Ability of Non-Pathogenic *Fusarium oxysporum* Strain Fo47 to Suppress Rhizomania Disease of Sugar Beets in Morocco

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Abstract

Rhizomania is one of the most devastating diseases of sugar beet worldwide. The disease poses a serious threat to Moroccan production and it is capable of significantly decreasing quality and yield of sugar beet plantations. The long-term survival of its fungal vector (*Polymyxa betae*) in soil makes it a very difficult disease to manage. Therefore, this study investigated the potential of a non-pathogenic fungal *Fusarium oxysporum* strain Fo47 to control *Polymyxa betae*. This biocontrol agent was applied as soil treatment, seed treatment, or a combination of the both treatments. A bio-test was performed on treated soil. After four weeks of culture, the roots of sugar beet seedlings were retrieved and analyzed by the DAS-ELISA test. Results indicated that *F. oxysporium* Fo47 reduced the activity and survival of *P. betae* when compared to a reference biocontrol agent *Trichoderma harzianum*, which only revealed significant in reducing the viral load of Beet Necrotic Yellow Vein Virus (BNYVV) as seed treatment. The non-pathogenic *Fusarium oxysporum* Fo47 was more effective as soil treatment and allowed almost the same reduction of BNYVV virus concentration as *T. harzianum* 908. Therefore, our findings emphasizes that the performance of the biocontrol agent depends on the method of application.

Keywords: Beet Necrotic Yellow Vein Virus; biocontrol, *Fusarium oxysporum* strain Fo47; *Trichoderma harzianum* strain 908

Introduction

Sugar beet (*Beta vulgaris* L.) is considered one of the major important cultivated crops in many countries, including Morocco. This crop has gained popularity and importance over time due to its various uses as a food source for humans and animals. However, the occurrence of several diseases and outbreaks during recent years in Morocco resulted in economically important loss of sugar beet yield in many sites of production (Anonymous, 2005; Snaiki *et al.*, 2005). Among them, Rhizomania is one of the most destructive diseases affecting sugar beet in Morocco. This disease was caused by *Beet Necrotic Yellow Vein Virus* (BNYVV) and sprayed over nature via the fungus *Polymyxa betae* Keskin (Brunt and Richards, 1989; Richards and Tamada, 1992; Van Regenmortel *et al.*, 2000). Rhizomania has been reported to occur in many countries (Putz *et al.*, 1990), and it is currently one of the most destructive sugar beet diseases in the world (Rush and Heidel, 1995; Scholten and Lange, 2000). The losses caused by rhizomania are usually over 30% and may even reach 100% in some cases (Asher, 1993).

To the best of our knowledge, this disease is difficult to control when it was declared and no such effective control method was found to date. Thus, common control strategies such as cultural control practices and soil disinfection showed their failures and the only way to control rhizomania is still the use of tolerant sugar beet varieties to the disease, however, the available tolerant varieties in the market were started showing their limitations. In addition, the manifestations of rhizomania on tolerant varieties were reported at several sites (ITB, 2010). This situation was partially explained by the lack of tolerance of some varieties, or higher pressure of the disease, due to one hot and wet spring and a more important presence of the *Polymyxa*, or even the appearance and increase of virulent pathotypes in the pathogen population (Schmit *et al.*, 2002; Rush, 2003; Bornemann and Varrelmann, 2011).

During last decades, biological control has increased and gained more attention of researchers in order to overcome the resistance problems against common used synthetic...
substances fueled by public concerns over the long-term impact of these substances on the environment and human health, and the need to develop eco-friendly alternatives for disease control (Brunner et al., 2005; Hagagg et al., 2006; Jin and Custis, 2011; Kakan et al., 2013). In most cases, biological control of plant diseases relies on the use of effective bacterial and fungal antagonists (Heydari and Pessarakli, 2010; Naraghi et al., 2010a, b and c; Jorjani et al., 2011; Naraghi et al., 2012a and b; Mansouri et al., 2013). These microorganisms usually controlled their target pathogens by different mechanisms including antibiotics, mycoparasitism, and competition for space and nutrient (Whipp's, 2001). Trichoderma spp. are among the most common saprophytic fungi extensively investigated for their biological control effects against fungal plant pathogens (Nelson, 2004; Woo and Lorito, 2007; Schuster and Schmoll, 2010; Kumar, 2013). Trichoderma members act on their targets by producing extracellular enzymes and antifungal substances, but they also may compete with pathogens for nutrients and space to delay or prevent infection and subsequently enhance plant growth and development and induce plant resistance to disease (Shalini et al., 2006). However, many of F. oxysporum species are among pathogens threatening a wide range of cultivated plants, but few strains of this fungus are well recognized as biological control agents (BCAs). For example, the non-pathogenic strains Fo47, have the particularity to protect plants from pathogenic infection of the same fungus. The biocontrol activity of Fo47 strains is relatively specific and they can be used to manage Fusarium wilt (Fravel et al., 2003; Alabouvette et al., 2009). The role played by non-pathogenic Fusarium species in controlling Fusarium wilt was first reported by Smith and Snyder (1971) on sweet potato plants. In addition, Kaur et al. (2010) found that Fusarium-suppressive soil contain a higher level of non-pathogenic Fusarium population; suggesting their eventual role in reducing the pathogenic Fusarium population.

Although, numerous reports underlined the possibility of applying beneficial microorganisms to control plant diseases pathogens, the biocontrol of soil-borne pathogens was always difficult than that of aerial diseases (Divya Rani and Sudini, 2013). In addition to the two extensively studied groups of BCAs Pseudomonas spp. and Trichoderma spp., the non-pathogenic strains of Fusarium oxysporum represent an original model, which may lead to the development of an effective and reliable biofungicide for the control of soil-borne pathogens both in greenhouse and field conditions. However, to our knowledge, until now there were few investigations dealing with biocontrol of the fungal vector of rhizomania (Resca et al., 2001; Naraghi et al., 2014). Therefore, the main objectives of the present study were to investigate the ability of the non-pathogenic fungus Fusarium strain Fo47 to suppress fungal vector P. betae and to evaluate the impact of different application methods of BCA on the biocontrol efficacy of sugar beet rhizomania disease in Morocco. The Trichoderma harzianum strain 908 was used as BCA reference. This study is a part of the development of a biocontrol strategy against this devastating rhizomania disease.

**Materials and Methods**

**Laboratory experiments**

**Soil sampling for the greenhouse experiment**

Soil samples were collected from infected sugar beet fields with rhizomania in Souk Sebh Ouled Neema district, Beni Mellal, province. Field Sampling procedure was performed according to the standards recommended by the official method of virus detection rhizomania in sugar beet (Anonymous, 2006). To ensure the infestation of the field soil by rhizomania, sugar beet tubers suspected to have the disease were removed from the fields and their thin rootlets were analyzed using an Enzyme Linked ImmunoSorbent Assay (ELISA) test. To ensure a homogeneous and important level of infestation, soil was mixed with sterile soil (1:1) and then soil was mixed with activated carbon (0.673 g/pot). Infested soil samples will be used further for the subsequent greenhouse experiments.

**Preparation of fungal biocontrol agents**

In this study, the hyperparasitic fungi F. oxysporum strain Fo47 and the BCA reference Trichoderma harzianum strain 908 were evaluated for their ability to reduce both rhizomania disease and fungal vector survival. Both fungi F. oxysporum strain Fo47 and T. harzianum strain 908 were applied at a concentration of 1×10⁶ CFU/g and 1×10⁷ CFU/g respectively.

**Greenhouse trial**

The trapping test was conducted according to the recommendations noted in “The official method of Detection of the virus in Rhizomania of beet by the biological test followed by the ELISA test” (Anonymous, 2010). To avoid contamination between samples, all sampling involved aseptic precautions were undertaken, including the use of sterile and disinfected instruments and disposable rubber gloves between each sample.

**Effectiveness of fungal biocontrol agents in suppressing P. betae**

The experiment was laid out in a randomized complete block-split plot design using the main factor (biocontrol agent) in four levels (1 – T. harzianum; 2 – F. oxysporum; 3 – the uninfested control; and 4 – the infested control) and replicated at 6 different times, and the sub-factor (method of BCA application) in three forms (1-seed treatment, 2-soil treatment, and 3-a combination of the above-mentioned methods). Each replication consisted of a pot containing 150 ml of infested sugar beet field soil with rhizomania. 20 seeds of the sugar beet susceptible cultivar (“Rhizopol”) were sown in each pot. The negative control (disease-free) was made with three pots of 150 ml of sterilized soil, while other three pots filled with infested soil were served as positive control. The plants were maintained for four weeks in greenhouse with a 16 h daily photoperiod at 25 °C and 50% relative humidity.

**Virological analysis**

Detection of BNYVV in sugar beet roots was performed using Double Antibody Sandwich - Enzyme Linked
Immuno Sorbent Assay (DAS-ELISA) according to the protocol provided by the manufacture (SEDIAG, France). Each sample was tested in two replicates. Reading was done after incubation periods of 1 h and 2 h with substrate pNPP (p-nitrophenyl phosphate) at 37 °C. The microplates were analysed using a BioTek Elx 800™ microplate reader.

Statistical analysis of data
All experiments were at least repeated twice and the collected data were subjected to analysis of variance (ANOVA) and the means were compared using Duncan’s Multiple Range Test with statistical software SPSS (SPSS version 20). The level of significance was determined in different treatments at 1% probability.

Results and Discussion
In this study, the suppression of the occurrence of *P. betae* in form of ‘cystosori’ in beetroots was considered as a rough indication of the biocontrol efficiency of BCAs. On the other hand, the ELISA results of BNYVV in the roots were considered as main objective and were statistically evaluated. After four weeks of planting, the virological analysis of beet roots samples was made through DAS-ELISA test. The obtained results showed that the activity of the fungal vector *P. betae* was negatively affected by BCAs *F. oxysporum* and *T. harzianum*.

The effect of the main factor of the BNYVV multiplication was significant at P < 0.01. When compared to the infested control, all the treatments containing antagonists showed a significant decrease in the proliferation of BNYVV (Table 1). There is no significant difference between the two antagonists. The treatments based on Fo47 and on Th908 demonstrated a higher significant decrease in the viral load of BNYVV, in comparison with those the infested control (Table 1). Th908 allowed a reduction of 47.6% of the proliferation of BNYVV compared to the positive control. As for the Fo47, a reduction of 44.7% was observed. Thus, it is obvious that the antagonistic effect of both fungi allows a decrease in the viral concentration (Table 1).

The effect of the sub-factor (method of antagonist application) on the proliferation of *P. betae* and multiplication of BNYVV was significant at P < 0.01. By considering that the optical density is proportional to the viral concentration, the results of the ANOVA showed that the proliferation of BNYVV was significantly reduced by the method of antagonist application as well as by both studied antagonists (Table 1 and 2).

Similarly, the effect of the combination of the main factor and sub-factor on sugar beet root weight was significant (P < 0.01). There were four statistically different groups (Table 3). Among the treatments, the soil treatment based on Fo47 and the seed treatment based on Th908 showed the lowest optical density (OD). However, the highest OD was recorded for both seed and soil treatment and as affected by Th908 (Table 3). Furthermore, compared to the positive control, all antagonist treatments showed a significant decrease in the viral load (Table 3).

Amongst the different methods of antagonist application mentioned above, soil treatment by the fungus *F. oxysporum* resulted in a higher reduction of the proliferation of BNYVV. However, the seed treatment with *T. harzianum* allowed the most reduction of disease.

Table 1. Efficacy of the main factor (two antagonists: *F. oxysporum* and *T. harzianum*) in the suppression of BNYVV multiplication in greenhouse conditions

<table>
<thead>
<tr>
<th>Main factor</th>
<th>Optical density</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. harzianum</em> strain 908</td>
<td>0.55 b*</td>
</tr>
<tr>
<td><em>F. oxysporum</em> strain Fo47</td>
<td>0.58 b</td>
</tr>
<tr>
<td>The infested control</td>
<td>1.03 a</td>
</tr>
<tr>
<td>The healthy control</td>
<td>0.13 c</td>
</tr>
</tbody>
</table>

*Values marked with different letters in the columns are statistically different according to Duncan’s multiple range test (p ≤ 0.01).*

Table 2. Efficacy of the sub-factor (different application methods of *T. harzianum* strain 908 and *F. oxysporum* strain Fo47) in the suppression of BNYVV multiplication in greenhouse conditions

<table>
<thead>
<tr>
<th>Sub-factor</th>
<th>Optical density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil treatment</td>
<td>0.49 b*</td>
</tr>
<tr>
<td>Seed treatment</td>
<td>0.53 b</td>
</tr>
<tr>
<td>Soil and seed treatment</td>
<td>0.72 a</td>
</tr>
</tbody>
</table>

*Values marked with different letters in the columns are statistically different according to Duncan’s multiple range test (p ≤ 0.01).*

Table 3. Efficacy of a combination of the main (different application methods of *T. harzianum* strain 908 and *F. oxysporum* strain Fo47) and the sub-factor (two antagonists: *T. harzianum* and *F. oxysporum*) in the suppression of BNYVV multiplication in greenhouse conditions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Optical density</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. harzianum</em> soil</td>
<td>0.49 b*</td>
</tr>
<tr>
<td><em>F. oxysporum</em> soil</td>
<td>0.42 b</td>
</tr>
<tr>
<td><em>T. harzianum</em> seed</td>
<td>0.42 b</td>
</tr>
<tr>
<td><em>F. oxysporum</em> seed</td>
<td>0.65 c</td>
</tr>
<tr>
<td><em>T. harzianum</em> soil and seed</td>
<td>0.75 c</td>
</tr>
<tr>
<td><em>F. oxysporum</em> soil and seed</td>
<td>0.69 c</td>
</tr>
<tr>
<td>The infested control</td>
<td>1.05 a</td>
</tr>
<tr>
<td>The healthy control</td>
<td>0.13 d</td>
</tr>
</tbody>
</table>

*Values marked with different letters in the columns are statistically different according to Duncan’s multiple range test (p ≤ 0.01).*
The performance of the biocontrol agent depends on the method of application. The non-pathogenic strain of *Fusarium oxysporum* Fo47 allowed a comparable concentration of BNYVV virus in the roots of the sugar to that recorded with *T. harzianum* 908.

Until now, no study was done on the biocontrol of the fungal vector of sugar beet rhizomania in Morocco. Our study is the first report on the ability of the non-pathogenic fungus *Fusarium oxysporum* in suppressing rhizomania disease. In fact, there was no report about the use of this fungus as BCA against this devastating disease. The reduction of resting structure of the fungal vector by antagonist treatments was in line with previous findings reported in few studies with bacterial and fungal agents. For example, using antagonistic isolates of *T. harzianum*, D’Ambra and Mutto (1986) found that *Trichoderma* isolates were able to parasitize and decompose the resting structures of the fungal vector. Similarly, Aksoy and Yilmazz (2008) demonstrated that *Pseudomonas putida* biotypes A and B reduced the population of disease fungal vector by 23% and 75%, respectively. Two non-pathogenic strains of *Fusarium oxysporum* CS-20 and Fo47 have demonstrated their ability to protect the plants from the infection by the aggressive isolates of the same fungus (Kaur et al., 2010; Shcherbakova et al., 2015). Our results showed also the capability of *Fusarium oxysporum* strain Fo47 to control the fungus *P. betae*, Alabouvette et al. (2009) reported the effectiveness of non-pathogenic strains of *Fusarium* in controlling *Fusarium* wilt.

Among the different methods of antagonist application mentioned above, soil treatment by the fungus *Fusarium oxysporum* resulted in a higher reduction of the proliferation of BNYVV. However, the seed treatment with *T. harzianum* allowed the most reduction of disease. Camporta et al. (1988) have disclosed that seed-coating preparations with a conidial suspension were the best solution because it allows economies the amount of the biological agent. The technique of coating allows the antagonist to colonize the rhizosphere and the root system. Naraghi et al. (2014) evaluated the impact of applying *T. harzianum* as soil and seed treatment in controlled conditions and highlighted that the soil treatment was more effective in reducing the *P. betae* population.

In the present study, the soil treatment method was shown to be far effective than the combination of seed and soil treatments in reducing the infestation of disease. This can be explained by a “crowding effect” phenomenon as previously reported by Chitarra (2003) on *Aspergillus* and *Penicillium* fungi. Under greenhouse conditions, Jakubikova et al. (2010) pointed out that *T. harzianum* isolates with an inhibition rate of 21-68% toward the pathogenic virus (BMYVV). The results of the previous studies have also shown that BCAs are effective against damping-off, root rot, and wilt diseases of ornamental plants, vegetables and cereals (Whipps, 2001; Assef et al., 2008; Naraghi et al., 2010a, b, c; Godhani, 2011; Ojahian, 2011).

Conclusions

Overall, the results reported in this study indicated the possibility of using beneficial microorganisms to decrease rhizomania disease on sugar beet by lowering the population of its fungal vector as well as the multiplication of BNYVV. The non-pathogenic strain of *Fusarium oxysporum* Fo47 provided the same concentration of BNYVV virus in the roots of the sugar beet as Th908. The performance of the biocontrol agent depends on the method of application. The soil treatment method was revealed most efficient in suppressing the multiplication of BNYVV and the proliferation of *P. betae* population.

Our study is the first report on the ability of the non-pathogenic fungus *F. oxysporum* strain Fo47 in suppressing rhizomania disease. In fact, there was no report about the use of this fungus as BCA against this devastating disease. Therefore, results of the present study may have a practical application in the formulation of an integrated and eco-friendly management strategy for the control of sugar beet rhizomania in sugar beets growing areas, including Morocco.

Acknowledgements

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