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Effect of compost and antagonistic fungi on suppression of Tomato Grey Mold

Btissam MOURIA[1], Amina OUAZZANI-TOUHAMI[2], Afifa MOURIA[3], Rachid BENKIRANE[4] and Alla Douira[5]*

[1-5]Laboratoire de botanique et de protection des plantes, Kénitra, Maroc (Morocco)

E-mail: douiraallal@hotmail.com

ABSTRACT

The use of municipal solid waste compost in agriculture has known a particular interest because of its favorable effects on soil, on cultures and on environment. Treatment of plants with compost or compost extract or even their root or foliar inoculation with *Trichoderma harzianum* Tcomp, isolated from compost, were the most three efficient treatments against three strains of *Botrytis cinerea* agent of gray mold of tomato. These treatments induced similar suppressive effects in term of severity for the three strains of *B. cinerea* (B2, Bf3 and BP1), ranging between 0.9 and 1.3 for B2, or between 1 and 1.3 for Bf3 or even between 1.1 and 1.2 for BP1. Conidia productions for these three treatments are also similar for the three strains of *B. cinerea*, ranging between 1.02 and 1.09 10^4 conidia / cm^2 for B2, or between 1.06 and 1.08 10^4 conidia / cm^2 for Bf3 or even between 1.13 and 1.27 10^4 conidia / cm^2 for Bp1. The antagonists used in this study have efficiently suppress gray mold of tomato to varying degrees according to the antagonist itself (*Trichoderma harzianum* or *Ulocladium atrum*) and also to the inoculation method for *T. harzianum*, since each strain acts using one or more modes of action. Thus, TH_1 and Tcomp strains act primarily by inducing the SR (systemic resistance) and LR (local resistance). *Ulocladium atrum* isolated from compost reduced *B. cinerea* by direct inhibition of conidia production. Whereas for *T. harzianum* Ttom, other mechanisms are involved. The effect of compost on *B. cinerea*, which is a foliar pathogen, is unusual and is due to the induction of tomato systemic resistance plants that is associated with the presence of a population of *T. harzianum* Tcomp in this compost able to induce this systemic effect. However, unidentified chemical factors in compost are also responsible for this suppressive activity.

Key words: Compost, *T. Harzianum*, Extract, *B. cinerea*, Induction of systemic resistance.

INTRODUCTION

Botrytis cinerea Pers.: Fr. is one of the most redoubtable of tomato crop in Morocco. It causes significant yield losses (Besri and Afailal, 1993; Chibane, 1999) and persists in the soil for several years.

Antagonistic microorganisms have been used successfully to control gray mold, including bacteria (Ait Barka *et al.*, 2002. Daulagala and Allan, 2003), yeast (Saligkarias *et al.*, 2002. Zhang *et al.*, 2005) and fungi (Schena *et al.*, 2003; Yohalem and Kristensen, 2004).

The effect of compost as spraying against foliar pathogens has boomed (Elad and Shtienberg 1994; Welke, 2004; Hmouni *et al.*, 2006.). However, the effect of compost on foliar disease is uncommon.

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The most pertinent results against *Botrytis cinerea* were obtained with *Trichoderma* (Elad, 2000, Hanson, 2000; Batta, 2004; Hmouni *et al*., 2006). However, the disadvantage of local treatment is the biocontrol agent applied most often comes into direct contact with fungicides sprayed on the foliage. This implies that it must be resistant to these compounds. The solution to this potential problem is the use of microorganisms that induce systemic resistance in plants.

Specific bacterial and fungal antagonists of the rhizosphere can induce this systemic effect in plants (De Meyer *et al*., 1998; Harman *et al*., 2004a, b; Yedidia *et al*., 2003) and ensure as well telluric that leaf disease control (Raupach and Kloepper, 1998; Pieterse *et al*., 2003).

The objective of this paper is to study the effects of (i) a compost produced by a unit of Co-treatment of urban waste, its aqueous extracts and of (ii) *T. harzianum* and *U. atrum* antagonistic strains against gray mold caused by *B. cinerea* on tomato crops, as well as (iii) comparing the effect of inoculation method of *T. harzianum* strains on the reduction of this disease.

MATERIALS AND METHODS

Compost:

The used compost is produced by a Unit of Co-treatment (UCT) of urban solid waste in the region of Missour in southeast of Morocco, which is a non-industrial area where the waste are predominantly domestic and building, minimizing the presence of heavy metals in the final product (Anonymous, 2004). This is a mature compost that have a good quality as a plants biofertilizer amendment (Mouria *et al*., 2010).

Pathogens:

Botrytis cinerea strains, B2, Bf3 and Bp1, are selected as the most pathogenic strains of tomato (Mouria *et al*., 2013a). These strains were grown on PSA medium and incubated at 22°C for 14 days in the dark.

Conidial suspensions were obtained by washing the mycelium with sterile distilled water and the concentrations were adjusted to 10^5 conidia.mL^{-1} using a hemacytometer with sterile distilled water containing 0.02% of Tween 20 and 0.5% of gelatin. The obtained conidial suspensions were added with 0.02 M of glucose and 0.04 M of KH_2PO_4 to promote infection (Leone and Tonneijck, 1990).

Antagonists:

Three strains of *Trichoderma harzianum* TH_1, isolated from rice leaves (Hmouni *et al*., 2006) and Ttom, isolated from tomato leaves and Tcomp, isolated from UCT compost of Missour (Mouria *et al*., 2013b) and whose antagonist ability has been verified (Mouria *et al*., 2010), and also an *Ulocladium atrum* (Uat) strain, isolated from UCTcompost of Missour were tested for their antagonistic ability against *B. cinerea*.

These strains were grown in darkness at 28°C on PSA plates (Potato: 200 g; sucrose: 15 g; Agar-agar: 20 g; distilled water: 1000 mL) for ten days. The conidia suspensions were obtained by scraping the surface submerged with sterile distilled water and concentrations were adjusted to 10^6 conidia.mL^{-1} using a Malassez slide by adding sterile distilled water supplemented with 0.02% of Tween 20 and 0.5% of gelatin.

Plant Material:

Seeds of tomato belonging to the variety Campbell 33 are superficially disinfected with sodium hypochlorite diluted to 1%, rinsed thoroughly with sterile distilled water, dried on the filter paper during 15 minutes. These seeds were then germinated in perforated alveoli containing peat. The alveoli were covered with a plastic film of 20 microns thick for two to three days to ensure good germination and placed in a greenhouse during four weeks.

The planting is performed when the seedlings were 2 true leaves well spread (Woo *et al*., 1996) in polyethylene pots (17 cm × 14 cm) perforated at the base after performing out the different treatments.

Treatments:

Tomato plants undergo two types of treatments:

Soil amendment with compost: Tomato plants were planted in a substrate consisting of a mixture of compost and soil at a rate of 20%, this rate has been retained in a previous work (Mouria *et al*., 2010).

● Compost extracts spraying: Tomato seedlings planted only in soil are sprayed every two days with a compost extract prepared at 10%. This extract allowed suppression of foliar symptoms of non-inoculated plants (Mouria *et al*., 2010).

Treated plant with Compost or with compost extract has undergone no inoculation with antagonists.

Inoculation:

In the age of 32 days old, Tomato plants grown in soil are sprayed preventively with conidial suspensions of *T. harzianum* or *U. atrum* 24 hours before inoculation with strains of *B. cinerea*.

In another experiment, the roots of tomato seedlings, surrounded by peat, were inoculated with strains of *T. harzianum* and transplanted into soil. 21 days after planting (DAP), the plants were inoculated with suspensions of different strains of *B. cinerea*. Plants treated with compost or with compost extract, are also inoculated with strains of *B. cinerea* 21 DAP.

After inoculating plants with pathogens, the pots are covered with transparent plastic bags to ensure high humidity, favoring infection with *B. cinerea*, while allowing light passage to avoid the withering plants (Elad and Shtienberg, 1994).

Experimental plan:

The pots were arranged in randomized blocks under a greenhouse. Each variant has been five replicates and the experiment was repeated twice. Five types of controls were performed:

- Plants inoculated with pathogens and grown in soil (positive control);
- Plants not inoculated pathogens and cultivated in soil;
- Plants not inoculated pathogens and grown in soil amended compost;
- Plants not inoculated pathogens and treated with compost extract;
- Plants not inoculated pathogens and treated with antagonists.

Evaluation of symptoms:

The severity of symptoms is noted 5 days after inoculation with *B. cinerea* by adopting the rating scale of Elad and Shtienberg (1994) below:

Notes	DLA* (%)
0	0
1	1-5
2	6-15
3	16-50
4	51-95
5	100

* DLA: Decayed Leaf Area

Tomato leaves from different treatments are cut into fragments of 1 cm^2 which are deposited in sterile moist rooms and incubated in the dark at 22°C to estimate the conidia production of *B. cinerea*. Ten fragments are deposited per dish and each treatment was repeated five.

After 10 days of incubation, the fragments are introduced into test tubes containing 3 ml of sterile distilled water. After mechanical agitation for two minutes, the counting is performed using a Malassez slide.

Other fragments of tomato leaves from different treatments are placed in sterile moist rooms and incubated 7 days at 28°C in order to verify the presence of *Trichoderma* and *U. atrum*. Ten fragments are deposited per dish and each treatment was the object of five repetitions.

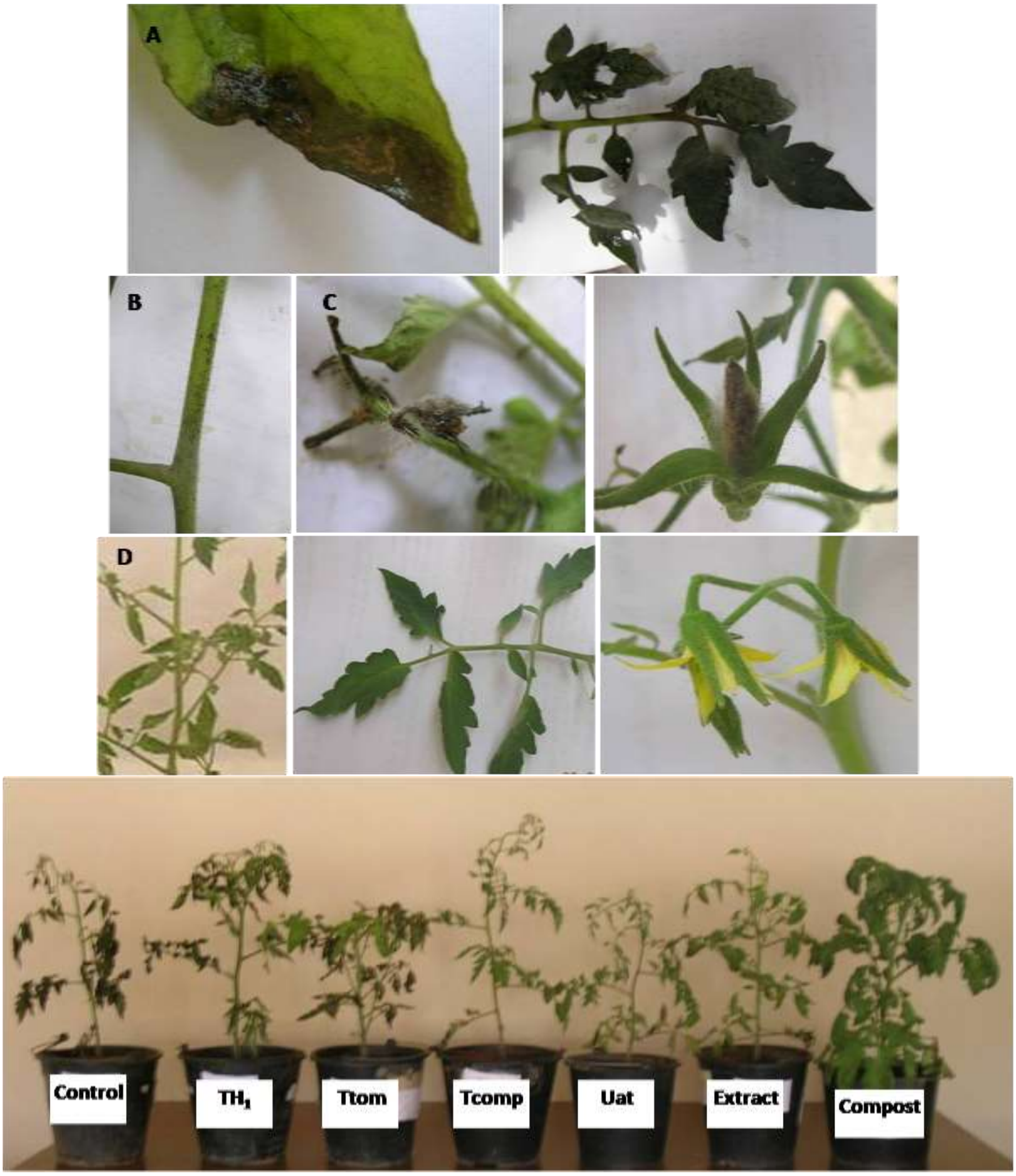
Statistical Analysis:

Analyses of variance were carried out after transformation of percentages to ArcSin $\sqrt{P}$ and averages are compared with Newman & Keuls test at 5%.

RESULTS

Severity notes of different strains of *B. cinerea* according to different treatments are shown in Table 1. *B. cinerea* strains sprayed on tomato plants, grown in soil, induced brownish lesions on the leaves soon become moldy and decayed. Brown and viscous spots appear on the stems and there is also a browning of flowers, often accompanied by the formation of a grayish down (Plate 1).

Plate 1: Induced symptoms on tomato plants of Campbell 33 variety after inoculation by B. *Cinerea* B2.



A: On leaves; **B:** On stem; **C:** On flowers.
D: Non-inoculated control with healthy stem, leaves and flowers.
E: Effect of different treatments on the severity of symptoms of *B. cinerea* B2 on tomato plants. Inoculation with *T. harzianum* strains is achieved via the roots.

Severity notes are high for all three strains in comparison with control plants. They were significantly reduced in plants treated with compost, compost extract or antagonistic fungi (Table 1; Plate 1).

Figure 1, which records the percentage of inhibition of severity of the strains of *B. cinerea* in different treatments shows that soil amendment with compost and plants inoculation with *T. harzianum* (Tcomp) are the two treatments which resulted in the greatest reduction in the severity of *B. cinerea* strain B2, the reduction was 75%.

Table 1: Severity notes of gray mold on tomato plants inoculated with three strains of *B. Cinerea* according to different treatments.

	B2	Bf3	Bp1
Control – (soil)	0 d	0 f	0 e
Control + (soil+Bc)	3.7 a	4.5 a	4.1 a
Bc + TH_1 R	1.6 b	1.6 c	1.4 cd
Bc + TH_1 L	1.7 b	1.5 cd	1.5 cd
Bc + Tcomp R	0.9 c	1 e	1.1 d
Bc + Tcomp L	1 c	1 e	1.2 cd
Bc + Ttom R	1.9 b	2.1 b	2.3 b
Bc + Ttom L	1.3 bc	1.3 cde	1.7 c
Bc + Uat F	1.3 bc	1.3 cde	1.3 cd
Bc + Comp	0.9 c	1.1 de	1.1d
Bc + E2	1.3 bc	1.3 cde	1.2 cd

Bc: *B. cinerea*; R: Root inoculation with *Trichoderma*; L: Leaves spraying with *Trichoderma*.
Each value is the average of two independent experiments with five replicates per experiment. Two values in the same column followed by the same letter are not significantly different at 5% according to the Newman & Keuls test.

U. atrum (Uat) has reduced the severity similar to that provided by spraying the compost extract level. Whereas, this inhibition has not exceeded 56% for *T. harzianum* TH_1.

As for *B. cinerea* B2, Tcomp induced the highest protection of plants against *B. cinerea* Bf3, while all treatments, except root inoculation with Ttom were comparable for *B. cinerea* BP1 (Figure 1).

Only *T. harzianum* Ttom showed a difference in its ability to suppress *B. cinerea* according to inoculation method. Indeed, this strain is more effective when sprayed on plants than when inoculated to the roots of tomato plants, especially on Bf3 strain.

Leaves spraying with Ttom led to 71% reduction in the severity of symptoms (Plate 2), while root inoculation induced reduction percentages lowest (53.32% and 43.89% respectively against Bf3 and Bp1). The other strains of *T. harzianum* (TH_1 and Tcomp) showed no significant difference between the two modes of inoculation.

Potential of conidia production of *B. cinerea* on tomato leaves inoculated with different strains are shown in Table 2.

Plants leaves inoculated with *B. cinerea* and have undergone no treatment have abundant conidia production of pathogen in wet rooms (Plate 3), the number of conidia observed varied between 3.99 and $4.91.10^4$ conidia/mm^2 for strains B2 and Bp1 respectively. This number has decreased by more than half in plants co-inoculated with the biocontrol agents or treated with compost or with aqueous extract (Table 2).

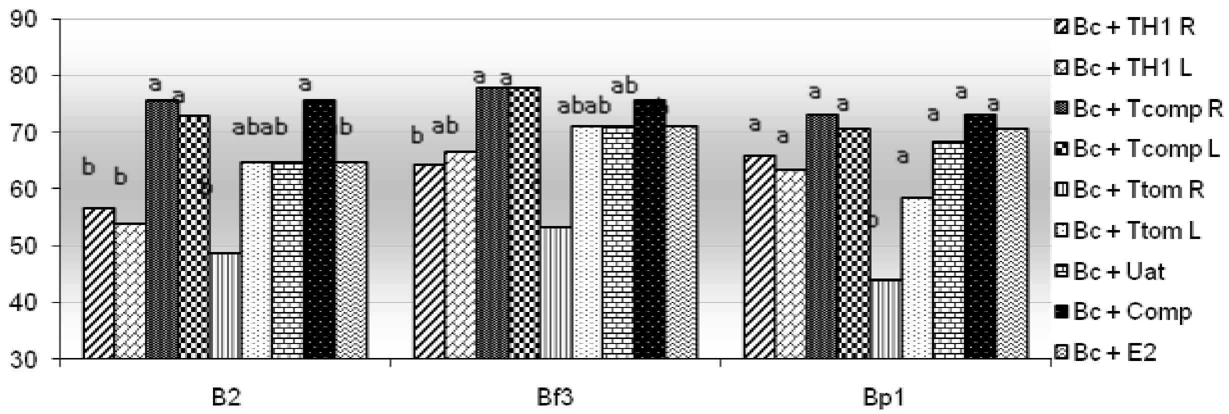
In fact, compost, extract and Tcomp strain of *T. harzianum* induced the highest percentages of inhibition of conidia production of Bf3 and B2 strains of *B. cinerea* (up to 76.98% reduction), while for Bp1, the highest inhibition was obtained only in plants treated with compost or inoculation with Tcomp. Compost extract leads to a similar inhibition of that induced with Uat (Figure 2).

Tomato plants inoculated with Ttom into root have weakly inhibited conidia production of *B. cinerea* strains and therefore induced the lowest percentages of inhibition of conidia production between 54.57 and 58.35%.

TH_1 and Uat have lead to intermediate percentages of inhibition of conidia production, between 59.67 and 70.93% for TH_1 and between 62% and 69.16 for Uat. Moreover, the presence of *Trichoderma* has been highlighted in tomato leaves inoculated by spraying. The conidiophores of the antagonists are observed with those of *B. cinerea* strains on the same leaf fragments (Plate 3).

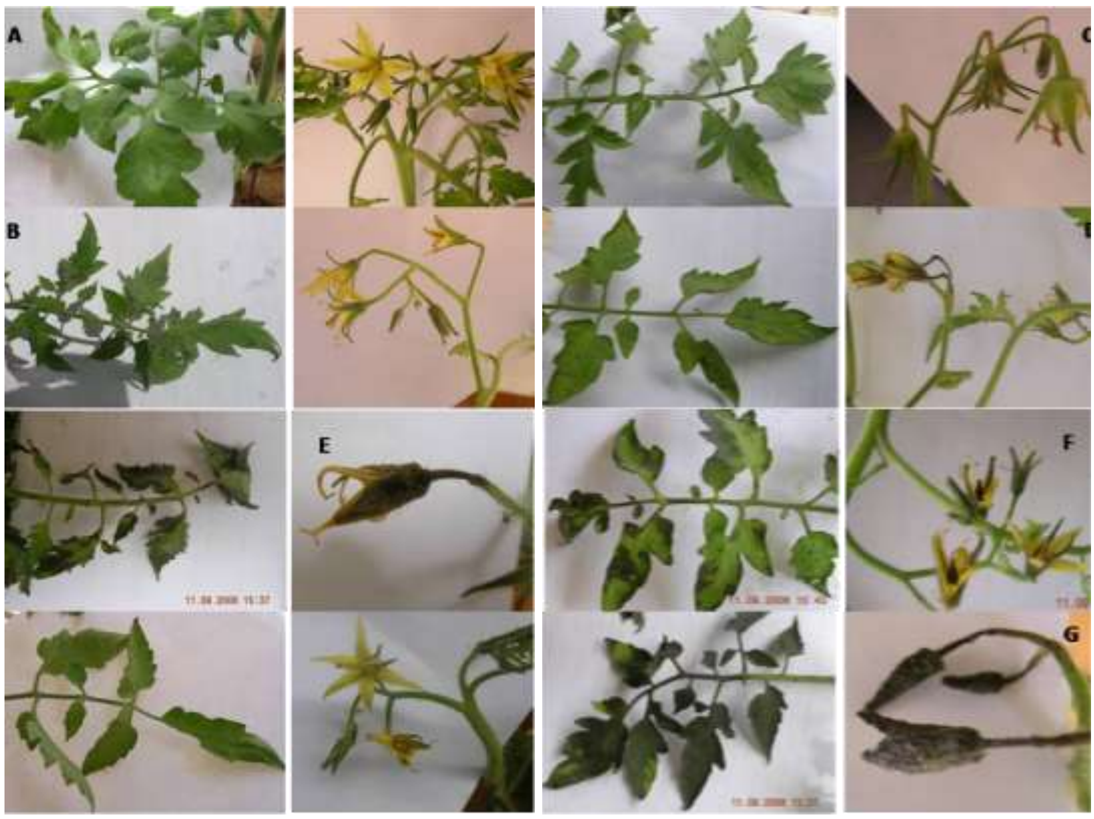
By against, leaves collected on tomato plants inoculated to root with antagonists or treated with compost or with extract showed no conidiophores of *Trichoderma* (Plate 3). As for *T. harzianum*, conidiophores of *U. atrum* developed on tomato leaves with those of *B. cinerea* (Plate 3).

Figure 1: Percentages of inhibition of severity on tomato leaves inoculated with strains of *B. Cinerea* according to different treatments.



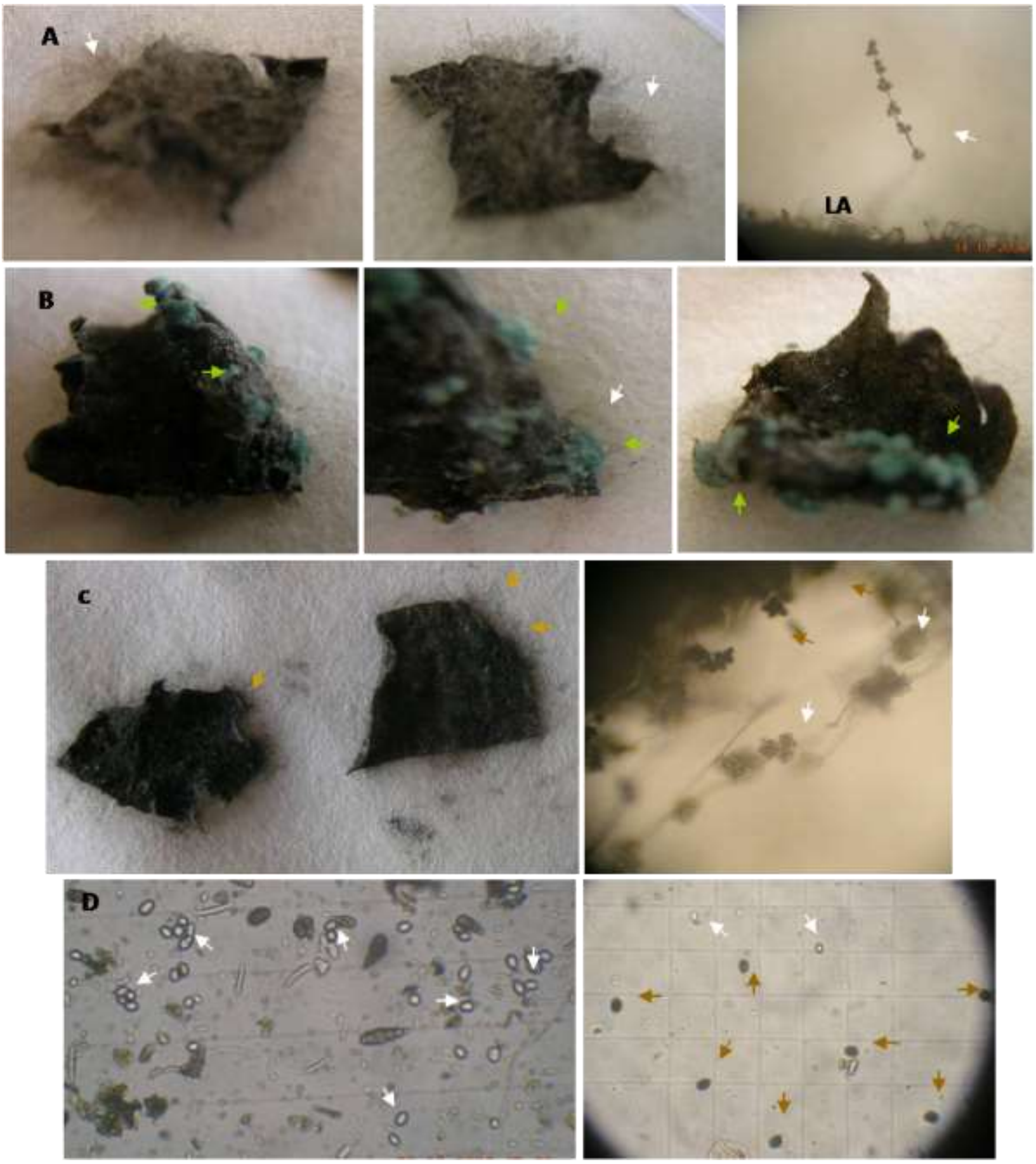
Bc: *B. cinerea*; R: Root inoculation with *Trichoderma*; L: Leaves spraying with *Trichoderma*.
Each value is the average of two independent experiments with five replicates per experiment.
For a given strain, two results affected by the same letter are not significantly different at 5% according to the Newman & Keuls test.

Plate 2: Reducing of symptoms of *B. cinerea* on leaves and flowers according to different treatments.



A: Soil amendment with compost; B: Spraying with compost extract; C: Inoculation with Tcomp; D: Inoculation with Uat;
E: Inoculation with Ttom via the roots (top) and by spraying (bottom); F: Inoculation with TH1; G: No treatment (control +).

Plate 3: Leaves fragments from plants: A: Inoculated only with *B. cinerea* ; **B:** Inoculated with *T. harzianum* Ttom and *B. cinerea* ; **C:** Inoculated with *U. atrum* Uat and *B. cinerea*. **D:** Example of microscopic comparison of number of *B. cinerea* conidia on leaves inoculated only with pathogen and leaves co-inoculated with *U. atrum*. → = Conidia of *B. cinerea;* LA = Leaf Area; → = Conidia of *T. harzianum* Ttom; → = Conidia of *U. atrum*.



DISCUSSION

Grey mold causes severe yield losses in tomato crops, especially in moist greenhouse atmosphere (Elad, 2000, Lee *et al*., 2006.). The three strains of *B. cinerea* studied induced mold symptoms on all control plants parts. Indeed, on the leaves, the symptoms are brownish lesions which immediately transformed into mold. Leaf tissues then become moldy and decayed. Brown and sticky spots appear on the stems, and there is also a browning of flowers often accompanied by the formation of a grayish down. This confirms the results of tests on tomato leaves slices of these strains pathogen ability on the variety Campbell 33 of tomato (Mouria *et al*., 2013a).

All strains of *T. harzianum* have significantly reduced the severity and the ability of strains of *B. cinerea* to sporulate on tomato leaves, but at varying degrees depending on the strain and on the mode of application.

Several studies have reported the antagonistic effect of *Trichoderma* spp. against *B. cinerea* both *in vitro* (Hmouni *et al*., 1999; Hjeljord *et al*., 2001.) and *in vivo* (Batta, 2004; Hmouni *et al*., 2006.). Based formulations of this biocontrol agent were commercialized against gray mold of tomato as Trichodex (Elad, 2000; Brunner *et al*., 2008; Tijerino *et al*., 2011).

Tcomp strain, isolated from UCT compost of Missour, was the most effective against all strains of *B. cinerea*, followed by Ttom strain isolated from the leaves of tomato plants of the same variety, but only when inoculated by plant spraying.

The relatively higher antagonism of Ttom against *B. cinerea* could be attributed, in part, to its inoculation at its original biotope. Indeed, Howell (2003) suggested that the best method to obtain a potential biocontrol agent (BCA) could be where the candidate species of *Trichoderma* are isolated from plant surfaces and the soil where it is expected to operate in the fight against diseases.

Hajlaoui *et al.* (1997) also showed that *Trichoderma* species isolated from tomato leaves suppressed the development of gray mold of the same culture better than iprodione. The difference between the severity and conidia production of *B. cinerea* in plants inoculated with this strain (Ttom) sprayed on plants and those inoculated through the roots confirms this eventuality. Indeed, Ttom applied to leaves reduced the severity of *B. cinerea* more strongly than when inoculated roots, while for the other two strains, the two application modes have almost the same effect.

The spatial separation between strains of *T. harzianum* inoculated roots of tomato plants and the pathogen was maintained, because these strains have not been found in leaf tissues of plants inoculated to roots. By against, these strains were re-isolated from plants inoculated by spraying, although disease development of the disease has been greatly reduced by TH_1 and Tcomp relatively by Ttom in two types of treatments.

Based on the proposed criteria for induced systemic resistance by BCA (Pieterse *et al*., 2003), suppression of gray mold by *Trichoderma*, particularly Tcomp and TH_1 strains is certainly due to the induction of systemic resistance that has played a major role. Whereas for Ttom, other mechanisms are also involved in leaves (competition, mycoparasitism...).

Similar responses were observed by De Meyer *et al*. (1998) and Horst *et al*. (2005), by inoculating peat with *T. harzianum* and *T. hamatum*, they observed a decrease in the severity of *B. cinerea* on the leaves and stems of several plants.

Indeed, *Trichoderma* spp. is a highly effective inducer of systemic resistance (SR) and local resistance (LR) in plants. This effect was demonstrated for the first time in 1997 by Bigirimana *et al*. Although suggested by several authors before that date, and does not replace other mechanisms previously reported (Harman *et al*., 2004 a, b).

Suppression of *B. cinerea* strains on tomato plants sprayed by Tcomp and TH_1 was similar to that provided by the two strains inoculated to roots, indicating that the induction of LR is also involved in the control of *B. cinerea* on plants sprayed with these two strains (De Meyer *et al*., 1998).

Uat strain of *U. atrum* isolated from compost, sprayed to tomato plants, reduced the severity and especially conidia production of *B. cinerea* strains better than TH_1 and Ttom strains of *T. harzianum*.

Indeed, several studies have reported the antagonistic ability of *U. atrum* against *B. cinerea* on several crops such as rose (Yohalem and Kristensen, 2004; Köhl and Gerlagh, 1999), cyclamen (Köhl *et al*., 2000. Köhl and Molehoek, 2001), geranium (Gerlagh *et al*., 2001) and strawberries (Boff and Köhl, 2002). Köhl *et al*. (1997) found that *U. atrum* competes with *Botrytis* sp. for necrotic tissue.

From another angle, compost and aqueous extract constituted with Tcomp, the most effective treatments on the inhibition of the development of *B. cinerea* strains. Since Weltzien and Ketterer (1986) found suppressive characteristics of compost extract, several authors have reported the effect of spraying of these extracts on the elimination of foliar diseases, both bacterial (Zhang *et al*., 1998; Al Dahmani, *et al*., 2003) and fungal (Elad and Shtienberg, 1994; Welke, 2004; Hmouni *et al*., 2006).

Hmouni *et al*. (2006) and V Pranitha et al (2014) compared the effect of TH_1 strain of *T. harzianum* used in this study, to that of three extracts prepared from different composts, on gray mold of tomato rot. One compost extract was more effective than *T. harzianum*, while the two other were less or as effective as TH_1.

However, the effect of compost amendment in substrate on foliar pathogens suppression is unusual because compost generally induces SR only when inoculated by BCA able to induce this effect (Hoitink and krause, 2003; Krause *et al*., 2003; Khan *et al*., 2004).

Very few studies have reported the effect of the amendment of substrate with composts on suppression of foliar fungal pathogens, such as *Colletotrichum lindemuthianum* on bean (Stone *et al*., 2003), *B. cinerea* on Begonia (Horst *et al*., 2005) and *B. cinerea* on cucumber (Segarra *et al*., 2007, 2013). They have concluded that the induction of defense responses in plants was the main mechanism of biological control used by the compost they used.

Induction of SR in plants with compost in this study is strongly related to the population of *T. harzianum* which has the same mode of action. Indeed, the suppressive ability of compost has often been attributed mainly to the increase in activity levels of its beneficial microflora in general, serving as a nutritious basis for indigenous microorganisms or introduced BCA (Hoitink and Boehm, 1999; Steinberg *et al*., 2004) and of *Trichoderma* in particular (Hoitink *et al*., 1997; Bulluck and Ristaino, 2002).

In addition to *T. harzianum* and *U. atrum*, other fungi species isolated from compost of UCT of Missour (Mouria *et al*., 2013b) have been reported as antagonists against *B. cinerea* such as *Aureobasidium pullulans* (Ippolito *et al*., 2000, 2005; Castoria *et al*., 2001; Schena *et al*., 2003) and *Chaetomium globosum* (Khadija Ahayoun et al, 2015 and Köhl *et al*., 1995, 1997) and probably other. Janisiewicz (1998) reported that the use of multiple BCA, with different modes of action, in a combined approach could allow a better performance against plant diseases, which explains the high ability of this compost to control pathogens.

Otherwise, a positive correlation was found between reduction of symptom severity of *B. cinerea* and inhibition of conidia production on tomato leaves. Indeed, treatments that reduced more the severity of gray mold are the same ones who most strongly inhibited conidia production of pathogenic strains. In this sense, the suppression of pathogen conidia production has been proposed as a potential strategy of control to mitigate the epidemics of *B. cinerea* (Köhl and Fokkema, 1998).

CONCLUSION

Treatment of plants with compost or compost extract or even their root or foliar inoculation with *T. harzianum* Tcomp, isolated from compost, induced similar suppressive effects on *B. cinerea* strains.

It is interesting to note that the BCA used in this section have efficiently suppress gray mold of tomato to vary degrees according to the antagonist itself (*T. harzianum* or *U. atrum*) and also to the inoculation method for *T. harzianum*, since each strain acts using one or more modes of action.

Thus, *U. atrum* isolated from compost reduced gray mold by direct inhibition of conidia production of *B. cinerea*. TH_1 and Tcomp strains act primarily by inducing the SR and LR. Whereas for Ttom, other mechanisms are put in play.

The effect of compost on *B. cinerea*, which is a foliar pathogen, is unusual and is due to the induction of systemic resistance of tomato plants that is associated with the presence of a population of *T. harzianum* Tcomp in this compost able to induce this systemic effect.

The presence of several antagonists having different modes of action in the compost

(Mouria *et al*. 2013b) also justifies its high suppressive ability which is related to the total microflora, called "general" suppression and to *T. harzianum* in particular called "specific" suppression.

However, unidentified chemical factors in compost are also responsible for this suppressive activity (Mouria *et al*., 2013a). This suppression was often attributed itself to the compost microflora.

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