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Verticillium dahliae-Eggplant as the Pathosystem Model to Reveal Biocontrol Potential of three *Trichoderma* spp in Greenhouse Conditions

Wafaa Mokhtari[1*], Mohamed Achouri[1], Abdellah Remah[1], and Hassan Boubaker[2]

[1] Plant Protection Department, Institut Agronomique et Vétérinaire Hassan II, Agadir, Morocco; [2] Biology Department, School of Sciences, University Ibn Zohr, Agadir, Morocco

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Abstract

In this study three *Trichoderma* species isolated from Moroccan soil (natural and agricultural habitats) were investigated for their biocontrol potential against virulent *Verticillium dahliae* on eggplant in green house conditions based on dipping root approach. Evaluation of biocontrol efficacy of *Trichoderma* spp. demonstrated effective potential of *Trichoderma* on reducing Verticilium disease on eggplant cultivars. Disease assessment was established by measuring disease incidence in root units (DI-RU) and in the above ground of eggplant cultivars (DI- AU). DI-RU% was recorded at 12.5%, 25.0%, 31.3% and 37.5% in T3, TC, T1 and T2 treatment respectively where DI-RU recorded in Tm2 controls; eggplants inoculated with *Verticillium* only was equal to 100.0%. Whereas, disease incidence outcomes in aerial part was DI- AU = 100.0% in treatments TC and T2, 87.5% in T1 and 96.0% in T3 treatment where Tm2 controls were assessed with 100.0%.

Keywords: Trichoderma, Biocontrol, *Verticillium dahliae*-eggplant pathosystem.

Abbreviations: g; gram, cm^2; centimeter area, cm; centimeter, DI; Disease Incidence, DI-AU; Disease incidence in Above ground units, DI-RU; Disease incidence in roots units; w/w; weight per weight, NPK; Nitrogen, Phosphor, Potassium, g/hl; gram per hectoliter, °C; Celsius degree, ml; milliliter.

Introduction

Verticillium dahliae is considered a highly polyphagous soil borne plant pathogen that attacks more than 400 vegetable species including Solanacea and Cucurbitaceae cultures and olive trees. Its polyphagous characteristic causes the attack of wide host range other than the original hosts what makes determination of host specificity and virulence of *Verticillium* isolates of paramount importance regarding its management (Tjamos, 1981). *Verticillium* is considered a chronic economic soil borne pathogen that causes vascular wilt and death in many plants in field and green house. No economic losses measure has been done till now in the cultural infestation with Verticilium in Morocco. However, it is estimated of billions of dollars annually losses all around the world (Pegg and Brady, 2002). Soil disinfection and genetic resistance are the major management strategies used to control Verticilium diseases. However, application of biocontrol agents like *Trichoderma* have become more and more used to control this soil borne pathogen due to negative effect of disinfectant on human health and environment (Cook and Baker, 1983).

Eggplant is a highly susceptible cultivar wilt and dies shortly after *Verticillium dahliae* invading and expressing first symptoms in it. In fact, *Verticillium* wilt has been reported to be the most destructive and prevalent diseases of eggplant. Therefore, eggplant-*Verticillium* is considered one of the best pathosystem models to evaluate resistance and/or tolerance to Verticilium wilt for designing the adequate and required disease management strategy (i.e. grafting, biocontrol application) in crop system. For instance, testing biocontrol potential of *Trichoderma* spp. using alike susceptible host controls would allow to iden-

* Corresponding author: w_mokhtari@yahoo.fr

tify the effective biocontrol potential among *Trichoderma* isolates especially when selecting antagonistic candidates during biocontrol agent screening (Huisman and Gerik, 1989; Blestos et al., 2003).

Selecting antagonists for specific biocontrol of *Verticillium* diseases was based on screening potential antagonistic candidates like *Trichoderma* spp. Many studies demonstrated that *Trichoderma* spp. can inhibit growth or kill soil borne pathogens and revealed different antagonistic interactions between the antagonist *Trichoderma* and the pathogen in vitro and in vivo (Lumsden et al., 1993; Monte, 2001; Yang et al., 2010). Actually, few research work have focused on the biocontrol efficacy of *Trichoderma* spp against *Verticillium* in vivo though important antagonistic potential were involved against *Verticillium dahliae* when in vitro assays were applied.

In this study, we attempt to evaluate biocontrol potential of three *Trichoderma* species isolated from Moroccan soil (natural and agricultural habitats) in green house conditions based on root dipping technique.

Material and Methods

Experimental Design for Biocontrol Treatments in Greenhouse Conditions

Three species of *Trichoderma* identified at the species level (Mokhtari et al., 2017); *Trichoderma afro-harzianum* (T8A4), *Trichoderma reseei* (T9i12) and *Trichoderma guizouhense* (T4) were tested for their biocontrol efficacy against *Verticillium dahliae*-eggplant pathosystem. Eggplant cultivars were grown for three weeks on 77 peat trays. Seedlings were then transplanted into pots after their inoculation with fungi. Eggplant seedlings were transplanted in 3 L pots filled with sterile substrate at 3:1 w/w peat to sand ratio. Substrate was fertilized using NPK and oligo-elements composition at 250 g/hl.

Experimental design was organized in four randomized complete blocs with four replicates in each experimental unit. That is, four pots were used in each experimental unit. Four treatments were tested; T1 was designated for treatment of cultivars inoculated with *Trichoderma afro-harzianum* (T814) and *Verticillium dahliae*, T2 was designated for treatment of cultivars inoculated with *Trichoderma guizouhense* (T4) and *Verticillium dahliae* and T3 was designated for treatment of cultivars inoculated with *Trichoderma reesei* (T9i12) and *Verticillium dahliae*. Controls were respectively; Tm1 was designated for healthy cultivars with non inoculated plants (negative controls), Tm2 was designated for cultivars inoculated with pathogen only (positive control). *Verticillium* has been known of its virulence and lethality towards woody and other crops. Pathogenicity test investigated in Tm2 controls allowed the exhibition of *Verticillium dahliae* virulence on susceptible eggplants (Schnathorst and Sibbett, 1971).

TC designated for treatment of cultivars inoculated with *Trichoderma afro-harzianum* extracted from a commercial product to be compared with other *Trichoderma* isolates.

Obtaining Verticillium isolate for Pathogenic Test

Branches and roots of olive trees diagnosed with wilt symptoms were collected, washed with tap water and disinfected one minute in 10% sodium hypochlorite.

Six small fragments from wilt branches and roots were washed in distilled di-ionized water for one more minute then inoculated in PDA and incubated at 19 °C in the dark for 10 days.

To obtain pure culture of *Verticillium dahliae*, mycelium of the fungus was collected from tissues baits with sterilized scalpel and inoculated into fresh PDA.

Verticillium dahliae Microsclerotia Inoculum and Eggplant Root- Dipping Inoculation

Microsclerotia were used exclusively as inoculum propagules to infest eggplant roots. Therefore, we used cellophane layer to produce uniform microsclerotia layer on potato dextrose agar (PDA, Difco) plate.

Cellophane plates with *Verticillium microsclerotia* were thereafter flooded with sterile di-ionized water and poured through 45 µm stainless steel sieve to remove spores and hyphae. The contents on the sieve were transferred to sterile glass petri dishes and comminuted with a sterile razor. To prepare microsclerotia suspension, microsclerotia obtained were transferred in beaker filled with distilled di-ionized water. After deep vortex, number of microsclerotia was determined with serial dilution ranged from 10^{-1} to 10^{-6} to a final microsclerotia concentration of 10^4 to 10^5/ml (Atibalentja and Eastburn, 1997; Shiraishi et al., 2014; Dongfang et al., 2014). Eggplant roots cuts of 2 to 3 cm diameters long were inoculated in 10^4 to 10^5/ml of microsclerotia of *Verticillium dahliae* (Atibalentja and Eastburn, 1997; Gray et al., 1998).

Disease Evaluation and Measurement of Leaves Surface Area (LSA)

Disease assessment was estimated by measuring Disease Incidence (DI) of *Verticillium dahliae* as reported by Campbell and Neher (1994). Disease Incidence (DI) was measured as the percentage of number of plant units that are visibly diseased. Therefore, DI percentage was calculated as shown in the equation (1) (Campbell and Neher, 1994).

Disease Incidence = [Number of infected plant units] / [Total number of plants in the experiment] x 100   Equation 1

Disease incidence was measured in above ground units corresponding to aerial parts of cultivars (DI-AU) and root units (DI-RU). DI- above ground Units was measured based on visible symptoms detected in the above-ground plant area. Therefore, symptoms were identified and measured in symptomatic vegetative tissues in leaves (i.e. typical bronze-yellowing, necrosis) and vascular tissues wilt. DI-RU was measured based on visible symptoms and signs in the diseased roots and crown (rots and/or discoloration) of related host plant. In fact, this

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method requires destructive sampling of plant, therefore, applied at the end of each experiment. Signs were basically examined based on the presence of pathogens' components like mycelium and propagules under microscopic observation (Campbell and Neher, 1994). In addition, plant parameters like Plant Height (PH) in centimeter (cm) and Leaf Surface Area (LSA) in square centimeters (cm^2) were also measured. These three parameters were assessed at the end of each experiment yet with non destructive method. Whereas, Root Dry Weight (RDW) and Plant Dry Weight (PDW) in gram (g) were measured at the end of each experiment with destructive sampling method (Benson and Baker, 1974; Campbell and Neher, 1994). In order to measure PDW, all plants were excavated from pots, roots were washed under running tap water to discard adhering substrate then exposed to the air at ambient temperature (25-30 °C) until they dry. When PDW was measured, roots were cut at crown level and dried in drying chamber at 60 °C for three to four days to measure RDW. In order to measure leaves surface area (LSA) technique described by Breda (2003) was used. Since leaves are irregular, LSA was measured based on constant mass ratio calculation (Breda, 2003). At the end of each experiment leaves were collected, laid on the surface of A4 papers then their outlines were traced. Each leaf drawn on A4 paper was cut at the level of outline and A4 leaf drawing pieces were kept to be weighed. Surface area (cm^2) and the mass (g) of a whole A4 paper were measured too. To calculate LSA (cm^2) of each A4 leaf drawing piece mass ratio was correlated to surface area (cm^2) of A4 paper as detailed in equation (2).

[surface area of A4 paper (cm^2)] [mass of A4 leaf drawing piece (g)]/[mass of A4 paper (g)] Euation 2

Results and Discussion

Reducing Verticillium Infestation on Eggplant Cultivars

As mentioned in material and methods in vivo antagonistic assay was tested on eggplant. Similarly to previous antagonistic assay in green house, disease responses of *Verticillium* were assessed by measuring disease incidence in above ground units (DI- AU) and root units (DI-RU). For eggplant, disease assessment and antagonistic evaluation were performed three months after *Trichoderma* spp. treatments. On the whole, *Trichoderma* spp. treatments exhibit biocontrol efficacy reducing *Verticillium* disease in eggplant cultivars yet could not control effectively *Verticillium* severe symptoms on eggplant. As detailed in Figure 1 disease incidence in above ground Units = 100.0% in treatments TC and T2, 87.5% in T1 and 96.0% in T3 treatment.

It can be inferred from DI- above ground Unit results that *Trichoderma* spp. treatments each containing T1; *T. afroharzianum*, T2; *T. guizouhense, T. reesei* and TC; commercial *Trichoderma* were not able to suppress definitively the pathogen. In fact, different symptoms on the above-ground eggplant cultivars were detected; typical bronze-yellowing and necrosis of leaves veins and wilting as illustrated in Figures 2 and 3.

Moreover, significant decrease was recorded in leaves surfaces area (LSA) of cultivars in different treatments relatively

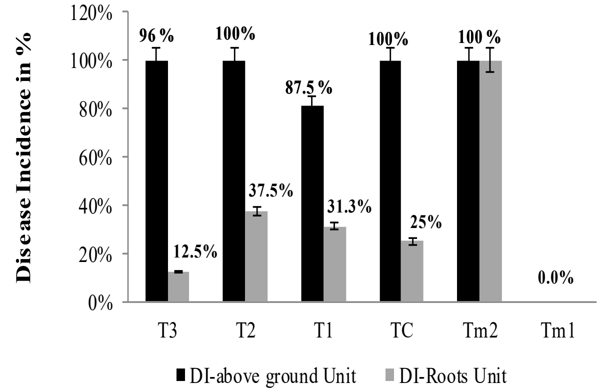

Figure 1. Disease incidence assessment in (%) on *Verticillium*-eggplant pathosystem towards evaluation of effect of three antagonists; *Trichoderma afroharzianum* T1, *Trichoderma reseei* T2 and *Trichoderma guizouhense* T3. TC *Trichoderma* extracted from commercial product used as reference. Disease incidence was assessed in above ground units DI-AU and in roots; DI-RU.

Figure 2 and 3. *Trichoderma* treatments of eggplant artificially infested with *Verticillium microsclerotia*; both figures represent above ground symptomatic eggplant treated with different *Trichoderma* spores suspenssions; *T. afro-harzianum* in T1 treatment, *T. reseei* in T2 and *T. guizouhense* in T3 and TC *Trichoderma* extracted from commercial product compared to healthy eggplants Tm1 and infested eggplant Tm2 controls.

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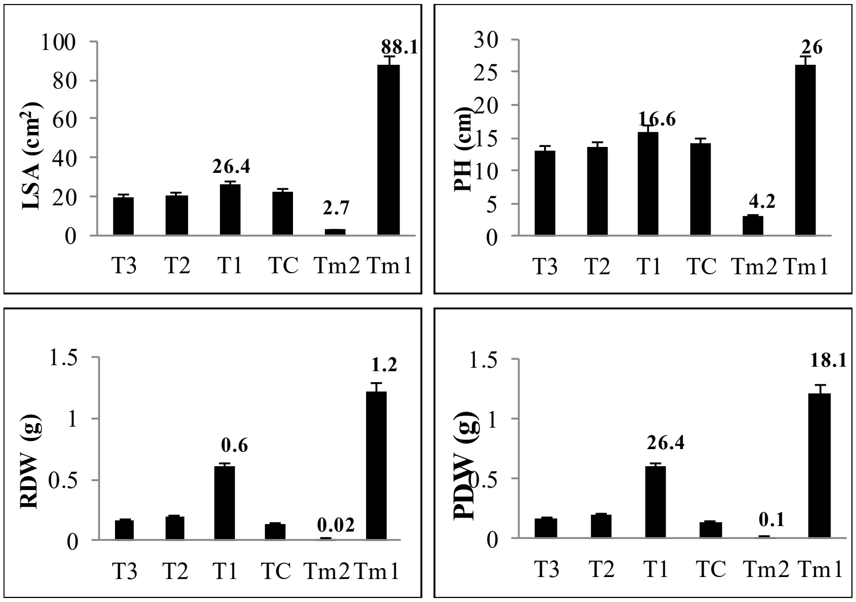

Figure 4. Evaluation of biocontrol efficacy of *Trichoderma* spp. on plant development parameters on Verticillium-eggplant pathosystem. Plant development parameters measured were; Leaves surface Area (LSA), Plant Height (PH), Root dry weight (RDW) and Plant dry weight (PDW).

compared to healthy plant Tm1. For instance LSA = 19.5 cm^2 of eggplant occurred in T3 treatment while LSA = 88.1 cm^2 occurred in healthy plant Tm1, (P = 0.000). LSA dramatically decreased to 2.7 cm^2 in Tm2 controls (Figure 4).

Interestingly, there seems to be different asset between disease incidence assessed above-ground (DI-AU) and in root unit (DI-RU). DI-RU was recorded at 12.5%, 25.0%, 31.3% and 37.5% in T3, TC, T1 and T2 treatment respectively (Figure 1). Root Dry Weight (RDW) recorded in eggplant with different treatments was respectively 0.1 gram (g) in TC, 0.2 g in T2 and T3 and 0.6 g in T1 compared to 1.2 g RDW for healthy eggplant controls Tm1. It seems that roots weight was significantly affected by disease responses when infested with *Verticillium* with only RDW = 0.2 g in Tm2 controls (for more details see Figures 4 and 5). In fact, Root dry weight recorded support the overall results obtained in DI-RU. It may be reasonable to suppose from DI-RU values that *Trichoderma* treatments were able to alleviate *Verticillium* root disease in eggplant. Moreover, from plant height (PH) results, it can be deduced that eggplant height decreased at some extent. Yet, PH was maintained at somewhat remarkable level in treated eggplants compared to Tm2 controls. That is, Tm2 infested controls hardly reached their 4.2 centimeters (cm) height whereas eggplant cultivars in T1 treatment containing T. afro-harzianum maintained their height at 16.6 cm (P = 0.000). PH in eggplants in T2, T3 and TC treatment noticeably reached 12.6, 13 to 13.5 centimeters respectively compared to Tm1 with PH = 26 cm, (P = 0.000).

These results were in line with some of previous work on *Verticillium* biocontrol efficacy test in many crop-systems in green house and field conditions. For instance, Zheng et al (2011) tested biocontrol potential and efficacy of 105 antagonists including *Trichoderma* spp. in the *V. dahliae*-cotton pathosystem in green house conditions. In their work, Zheng et al (2011) assessed 33 fungal antagonists including *Trichoderma* spp. with biocontrol potential in vitro which displayed efficacy under green house (Zheng et al., 2011). Carrero-Carron et al. (2016) have demonstrated that two of T. asperellum strains reduce significantly the severity of defoliating Verticilium disease on olive plants and promoting growth in infested and non-infested olive plants (Carrero-Carron et al., 2016). These interesting biocontrol properties and potential of *Trichoderma* spp. on root disease like *Verticillium* investigated in the previous and the present studies may be due to a number of reasons including *Trichoderma* antagonistic traits and inoculation method applied.

Figure 5. Roots of eggplant artificially infested with Verticllium and treated with different *Trichoderma* spores suspenssions; T. afro-harzianum in T1 treatment, T. reseei in T2, T. guizouhense in T3, and TC *Trichoderma* extracted from commercial product compared to controls; healthy eggplants Tm1 and eggplant inoculated with Verticillium only Tm2.

As mentioned in the literature investigating the naturally occurring highly susceptible eggplant and virulent *Verticillium* interactions allowed us to reveal the congruent potential of *Trichoderma* species like *T. afro-harzianum* in this work and exhibiting the antagonistic potential in reducing virulence of such pathogen. Another point, *Trichoderma* has been previously demonstrated to be used as compatibly as efficacious in biocontrol when combined with other BCAs.

Thaloromyces flavus and non pathogenic *Fusarium oxysporum* have been recognized as the most effective fungal biocontrol agents against *Verticillium dahliae* disease. In fact, different research work demonstrated the potential of different antagonists like *Thalaromyces flavus, Fusarium oxysporum* and *phomopsis* sp. to control different *Verticillium* wilt in tomatoes, pistachio, cotton and eggplant (Marois et al., 1982; Tjamos et al., 2004; Zheng et al., 2011; Angelopoulou et al., 2014). Recently Yuan et al (2017) research work on biocontrol efficacy of Penicillium simplicissimum with DI = 41.4%, Acremonium sp with DI = 39.2%, Leptosphaeria sp. with DI = 32.4%, and Talaromyces flavus with DI = 36.9% against *Verticillium* disease showed effective control of the disease when seed soaked with these fungal antagonists (Yuan et al., 2017).

Therefore, combination of *Trichoderma* spp. with *Thaloromyces flavus*, non-pathogenic *Fusarium oxysporum* or *Penicillium simplicissimum* may be used as an integrated biocontrol application against *Verticillium dahliae*.

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