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Biological control of *Polymyxa betae*, fungal vector of rhizomania disease of sugar beets in greenhouse conditions

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Abstract: Rhizomania is one of the most important diseases of sugar beet around the world – including in Iran. The disease causes a severe decrease in sugar yield and is a limiting factor in sugar beet cultivation. Control of the disease is very difficult due to the long-term survival of its fungal vector (*Polymyxa betae*) in the soil. In this study, we investigated the effects of antagonistic fungal isolates on the population of the resting structure (cystosorus) of *P. betae*, under greenhouse conditions. Antagonistic fungi, including *Trichoderma harzianum* and *Talaromyces flavus*, were isolated from soil samples collected from sugar beet infested fields in the Semnan Province of Iran. In the next step, their inocula were prepared through reproduction on rice bran. For evaluation of the efficacy of antagonists in greenhouse conditions, a split plot trial was conducted and performed. The main factor was three different methods of application of *T. flavus* as the soil treatment, seed treatment, and a combination of both methods. The sub-factor was the use of different fungal isolates. To determine the cystosorus population of the fungal vector, seedling roots in all treatments were stained with lactic acid and fuchsine (lactofushine), 60 days after sowing. The number of cystosorus in one gram of root was counted using a light microscope and hemocytometer. At the end of the study, average root weight in different treatments was also measured to select and introduce the best treatments in regard to their effects on root weight. According to the results, the number of cystosorus in 1 g of root was different in various treatments and those treatments containing TF-Su-M-1, TF-Su-M-2, TH-Su-M-1, and TH-Su-M-2 used as a soil application method were more effective in the reduction of the cystosorus population and root weight increase. Among the above-mentioned treatments, maximum reduction of cystosori population and the increase in root weight were observed in TH-Su-M-1 and TF-Su-M-2 through the soil application method.

Key words: antagonistic fungi, biocontrol, *Polymyxa betae*, rhizomania, rhizomania disease, sugar beet

Introduction

Sugar beet (*Beta vulgaris* L.) is one of the most important cash crops, and its cultivation as a major crop is common in many countries, including Iran. The importance of this crop is due to its various uses as a food source for humans and animals. The cultivated area of sugar beet in Iran is about 200,000 ha, which is a considerable amount, but yield and production of this crop in Iran needs to increase. The occurrence of different diseases is an important reason for the low sugar beet yield in Iran. One of the most important diseases of sugar beet is rhizomania caused by *Beet necrotic yellow vein virus* (BNYVV) and transmitted by the fungus *Polymyxa betae* Keskin in nature (Brunt and Richards 1989; Richards and Tamada 1992; Van Regenmortel *et al*. 2000).

Interest in biological control has increased recently fuelled by public concerns over the use of chemicals in the environment in general, and the need to find alternatives to the use of chemicals for disease control (Brunner *et al*. 2005; Haggag *et al*. 2006; Jin and Custis 2011; Kakvan *et al*. 2013).

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). Biological control of plant diseases by antagonistic bacteria and fungi (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) is considered to be one of the most efficient method that researchers have focused on in recent years. This method is not very expensive and is not hazardous for the environment and non-target organisms.

The main mechanisms involved in biocontrol are antibiosis, mycoparasitism, and food competition (Whipps 2001). Trichoderma strains can produce extracellular enzymes and antifungal antibiotics, but they also could be

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competitors of pathogenic fungi, stimulate development and induce plant resistance (Shalini *et al*. 2006). On the other hand, 40% of non-volatile extracts (Talaron) of *Talaromyces flavus* are thought to affect production of hydrogen peroxide due to the glucose oxidase enzyme, and this gives this fungus antibacterial and antifungal characteristics (Kim and Fravel 1990).

So far, several studies have been conducted and executed in Iran in regard with the dispersion of rhizomania in different provinces (Farzadfar *et al*. 2007). However, there has been no considerable study on the biocontrol of the fungal vector of this disease. The objectives of this study were to investigate and evaluate the efficacy of *T. flavus* and *Trichoderma harzianum* isolates and their different application methods in biological control of sugar beet rhizomania disease in Iran.

Materials and Methods

Laboratory experiments

Preparation of antagonistic fungal isolates

To prepare the *T. harzianum* and *T. flavus* isolates, soil samples were collected from sugar beet fields in Meyamey, Semnan province, using Butterfield and De Vay's method (1977). The specific culture media of TSM2 (Koehl 1989) and TF (Marois *et al.* 1984) were used to isolate *T. harzianum* and *T. flavus*.

Preparing T. harzianum and T. flavus inocula

In this study, two isolates of *T. harzianum* and two isolates of *T. flavus* were used. The inoculum of each antagonistic fungal isolate was prepared separately. This meant that each isolate was proliferated on a rice bran bed (Naraghi *et al*. 2010a).

Collection of soil samples from infested sugar beet fields for use in the greenhouse experiments

Soil samples were collected from those sugar beet fields with a history of rhizomania in Meyamey, Semnan province of Iran. To ensure the infestation of the field soil, sugar beet tubers suspected of the disease were removed from the fields and their thin rootlets were removed for detecting cystosorus of the fungus *P. betae* (Abe and Tamada 1986). The rootlets were stained according to the following procedure:

1. The rootlets were washed and prepared in 3-cm long pieces from the terminal part, as the pieces included root caps.
2. The rootlet pieces were placed in fuchsine acid (0.25 g of fuchsine in 50 ml of lactic acid) effervescently for 2 min.
3. The root pieces were placed in lactophenol for bleaching.
4. The cystosori in the rootlet pieces were examined and counted using a light microscope.

Greenhouse experiments

Antagonistic effects of T. harzianum and T. flavus on P. betae and the sugar beet root weight

The experiment was performed four times with a randomized complete block-split plot design using the main factor (application of antagonist) in three forms (1-seed treatment, 2-soil treatment, and 3-a combination of the above-mentioned methods), and sub-factor (different antagonistic isolates) in six levels (1 and 2 – two isolates of *T. harzianum*, separately; 3 and 4 – two isolates of *T. flavus*, separately; 5 – the uninfested control; and 6 – the infested control.

Each replication consisted of a pot containing 3 kg of sugar beet field soil in which the soil had a history of rhizomania. Five seeds of the sugar beet susceptible cultivar (Shirin) were sown in each pot. After germination of the seeds and ensuring that the seedling were growing, two seedlings were kept in each pot to study the growth characteristics. Sixty days after sowing, one seedling in each pot was removed to determine the number of cystosorus of the fungal vector there was in one gram of root. The remaining seedlings were kept in the pots up to one year after sowing, to determine the root weight. The number of cystosorus in the seedling rootlets was determined as described above.

The plants were kept on a greenhouse bench at 21–23°C and 30% relative humidity (RH). Daylight was supplemented with fluorescent light to provide a 12 h day length.

Statistical analysis of data

The data were subjected to analysis of variance (ANOVA) and the means were compared using Duncan's Multiple Range Test with MS-TAT-C statistical software (Johannes Kepler University of Linz, Austria). The level of significance was determined in different treatments at 1% probability.

Results

Laboratory experiments

Preparation of antagonistic fungal isolates

In laboratory experiments, two isolates of *T. flavus* (TF-Su-M-1 and TF-Su-M-2) and two isolates of *T. harzianum* (TH-Su-M-1 and TH-Su-M-2) obtained from sugar beet field soil in the Meyamey region, Semnan province, were used.

Macroscopic observation of *T. harzianum* isolates showed that at first, the colonies on Potato Dextrose Agar (PDA) medium were white at the top, and then they turned to light green in the center after two days upon appearance of green conidia. Seven to eight days after incubation, the colonies turned dark green; 60% at the top, whereas the bottom of the colonies turned light green. Microscopic observation of the isolates showed that conidiophores had dual branches along the main axis. Phi-

alids with flask-like shapes and a swollen middle part had a cluster form at the end of the conidiophore branches. Moreover, colorless chlamydospores with a smooth wall were observed.

Macroscopic observation of *T. flavus* isolates showed that colonies on the PDA culture medium had a surrounding bright yellow aura and a green area in the center. In terms of microscopic specifications, the above isolates had hyphae and asexual reproduction organs (conidia and conidiophores) similar to *Penicillium*. Sexual organs including ascogonium, anteridium, ascocarp, ascus, and ascospore were observed in the above isolates.

Preparation of T. harzianum and T. flavus inocula

To add the fungal antagonistic isolate inoculum to pots containing 3 kg soil, based on 2×10^7 spores per gram of soil, the required amount of *T. flavus* and *T. harzianum* isolates was calculated as 15 g and 18 g per pot, respectively.

Examination of sugar beet samples

Symptoms of rhizomania in the sugar beet samples collected from the infested fields included tuber narrowing, beard-like roots on the tubers, and green veins with yellow intervals. Furthermore, in the analysis of the microscopic section of the roots, 224 cystosorus of the vector *P. betae* were counted in one gram of root.

Greenhouse experiments

Observation of disease symptoms

Sixty days after sowing, disease symptoms appeared in the plants. The symptoms included root narrowing and beard-like roots in the sugar beets infested with rhizomania. A minimum of severe symptoms was observed in all treatments affected by antagonistic isolates as the soil treatment. Moreover, the combination of two methods (soil and seed treatment) considerably reduced the severity of symptoms compared to the seed treatment alone.

Determining the number of resting structures (*cystosorus*) *of P. betae in sugar beet seedling roots*

The effect of the main factor (different application methods of antagonistic isolates) on the cystosorus count of the disease vector fungus in one gram of root, was significant at a probability level of 1.0%. Among the treatments, the minimum and maximum mean cystosorus count in one gram of root belonged to the seed treatment and the soil treatment, respectively (Table 1). The effect of a sub-factor (different antagonistic isolates of *T. flavus* and *T. harzianum*) on the cystosorus count of the disease vector fungus in one gram of root was significant at a probability level of 1.0%. Compared to the infested control, all the treatments influenced by antagonistic isolates showed a significant reduction in the cystosorus count of the disease vector fungus in one gram of root (Table 3). Among the treatments, TH-Su-M-2 treatment showed a significant increase in mean cystosorus count of the disease vector fungus (Table 2). However, there was no significant statistical difference among TF-Su-M-1, TF-Su-M-2, and TH-Su-M-1 (Table 2).

The effect of a combination of the main factor and the sub-factor on the cystosorus count of the disease vector fungus in one gram of root, was significant at a probability level of 1.0%. Based on the results of grouping the means of the cystosorus count in one gram of root, the treatments were put into eleven statistical groups (Table 3). Among the treatments, the soil treatment containing the TF-Su-M-2 isolate showed a minimum mean cystosorus count of the disease vector fungus in one gram of root. However, the maximum mean cystosorus count of the disease vector fungus in one gram of root, was observed in the seed treatment affected by the TH-Su-M-2 isolate (Table 3).

Effects of T. harzianum and T. flavus on sugar beet root weight

The effect of the main factor on the sugar beet root weight was significant at a probability level of 1.0%. Of the treatments, minimum and maximum mean sugar beet root weight belonged to the seed treatment and the soil treatment, respectively (Table 1). The effect of the sub-factor on sugar beet root weight was significant at a probability level of 1.0%. Compared to the infested control, all the treatments containing antagonistic isolates showed a significant increase in sugar beet root weight (Table 2). Of the treatments affected by antagonistic isolates, the treatments containing TF-Su-M-1 and TF-Su-M-2 showed a significant increase in sugar beet root weight, compared to treatments containing TH-Su-M-1 and TH-Su-M-2 (Table 2). However, there was not any significant statistical difference among treatments affected by different isolates related to the same fungal genus (Table 2).

Table 1. Efficacy of the main factor (different application methods of *T. flavus* and *T. harzianum*) in the reduction of the *P. betae* cystosori population and in increasing sugar beet root weight in greenhouse conditions

Main factor	Average cystosorus number in 1 g of root	Average root weight [g]
Soil treatment	30.83 c	68.26 a
Seed treatment	48.17 a	47.85 c
Soil and seed treatment	39.33 b	58.52 b

*values marked with different letter in the columns are statistically different according to Duncan's multiple range test ($p \leq 0.01$)

Table 2. Efficacy of the sub-factor (different isolates of *T. flavus* and *T. harzianum*) in the reduction of the *P. betae* cystosori population and in increasing sugar beet root weight in greenhouse conditions

Sub-factor	Average cystosorus number in 1 g of root	Average root weight [g]
TF-Su-M-1	41.00 c	62.31 b
TF-Su-M-2	39.67 c	61.71 b
TH-Su-M-1	38.00 c	56.38 c
TH-Su-M-2	47.00 b	56.44 c
The infested control	71.00 a	32.76 d
The healthy control	0 d	79.65 a

*values marked with different letter(s) in the columns are statistically different according to Duncan's multiple range test ($p \leq 0.01$)

Table 3. Efficacy of a combination of the main factor (different application methods of *T. flavus* and *T. harzianum*) and the sub-factor (different isolates of *T. flavus* and *T. harzianum*) in the reduction of the *P. betae* cystosori population and in increasing sugar beet root weight in greenhouse conditions

Treatment	Average cystosorus number in 1 g of root	Average root weight [g]
TF-Su-M-1 – soil	30.00 h	65.17 d
TF-Su-M-2 – soil	26.00 j	65.65 d
TH-Su-M-1 – soil	28.00 i	89.70 a
TH-Su-M-2 – soil	30.00 h	76.64 c
TF-Su-M-1 – seed	54.00 c	56.30 f
TF-Su-M-2 – seed	51.00 d	62.36 e
TH-Su-M-1 – seed	54.00 c	24.48 h
TH-Su-M-2 – seed	59.00 b	31.52 g
TF-Su-M-1 – soil and seed	39.00 f	65.47 d
TF-Su-M-2 – soil and seed	42.00 e	57.13 f
TH-Su-M-1 – soil and seed	32.00 g	54.97 f
TH-Su-M-2 – soil and seed	52.00 d	61.15 e
The infested control	71.00 a	32.76 g
The healthy control	0 k	79.65 b

*values marked with different letter(s) in the columns are statistically different according to Duncan's multiple range test ($p \leq 0.01$)

The effect of the combination of the main factor and sub-factor on sugar beet root weight was significant at a probability level of 1.0%. According to the results of the grouping means of the sugar beet root weight, the treatments were put into eight statistical groups (Table 3). Among the treatments, the soil treatment containing TH-Su-M-1 showed a maximum mean sugar beet root weight. However, the minimum mean sugar beet root weight was observed in the seed treatment affected by TH-Su-M-1 (Table 3). Furthermore, compared to the healthy control, only the soil treatment containing TH-Su-M-1 showed a significant increase in sugar beet root weight (Table 3).

Discussion

The overall results of this study indicate that it may be possible to decrease rhizomania disease by reducing its fungal vector population, using *Talaromyces* and *Trichoderma* fungal antagonists. In this study, these fungal antagonists were capable of both disease suppression and promotion of sugar beet growth factors, in greenhouse conditions.

Among the different methods for application of the above-mentioned antagonists, the soil treatment method was the most efficient in reducing the *P. betae* population. Under greenhouse conditions, the incidence of the disease in the plants sown in the infested soil revealed that the soil had a potential pathogenicity for the sugar beet. Results of a previous study on the dispersion of *P. betae* in Isfahan, West Azerbaijan, and the Khorasan-e-Razavi provinces of Iran, showed the presence of 164–480 cystosori in one gram of root in sugar beet fields infested with rhizomania. This amount was an indicator of the high probability of the disease occurrence (Jalali *et al*. 2009).

So far, no study has been done on biocontrol of the fungal vector of sugar beet rhizomania in Iran. However, results of the present study about the reduced resting structure of the disease fungal vector in treatments affected by *T. flavus* and *T. harzianum* agree with the results

of the few previous studies in other countries using antagonistic fungi and bacteria.

For example, in a study on the biocontrol of rhizomania using antagonistic isolates of *T. harzianum*, D'Ambra and Mutto (1986) showed that these isolates could parasitise and decompose the resting structure of the disease fungal vector. In a similar study on the antagonistic effects of *Pseudomonas putida* on the fungal vector, the bacterium biotypes A and B reduced the disease fungal vector populations by 23% and 75%, respectively (Aksoy and Yilmazz 2008).

Moreover, the results of the present study in the treatments affected by antagonistic isolates with different methods of application, showed that the resting structure of the fungal vector was reduced by all antagonistic isolates used as soil treatment. In the previous studies which used antagonistic bacteria and fungi only in the form of spore suspension, the results were somehow different compared with the results of the present study. It can be argued that the high effectiveness of antagonistic fungi used as a soil treatment compared to the seed treatment is due to the higher populations in the inocula added to the soil (Choi 2003; Adandonon *et al*. 2006).

In our study, the soil treatment method was more effective than the combination of the seed and soil application methods. This may be due to a "crowding effect" phenomenon that occurs when the spore populations of antagonistic fungi are too high, as was previously reported by Chitarra (2003) in regard to *Aspergillus* and *Penicillium* fungi. Based on a greenhouse experiment, Jakubikova *et al*. (2006) refereed certain *T. harzianum* isolates with an inhibition capability of 21–68%, for proliferation of the pathogenic virus (BNYVV), as the biological factors effective in the control of rhizomania in sugar beet fields.

The results of the previous studies have also shown that biological control agents are effective against damping-off, root rot, and wilt diseases of ornamental plants, vegetables and cereals, caused by *Rhizoctonia*, *Pythium*, *Phytophthora*, *Sclerotium*, *Fusarium* and some other fungi (Whipps 2001; Asef *et al*. 2008; Naraghi *et al*. 2010a, b, c; Godhani 2011; Ojaghian 2011).

Our results also revealed that maximum reduced cystosori populations of the fungal vector and a maximum increased weight of sugar beet roots were respectively obtained in TF-Su-M-2 and TH-Su-M-1 treatments, using the soil application method. Therefore, these treatments may be used in field studies. Based on the results of this study, recent biological formulations could be used in the field. In a field study, *T. harzianum* has been applied for soil and seed treatments as 2×10^{12} spores/ha (2 kg/ha) and 8 g/kg seed, respectively (Godhani 2011).

The overall results of the present study may have a practical application in the formulation of an integrated and non-chemical management strategy for the control of sugar beet rhizomania which is a serious and devastating disease in all sugar beets growing all over the world, including Iran.

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