%)									
Fungi	C1	C2	C3	C4	C5	C6	C7	C8	C9	Mean
Alternaria sp.	31.9±5.3	32.1±5.9	38.9±6.6	35.3±6.2	34.4±6.2	33.1±5.9	42.9±5.5	28.1±5.3	32.2±5.4	34.3
Colletotrichum coccodes	33.6±4.9	37.8±5.1	31.0±4.8	40.0±5.2	34.3±5,5	26.6±5.4	44.4±4.4	32.1±5.6	37.4±4.8	35.2

LDS (Compost extracts x Fungi) = 0.55 at $p \leq 0.05$, Each value represents the mean of 10 values±standard error

Table 8: Inhibition ratio of the mycelial growth of *Fusarium solani*, *Fusicoccum amygdalis and Verticillium dahliae* due to the compost extracts tested, after 9, 10 and 24 days of incubation at 25°C, respectively, on PDA

	Inhibition ratio (%)									
Champignons	C1	C2	C3	C4	C5	C6	C7	C8	C9	Mean
Fusarium	30.9±	36.5±	24.7±	32.3±	35.6±	29.1±	34.7±	27.5±	35.6±	31.87
solani	5.6ab	5.9a	5.4ab	5.4ab	5.7ab	6.0b	4.8a	5.2ab	5.3ab	
Fusicoccum	31.1±	27.5±	25.1±	42.6±	19.4±	26.6±	31.0±	30.2±	27.5±	28.99
amygdalis	6.1ab	5.4ab	5.4ab	2.9a	5.7b	5.9ab	5.9ab	5.0ab	5.9ab	
Verticillium	69.0±	72.5±	54.5±	50.5±	58.8±	69.5±	48.5±	68.9±	76.2±	63.17
dahliae	2.3ab	3.7a	2.0bc	1.6bc	3.5bc	3.6ab	3.6bc	5.7ab	3.4a	

Each value represents the mean of 10 values±standard error, Different letter (s) within columns represent values that are significantly different at p = 0.05 based on ANOVA and Duncan test

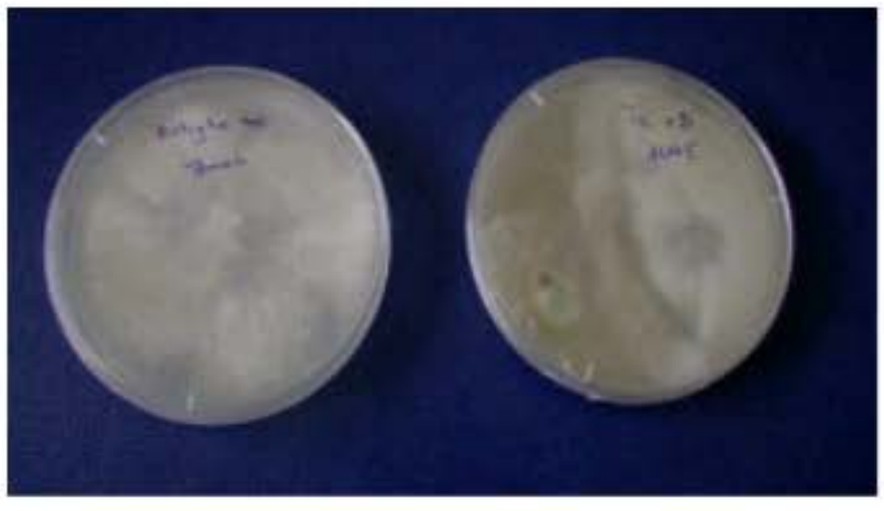

Fig. 1: Overlapping of the colony of *Botrytis cinerea* by the microorganisms of compost extracts (right) in comparison to the control (left) (PDA, after 6 days at 25°C)

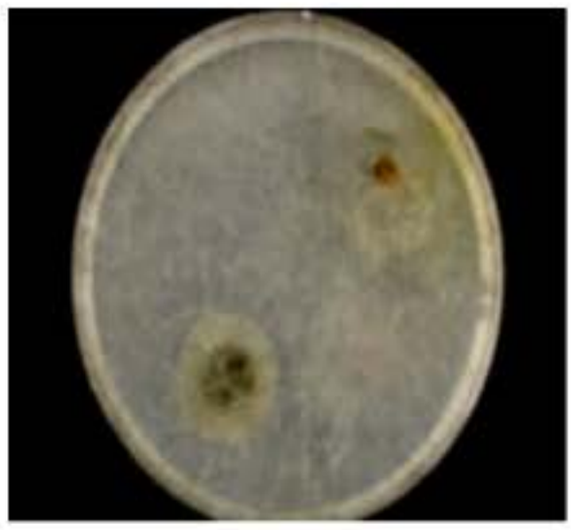

Fig. 2: Development of saprophytic fungi (*Rhizopus* sp.) From compost extract (PDA; 25°C)

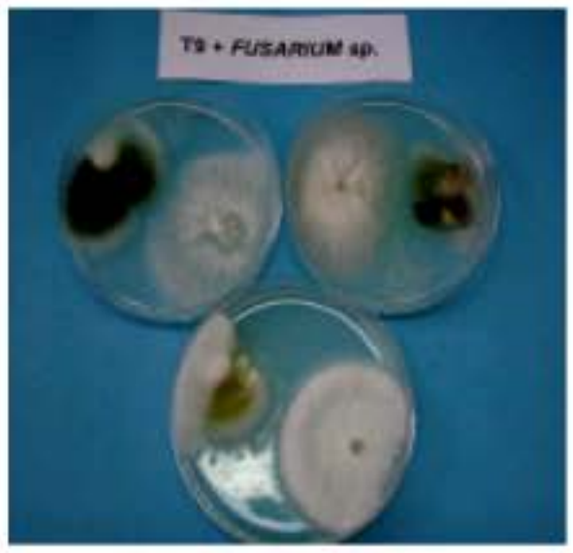

Fig. 3: Antibiosis zones formed after confrontation of *Fusarium solani* with the compost extract C9 (PDA; after 9 days; 25°C)

Table 9: Microbial populations of the tested composts (cfu g^{-1})

Composts	C1	C2	C3	C4	C5	C6	C7	C8	C9
Fungi ($\times 10^3$)	26a	33b	16.5de	8f	20cd	13.5e	24bc	14e	4f
Bacteria ($\times 10^7$)	6.5ab	7.4a	4.7abc	2.7bcd	2.7bcd	0.5d	4abcd	1.5cd	3.5abcd
Actinomycetes ($\times 10^5$)	20c	50b	63.5a	31.5c	28.5c	25c	2d	47b	23c

Each value represents the mean of 3 values. Different letter (s) within lines represent values that are significantly different at p = 0.05 based on ANOVA and Duncan test

Microbial Population of Compost Extracts

The results showed that the population of fungi, bacteria and actinomycetes in the tested compost extracts significantly differed (Table 9). The extract C2 had the highest population of fungi and bacteria with 33×10^3 and 7.4×10^7 cfu g^{-1}, respectively. The higher population of actinomycetes was recorded in extract C3 (63.5×10^5 cfu g^{-1}).

Some microorganisms present in compost extracts C2 and C7, which are showed to be the most effective against the tested phytopathogenic fungi, were identified as *Aspergillus* sp. and *Trichoderma* sp. for fungi and as *Serratia liquefaciens*, *Serratia odorofira* and *Aeromona hydrophylaa* for bacteria.

DISCUSSION

These results showed that the tested compost extracts, reduced the fungal growth by variable degrees. The inhibition ratio varied from 0 to 76.2%, according to this method of confrontation and

incubation conditions. These findings confirm previous studies showing that compost extracts, based on animal manures, had a inhibitory effect on some severe economic phytopathogenic fungi (Weltzien, 1992; Elad and Shtienberg, 1994; McQuilken *et al.*, 1994; Zhang *et al.*, 1998).

This *in vitro* antifungal activity varied according to the compost extracts and the tested fungi. All extracts showed an inhibitory effect on the mycelial growth of the majority of the fungi used except for *Aspergillus niger*. However, *Verticillium dahliae* seems to be the most sensitive probably due to its slow growth (24 days), comparatively to others which favored the development of beneficial microorganisms (antagonists) present in the compost extracts.

Compost extracts C2 and C7, based only on cattle and sheep manures and vegetable or straw wastes (Table 1), showed better inhibition ratios. This composition favored the development of effective and competitive microorganisms, which could be implicated in their broader suppressive effects. The higher microbial content of C2 extract seems to be in relation to its pathogen suppression potential. In contrast, the C7 extract was very effective despite its moderate population content and the recovered microorganisms seem to be very efficient. This result confirm the findings of Nelson and Boehm (2002) who reported that compost extracts with the highest microbial populations are not necessarily the most suppressive and conversely.

The inhibition ratios of the tested fungi were different compared with some previous studies. In fact, by the use of leaf fruit and garden or crop extract composts, El-Masry *et al.* (2002) obtained an inhibition ratio of 94.4% toward *Fusarium oxysporum* f. sp. *lycopersici*. Whereas, in our study, the pathogen inhibition was 38.12% by using animal manure extracts. Contrary to this result, Weltzien (1992) showed that animal manure composts were most efficacious comparatively to composts based on vegetative material. Brinton *et al.* (1996), Hoitink *et al.* (1997) and Fuchs (2003) showed that composts haven't the same capacity to protect plants against diseases. Difference was attributed to their initial ingredients and to their microbial activity. Consequently, as the compost extracts are the liquid version of initially solid composts (Ingham, 2002), they couldn't have the same degree of fungal suppression. Difference between the tested extracts, confirm the previous results obtained by Znaïdi (2002) and Khorcheni (2003) who also observed a variable activity in four types of animal manure compost extracts.

Several microorganisms (bacteria and fungi) were isolated from the tested compost extracts; this microflora plays a major role in the suppression of several plant pathogens (Bess, 2000; Camozzi, 2003; Hoitink *et al.*, 1991, 1997; Ingham, 2002; Quarles, 2001; Zhang *et al.*, 1998). Indeed, pasteurization of the extracts destroys their active microflora and nullifies their antifungal activity (Elad and Shtienberg, 1994). The identification of some microorganisms isolated from extract C2 and C7 showed the presence of some filamentous fungi such as *Trichoderma* sp. and A*spergillus* sp. and some bacteria such as *Serratia liquefaciens*, *Serratia odorofira* and *Aeromona hydrophylaa*. The both fungi mentioned were found in previous studies (McQuilken *et al.*, 1994; El-Masry *et al.*, 2002; Daami-Remadi *et al.*, 2006). Hoitink *et al.* (1997) indicated that some microorganisms isolated from compost extracts, such as *Trichoderma* sp., were identified as biocontrol agents. This is also the case of *Serratia* as indicated by Brinton *et al.* (1996).

The overlapping of the pathogen colony by the compost extract microorganisms, the antibiosis zone formation and the development of saprophytic fungi with a competitive potential were the main modes of suppression implicated and some of them were reported in several studies (Hoitink *et al.*, 1991, 1997).

The present study showed that water extracts of mature composts prepared from various animal manures had high inhibitory effects on *V. dahliae*, *F. oxysporum* f. sp. *radicis-lycopersici*, *F. solani*, *Fusicoccum amygdalis*, *Altenaria* sp. and *Colletotrichum coccodes*. Whereas, a lesser inhibition ratio was noted against *Rhizoctonia solani*, *Pythium* sp., *R. bataticola*, *Sclerotinia sclerotiorum*, *Botrytis cinerea* and *F. graminearum* and no effect was noted against *Aspergillus niger*.

These results demonstrated that some compost extracts are suitable products for the *in vitro* suppression of some plant pathogenic fungi. They could constitute a promising alternative for a biological control of some plant diseases and thus could reduce the need of the fungicides use. In a next step, *in vivo* and in natural conditions assays will be realized. Major microorganisms implicated in disease suppression will be individually tested for their potential as biocontrol agents.

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