Application of some fungal bioformulations for controlling garlic white rot disease in the field conditions

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Abstract

Two field experiments were conducted and executed during 2015 and 2016 to evaluate the effectiveness of six newly developed fungal bioformulations in controlling garlic white rot disease caused by *Sclerotium cepivorum.* The bioformulations were developed using three antagonistic fungi including *Trichoderma harzianum, T. asperellum* and *Talaromyces flavus* and rice bran as an organic carrier. They were selected based on their performance in the greenhouse conditions in a previous study, where they effectively controlled and reduced the garlic white rot disease. Field trials were conducted in a randomized complete blocks design with eight treatments (six bioformulations, an untreated control and a Carbendazim fungicide) each with four replicates. The garlic seeds (bulbs) were coated with each bioformulation and were sown in the field soil pre-inoculated with *S. cepivorum*. The incidence and the index of white rot disease severity were then determined in different treatments 90 days after sowing. Results showed that in the first year field experiment, most bioformulations were effective and reduced both disease factors significantly in comparison with the untreated control. The biofomulations R.B-T.h-1 and R.B-T.h-2, with 9.11% and 13.50% disease incidence, respectively, reduced the disease significantly and were as effective as the fungicide. However, in the second year due to the higher soil inoculum density, four out of the six applied bioformulations performed effectively in controlling the disease. The overall results of this study suggest that these newly developed bioformulations could replace harmful chemical fungicides in managing white rot disease in the garlic fields.

Keywords: biological control, *Sclerotium cepivorum*, fungal antagonists, Iran

Introduction

Continuous using and applying chemical pesticides in the modern agriculture has become the subject of environmental protection agencies and public concerns due to their negative impacts on the non-target organisms and contamination of the agricultural environment (Cook & Baker, 1988). In addition to the above-mentioned problems, the cost of production of chemical pesticides and the possibility of the appearance of resistant races among pest and pathogens should also be considered. The search for non-chemical and ecological friendly alternative strategies is unavoidable and has been the subject of numerous research projects among the agricultural scientists. Natural substances such as plant extracts have also been recommended as one of the suitable alternative choices to replace synthetic chemicals (Bahraminejad *et al*., 2010; Nikan & Khavari, 2014; Nikan, 2015).

Biological control using beneficial microorganisms including bacterial and fungal antagonists has been considered as a safe and viable strategy to replace or to reduce the use and application of the harmful chemicals (Ardakani *et al.*, 2009; Heydari & Pessarakli, 2010; Naraghi *et al.*, 2011; Kakvan *et al*., 2013; Naraghi *et al*,. 2013). In the biological control studies, different fungal antagonists including *Trichoderma* have successfully been used and have produced promising results. For example in a recent study, Naeimi & Zare (2013) used isolates of *Trichoderma* spp. against *Botrytis cinerea* on strawberry and controlled the gray mold disease caused by this fungal pathogen.

In addition to the above-mentioned studies, Kakvan *et al.* (2013) used two species of *Trichoderma* to control sugar beet damping-off and obtained positive results. Along with *Trichoderma* some other antagonistic fungi including *Talaromyces flavus* have also been used against several plant pathogenic agents in biological control studies. For example, *Verticillium dahliae* the causal agent of wilt disease on several plants including cotton, potato and tomato has successfully been controlled by the application of this fungal antagonist (Naraghi *et al.*, 2006; Naraghi *et al.*, 2010; Naraghi *et al.*, 2013). Moreover, in a recent study, Kakvan *et al.* (2013) used *T. flavus* to control *Rhizoctonia solani*-induced sugar beet seedling damping-off disease and produced promising results.

Results of the previous studies have indicated that most biocontrol-active microorganisms act effectively in the laboratory and greenhouse conditions but they lose their effectiveness significantly when transferred to the field which could be due to the several factors including environmental conditions and lack of proper method of application. Unsuitable formulations and delivery of microbial antagonists could be one of the reasons for the failure of biocontrol in the field conditions.

Seed coating or seed treatment is probably the most practical method for the application of microbial antagonists in the field which requires the development and preparation of powdery formulations of the microbial antagonists which can enable the farmers to use them as seed treatment particularly for controlling seed and root diseases (Naraghi *et al.*, 2010; Kakvan *et al.*, 2013; Samavat *et al.*, 2014).

According to the results of previous biocontrol studies, the effectiveness of some microbial antagonists was preserved after they were mixed with organic and inorganic carriers (Heydari & Pessarakli, 2010; Kakvan *et al.*, 2013; Samavat *et al.*, 2014). Garlic (*Allium sativum*) is an important multi-use crop around the world including Iran which is attacked by different pathogenic agents particularly soil borne fungal pathogens such as *Sclerotium cepivorum*, the causal agent of white rot disease (Mahdizadehnaraghi *et al.*, 2007; Francisco *et al.*, 2011). White rot is one of the most damaging and yield reducing diseases of garlic in the world including Iran (McLean & Kirstin, 2001; Saremi *et al*., 2010). Application of the chemical fungicides is the most common method for controlling this disease in the field, which mostly is not effective due to the long time application and appearance of resistant races of the pathogen (Heydari & Pessarakli, 2010). In addition, the high production cost of the chemical fungicides and the negative impacts on non-target organisms should be taken into account. In search for a non-chemical and environmentally safe strategy for managing white rot diseases, we conducted and executed this study to develop and prepare some new bioformulations containing three fungal antagonists (*T. harzianum, T. asperellum* and *Talaromyces flavus*) and an organic carrier (rice bran) and evaluate their effectiveness in controlling white rot disease of garlic in the field conditions.

Materials and Methods

Isolation of *Sclerotium cepivorum* from the garlic fields

Garlic fields in Hamadan province of Iran were surveyed during spring 2014 and diseased plants showing white rot symptoms were sampled and collected and were transferred to the laboratory for the isolation of pathogenic agents. Fungal isolation process was performed by cutting, removing, surface sterilizing and culturing infected pieces of the stems and bulbs on potato dextrose agar (PDA) culture medium. The colonies of grown fungus were purified and were identified as *Sclerotium cepivorum* using the standard identification keys (Barnett & Hunter, 1998). The role of the isolated fungal agent in the disease symptoms appearance was approved by conducting and performing pathogenicity test (Koch's postulate).

Fungal antagonists preparation

Isolates of three fungal species (*T. asperellum, T. harzianum* and *Talaromyces flavus*) were obtained from the microbial collection of the beneficial microorganisms research laboratory, Iranian Research Institute of Plant Protection. These isolates were previously obtained from the rhizosphere of the garlic and potato plants in the fields of Hamadan province of Iran. Antagonistic effects of the isolates on some pathogenic fungi including *S. cepivorum* were previously examined and approved in the laboratory conditions. Antagonistic fungal isolates used in the study and their characteristics are indicated in Table 1.

Table 1. Characteristics of the antagonistic fungal isolates used in this study.

Isolate identity	Isolate code	Isolation host	Isolation location	Isolation time
Trichoderma. harzianum (isolate 1)	T.h-1	Garlic	Hamadan province	Spring 2013
Trichoderma harzianum (isolate 2)	T.h-2	Garlic	Hamadan province	Spring 2013
Trichoderma asperellum (isolate 1)	T.a-1	Potato	Hamadan province	Summer 2013
Trichoderma asperellum (isolate 2)	T.a-2	Potato	Hamadan province	Fall 2013
Talaromyces flavus (isolate 1)	T.f-1	Garlic	Hamadan province	Fall 2013
Talaromyces flavus (isolate 2)	T.f-2	Garlic	Hamadan province	Fall 2013

Bioformulation development

Bioformulations were developed using an organic carrier (rice bran) and six isolates of the above-mentioned antagonistic fungi as follows:

Rice bran was steam-sterilized at 121°C for 30 min and dried aseptically in glass trays before the use. The fungal isolates were first grown on PDA culture medium for purification and were then incubated for about three weeks for sporulation. The spores in the Petri dishes were washed out by adding 10 ml of distilled water to each dish. A spore suspension of each fungal isolate was prepared with the concentration of 10^7 spore ml^{-1} using a hemocytometer. For the development of bioformulations, 10ml of each fungal spore suspension was added to a plastic bag containing 50 g of rice bran carrier. The bags were then incubated at 30°C for three weeks until the fungal hyphae covered the entire surface of the carrier (Kakvan *et al*., 2013). The contents of the bags were then evacuated and dried out in the laboratory conditions and were used for the garlic seed (bulb) treatment. A list of developed bioformulations and used in the field conditions is presented in Table 2. For the garlic seed (bulb) treatment, 5 g of each bioformulation was mixed with 15ml distilled water in a glass tray which was used for the treatment of 100 g of seed bulbs. The coating and treating process was performed by rolling the garlic bulbs in the bioformulation for 10 min and then allowing them to dry for 60 min.

Field trials

Based on the results of a previous greenhouse experiment (Mahdizadehnaraghi *et al*., 2015), six bioformulations were developed using rice bran and two isolates of each three antagonistic fungi (described above) and selected for the evaluation under the field conditions in a trial with randomized complete blocks (RCB) design. The field experiments were conducted and executed in 2015 and 2016 years in a garlic field with white rot disease history in Hamadan Agricultural and Natural Resources Research and Education Center. Each field experiment included eight treatments (six bioformulations, an untreated control and a carbendazim fungicide) each with four replicates. Each replicate consisted of three planting lines each, with two-meters length and the space between plants was 10 cm. In addition to the natural infestation, the soil in field plots was inoculated with the inoculum of *S. cepivorum* prepared on wheat grains (Mc Lean & Kirstin, 2001) at the rate of 10 g/m^2. The effectiveness of bioformulations in controlling garlic white rot disease was evaluated by determining the disease incidence and the disease severity index on the plants in different treatments 90 days after sowing. Disease incidence was determined by counting the number of plants showing white rot symptoms in each treatment. Disease index was calculated based on the observed white rot symptoms on the diseased plants and indexing them using 1–5 standard indexing scale where 1 means no disease, 2 means 1–10% disease, 3 means 11–25% disease, 4 means 26–50% disease, and 5 means more than 50% disease (Entwistle, 1990).

Statistical analysis

All data obtained in the field experiments were subjected to the analysis of variance (ANOVA) and means were compared using Duncan’s Multiple Range Test by Statistical Analysis System (SAS) software version 9.

Table 2. Developed bioformulations and their ingredients used in the field trials.

Bioformulation code	Bioformulation description (ingredient)
R.B-T.h-1	10 ml of spore suspension of *Trichoderma harzianum*-1 + 50 g of rice bran carrier
R.B-T.h-2	10 ml of spore suspension of *Trichoderma harzianum*-2 + 50 g of rice bran carrier
R.B-T.a-1	10 ml of spore suspension of *Trichoderma asperellum*-1 + 50 g of rice bran carrier
R.B-T.a-2	10 ml of spore suspension of *Trichoderma asperellum*-2 + 50 g of rice bran carrier
R.B-T.f-1	10 ml of spore suspension of *Talaromyces flavus*-1 + 50 g of rice bran carrier
R.B-T.f-2	10 ml of spore suspension o *Talaromyces flavus*-2 + 50 g of rice bran carrier

Results

Results of the field experiments are presented in Tables 3 and 4. Table 3 indicates the results of the field experiment in the first year (2015). As was mentioned previously, to evaluate the effectiveness of the bioformulations, two factors including white rot disease incidence and severity index were determined in different treatments. As the Table 3 shows, in the first year field experiment, almost all bioformulations performed effective in controlling and reducing garlic white rot disease. According to the results, the most effective bioformulations in reducing white rot disease incidence and severity index included R.B-T.h-1, R.B-T.h-2, R.B-T.a-2, R.B-T.f-1, R.B-T.f-2 and R.B-T.a-1, respectively. In regard with controlling the white rot disease severity, all bioformulations were placed in the same statistical group but regarding the control of disease incidence, they were placed in different groups. The biofomulation R.B-T.h-1, with 9.11% diseased plants, was the most effective one which along with the biofomulation R.B-T.h-1 and the fungicide treatment were placed in the same statistical group. In the second year field experiment (2016), results were somehow different and the applied bioformulations did not perform as effective as the first year (Table 4). As the Table 4 presents, in the second year, four out of six bioformulations including R.B-T.h-1, R.B-T.h-2, R.B-T.f-1 and R.B-T.f-2 performed effective in controlling white rot disease in comparison with the control treatment.

Table 3. Effects of different fungal bioformulations on garlic white rot disease incidence and severity index in comparison with the control and common fungicide in the first year field experiment

Treatment	Disease Incidence (%)	Disease Severity Index
Rice bran *T. harzianum* 1(R.B-T.h-1)	9.11 f	1.50 d
Rice bran *T. harzianum* 2 (R.B-T.h-2)	13.50 ef	1.75 cd
Control+(Inoculated)	52.06 a	4.75 a
Fungicide (Carbendazim)	13.18 ef	1.85 cd
Rice bran *T. asperellum* 2 (R.B-T.a-2)	17.01 de	2.06 bcd
Rice bran *T. asperellum* 1(R.B-T.a-1)	25.86 b	2.62 bcd
Rice bran *Talaromyces flavus* 1 (R.B-T.f-1)	19.80 cd	2.12 bcd
Rice bran *T. flavus* 2 (R.B-T.f-2)	22.41 bc	2.37 bcd

In each column, values indicated with the same letters are not statistically different according to Duncan's Multiple Range Test (P>0.05).

Table 4. Effects of different fungal bioformulations on garlic white rot disease incidence and severity index in comparison with the control and common fungicide in the second year field experiment.

Treatment	Disease Incidence (%)	Disease Severity Index
Rice bran *T. harzianum* 1(R.B-T.h-1)	41.25 c	2.38 bc
Rice bran *T. harzianum* 2 (R.B-T.h-2)	50.00 bc	2.55 bc
Control+(Inoculated)	80.00 a	3.75 a
Fungicide (Carbendazim)	43.75 bc	2.02 c
Rice bran *T. asperellum* 2 (R.B-T.a-2)	62.50 abc	2.88 abc
Rice bran *T. asperellum* 1(R.B-T.a-1)	75.00 a	3.23 ab
Rice bran *Talaromyces flavus* 1 (R.B-T.f-1)	50.00 bc	2.45 bc
Rice bran *T. flavus* 2 (R.B-T.f-2)	56.25 abc	2.70 bc

In each column, values indicated with the same letters are not statistically different according to Duncan's Multiple Range Test (P>0.05).

Discussion

White rot disease caused by the soil born fungal pathogen, *S. cepivorum*, was the subject of this study because it is an important and damaging disease of the garlic wherever it is grown and cultivated in the world including Iran (Mahdizadehnaraghi *et al*., 2007; Saremi *et al*., 2010; Bakony *et al*., 2011). Application and the use of chemical fungicides is the most common method for controlling this disease. Due to the environmental and health related negative impacts of the chemicals (Cook & Baker, 1988; Heydari & Naraghi, 2014), we developed some biofomulations and used them in conducting a biocontrol study to introduce a non-chemical and eco-friendly method for controlling this important disease.

The bioformulations we developed, were first screened and evaluated in greenhouse conditions for their effects on white rot disease on garlic plant (Mahdizadehnaraghi *et al.,* 2015) from which, the most effective ones were selected for the field experiments. The results obtained in the field experiments showed that most bioformulations performed effectively in reducing the garlic white rot disease incidence and disease severity. For example, in the first year field experiment, the biofomulations R.B-T.h-1 and R.B-T.h-2 could reduce the disease incidence by 42.9% and 38.5%, respectively in comparison with the untreated control. These results are in the agreement with the results of the green house experiment reported by Mahdizadehnaraghi *et al*. (2015) who indicated that, biofomulations reduced the disease incidence by 64.5% and 64%, respectively, compared to the control. However, in the second year field experiment (2016), the applied bioformulations did not perform as effective as the first year which was probably related to the higher disease occurrence which resulted from the higher soil inoculum density due to the first year remaining fungal pathogen inoculum. According to the overall results of this study, development and application of new bioformulations based on antagonistic fungi including *Trichoderma harzianum, T. asperellum* and *Talaromyces flavus* and rice bran organic carrier was an effective method in controlling white rot disease. We used four isolates of two species of *Trichoderma* including *T. harzianum* and *T. asperellum* in our study. This fungal genus has effectively been used in the biological control of different plant diseases in the previous studies (McLean & Kirstin, 2001; Francisco *et al*., 2011; Kakvan *et al*., 2013; Naeimi & Zare, 2013). In addition to the *Trichoderma* antagonistic fungus, we also used two isolates of *T. flavus* for the developing and preparing our bioformulations. *T. flavus* has also been successfully used in several recent biocontrol studies on various plant pathogens (Naraghi *et al*., 2006; Naraghi *et al*,. 2010; Kakvan *et al*., 2013; Naraghi *et al*., 2013).

To develop the bioformulations in our study, we used rice bran organic carrier. The selection and the use of rice bran was based on its use in the several previous biocontrol studies (Naraghi *et al*., 2006; Kakvan *et al*., 2013; Samavat *et al.*, 2014).

The effectiveness of the bioformulations could be mainly related to the antagonistic fungi used for their development. It may also be partially related to the rice bran which has an organic nature and its positive role in the effectiveness of developed bioformulations has previously been reported (Naraghi *et al*., 2006; Kakvan *et al*., 2013; Samavat *et al.,* 2014). Comparing the fungal antagonists used in the study, there were some differences among various species and isolates. Based on the results, *T. harzianum* performed more effective than *T. asperellum* and *T. flavus* which could be related to the differences in antagonistic mechanisms of different species which has been shown in the previous studies (Francisco *et al*., 2011; Kakvan *et al*., 2013). In addition to the differences in the effectiveness of various fungal species, various isolates of the same species also performed differently in the present study that is perhaps due to their genetic structure, isolation host and antagonistic mechanisms. Similar results in this regard have previously been observed and reported (Francisco *et al*., 2011; Kakvan *et al*., 2013; Naeimi & Zare, 2013).

In

this study, we developed some bioformulations and successfully applied them against white rot disease of garlic in the field conditions. This to our knowledge is the first attempt in biologiccal control of this important disease in the field conditions in Iran. The results of the present study are promising and may have practical application in the increase of garlic yield and production. Moreover, as a non-chemical and ecological friendly strategy, it can result in the reduction of harmful chemicals application and protect the agricultural environment and natural resources.

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کاربرد چند فرمولاسیون زیستی قارچی برای مهار بیماری پوسیدگی سفید سیر در شرایط مزرعه

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چکیده

دو آزمایش مزرعه‌ای برای ارزیابی تأثیر شش فرمولاسیون زیستی جدید در مهار بیماری پوسیدگی سفید سیر ناشی از قارچ *Scleotium cepivorum*، در سال‌های ۱۳۹۴ و ۱۳۹۵ به اجرا درآمدند. فرمولاسیون‌های زیستی به‌کارگرفته شده با استفاده از سه گونه قارچ آنتاگونیست (*Trichoderma asperellum*، *T. harzianum* و *Talaromyces flavus*) و یک حامل ارگانیک (سبوس برنج) تهیه شدند. ملاک انتخاب این فرمولاسیون‌ها برای آزمون مزرعه‌ای عملکرد مؤثرشان در مهار پوسیدگی سفید سیر در شرایط گلخانه در مطالعه قبلی بود. آزمایش‌های مزرعه‌ای در قالب طرح بلوک‌های کامل تصادفی با هشت تیمار (شش فرمولاسیون زیستی، تیمار قارچ‌کش کاربندازیم و تیمار شاهد) در چهار تکرار به اجرا درآمدند. اعمال تیمارها قبل از کاشت و به‌روش پوشش بذر (سوخچه) سیر با هر یک از فرمولاسیون‌های زیستی، قارچ‌کش یا حامل ارگانیک (برای تیمار شاهد) صورت گرفت. برای اطمینان از وجود آلودگی یکنواخت، علاوه بر این‌که از زمین آلوده به *S. cepivorum* برای کاشت آزمایش استفاده شد، هنگام کاشت بذور، مقداری مایه بیماری (تکثیر شده روی دانهٔ گندم) نیز به بستر کاشت اضافه شد. نود روز پس از کاشت، ارزیابی تیمارها با تعیین میانگین وقوع بیماری و شاخص شدت بیماری در هر تیمار، صورت گرفت. نتایج نشان داد که در سال اول آزمایش اغلب فرمولاسیون‌های زیستی مورد آزمون در مقایسه با تیمار شاهد، هر دو فاکتور بیماری را به‌طور معنی‌داری کاهش دادند. فرمولاسیون‌های زیستی R.B-T.h-1 و R.B-T.h-2 به‌ترتیب با داشتن ۹/۱۱ درصد و ۱۳/۵ درصد بوته بیمار مؤثرترین فرمولاسیون‌ها در مهار پوسیدگی سفید سیر بودند که از لحاظ آماری به اندازهٔ تیمار قارچ‌کش بیماری را کاهش دادند. در سال دوم به‌دلیل تراکم بالاتر مایه بیماری در خاک، از شش فرمولاسیون زیستی مورد استفاده، چهار فرمولاسیون به‌طور مؤثری بیماری را مهار کردند. به‌طور کلی نتایج این تحقیق نشان می‌دهد که این فرمولاسیون‌های زیستی جدید می‌توانند در مدیریت بیماری پوسیدگی سفید در مزارع سیر، جایگزین قارچ‌کش‌های شیمیایی زیان‌بار شوند.

واژه‌های کلیدی: بیوکنترل، قارچ آنتاگونیست، *Sclerotium cepivorum*، ایران