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CONTROLLING SUGAR BEET MORTALITY DISEASE BY APPLICATION OF NEW BIOFORMULATIONS

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Abstract: There is growing interests in the use of biological approaches to replace or reduce the application of chemical pesticides in modern agriculture. In this regard, antagonistic fungi and particularly bacteria have proved to be potential candidates. In the search for efficient alternative biofungicides, eight new Bioformulations were developed and prepared using two strains of *Pseudomonas fluorescens* (B1) and *Bacillus coagulans* (B2) isolated from different rhizospheric soils and plant roots of Iranian sugar beet fields. Bioformulations were developed using procedures described in the literature. Bioformulations included a talc-based powder and bentonite-based powder as inorganic carriers, and peat and rice bran as organic carriers. The results of our greenhouse experiment, where these bioformulations were applied to sugar beet seeds to control seedling mortality disease, showed that most of the treatments at different intervals (15, 30, 45 and 60 days after sowing) were effective in reducing the disease (compared to the untreated control). According to the results, six out of eight of the developed bioformulations, including Peat-B1, Peat-B2, R.B.-B2, Bent.-B1, Talc-B1 and Talc-B2, were more effective than commonly used fungicides (Carboxin-thiram) in controlling sugar beet mortality disease. Yet, two bioformulations (R.B.-B1 and Bent.-B2) were less effective than carboxin-thiram in the reduction of the disease incidence.

Key words: antagonistic bacteria, carriers, biofungicide, *Rhizoctonia solani*, sugar beet

INTRODUCTION

Indiscriminate use of chemical pesticides in agriculture has led to toxic residues, development of resistance among pests and pathogens, environmental contaminations, and negative impacts on non-target organisms including human (Weller 1998). In this context, biocontrol approaches may help to develop an eco-friendly control strategy for managing plant disease (Heydari and Misaghi 2003; Bharathi *et al.* 2004; Shahraki *et al*. 2008). Among biocontrol agents (BCA), antagonistic bacteria including *Pseudomonas* and *Bacillus* spp. have been shown to play very important roles in controlling several diseases (Collins and Jacobson 2003; Heydari and Misaghi 2003; Shahraki *et al*. 2008; Shahraki *et al*. 2009; Weller 1998).

Bacterial antagonists have employed several mechanisms to perform their antagonistic activities including competition for site and nutrients, antibiosis, siderophore production, and inducing resistance by systemically activating the latent defense mechanisms of the host plant, known as induced systemic resistance (Weller 1998; Heydari and Misaghi 2003; Shahraki *et al*. 2009). This mechanism operates via the activation of multiple defense compounds at sites distant from the point of pathogen attack (Dean and Kuc 1985; Bharathi *et al.* 2004).

The majority of antagonistic bacteria perform efficiently in controlled environmental conditions such as the laboratory and greenhouse, but fail to do so in the field due to many reasons including the impact of environmental factors. One of the most important reasons for the failure of bacterial antagonists in the field may have been related to the lack of proper formulations. The most practical method for application of biocontrol agents in the field is developing and preparing them as powdery formulations. Farmers will be able to use such formulations as a seed treatment particularly for controlling seed and root diseases. Studies have shown that the efficacy of bacterial antagonists in biological control of some plant diseases have increased after they have been mixed with some organic and inorganic carriers (Duffy *et al.* 1996; Viswanathan and Samiyappan 2001; Jayaraj *et al.* 2005; Ardekani *et al*. 2009).

Sugar beet is an important cash crop grown in many countries around the world including Iran (Shah-Smith and Burns 1997; Collins and Jacobson 2003; Shahraki *et al*.

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2008; Shahraki *et al*. 2009). Like many other crop plants, sugar beet is also susceptible to several plant pathogenic agents including soil-born fungi. Seedling mortality or damping-off is one of the most important sugar beet diseases in Iran as well as throughout the world. The use of chemical fungicides as seed treatment is the most common method for controlling this disease in the field, however, most of the time it is not effective due to the long time of application and appearance of resistant races of the pathogen (Shahraki *et al*. 2008; Weller 1998). In addition, the high production cost of the chemical fungicides and the negative impacts on non-target organisms should also be considered.

In order to test the efficacy of bacterial antagonists when mixing them with carriers, this study was conducted to develop some bioformulations using two bacterial antagonists and four organic and inorganic compounds. Their effectiveness was investigated against sugar beet seedling mortality disease in greenhouse conditions.

MATERIALS AND METHODS

Materials

Chemicals, microbial culture media and ingredients used for the development of different bioformulations were of laboratory chemical grade and were purchased from the Tehran chemical market. The culture media used in this study included Nutrient Agar (NA), King's B (KB) and Potato Dextrose Agar (PDA). Sugar beet seeds (Rasoul variety) were obtained from the Iranian Sugar Beet Research Institute.

Microbial cultures

Antagonistic bacterial isolates used in this study belonged to *Pseudomonas fluorescens* and *Bacillus coagulans*. *Rhizoctonia solani* was the causal agent of sugar beet seedling damping-off disease in the previous studies (Shahraki *et al*. 2008, 2009). The isolates selected for the present study were from the laboratory microbial collection and were based on their performance and antagonistic activities against *R. solani*.

Preparation of inorganic and organic carriers

Two inorganic powdery compounds (talc and bentonite) and two powdery organic compounds (peat and rice bran) were selected as carriers based on their use in previous studies. They were steam-sterilized at 140 kPa for 30 min. Then, they were dried aseptically in glass trays for 12 h at 50°C before use.

Preparation of bacterial suspensions

The bacterial strains and cells from stocks were first grown on KB medium to verify their purity. The inoculum was produced by transferring one loop from the culture to NA culture medium followed by incubation. Antagonistic bacterial cells were then harvested after five days of growth in NA culture medium, centrifuged at 6,000 rpm for 15 min and resuspended in a phosphate buffer (0.01 M, pH 7.0). The concentration was adjusted to an approximately 10^9 colony forming unit CFU/ml using a spectrophotometer.

Preparation and Development of bioformulations

One loopful of individual *Pseudomonas* strains was inoculated into KB broth and incubated on a shaker incubator at 150 rpm for 48 h at room temperature (28±2°C). After 48 hours, the suspension containing 10^9 CFU/ml was used for the preparation of the bioformulations as follows: for the 40 ml bacterial suspension, a mixture of 100 g of a purified talc, bentonite (Bent.), peat and rice bran (R.B.) powder, and 1 g carboxymethyl cellulose (CMC) was prepared under sterile conditions (Vidhyasekaran and Muthuamilan 1995). The product was shade-dried to reduce the moisture content, packed in polypropylene bags and sealed. In each g of bioformulations, the number of bacteria were $4x10^8$ CFU ($40x10^9/100$).

Seed treatment

For the seed treatment, sugar beet seeds were initially surface-sterilized with 1% sodium hypochlorite. Then they were washed with sterile water and transferred to 20 cm diameter petri dishes containing one of the prepared bioformulations. For each bioformulation, 80 seeds were selected (20 seeds for each green house pot). According to the results of our previous study (Shahraki *et al*. 2008) the optimum number of bacteria per sugar beet seed for effective control of sugar beet mortality disease was 10^7 CFU. Seeds were then placed in a petri dish containing 2 g of each powdery bioformualtion (calculated according to the above-mentioned study). Surface wet seeds were rolled in the bioformulation for about 10 minutes until they were completely coated with the bioformulations. The approximate number of bacteria for each of the seeds was 10^7 CFU. The above procedure was performed separately for each prepared bioformulation (Vidhyasekaran *et al*. 1997).

Greenhouse studies

Developed bioformulations (Table 1) were assessed for their effectiveness in controlling sugar beet seedling mortality disease in a greenhouse experiment. A pot culture study was undertaken with the following procedures: soil collected from sugar beet fields was air-dried, homogenized using a revolving jar mill, pasteurized with a steam heater for 3 h at 85°C, and mixed with 1% (w/w) of pooled inoculum of *R. solani*. Pots (30 cm in diameter) were filled with *R. solani*-infested soil (3.5 kg). Twenty mono-germ sugar beet seeds (Rasoul variety) treated with bioformulations (described previously) were sown in each pot. The experiment consisted of 10 treatments (8 bioformulations, the untreated control, and seeds treated with commonly used fungicide) and 4 replicates (pots) for each treatment. The number of emerged and healthy seedlings was recorded 15, 30, 45 and 60 days after sowing.

Statistical analyses

Statistical analyses were carried out for the results of the greenhouse experiment using Co-Stat statistical software (Cohort Company, CA, USA). Data for the number of healthy seedlings in different treatments and various

time intervals were subjected to analysis of variance (ANOVA). The means were compared using Duncan's Multiple Range test. The levels of significance were then determined which are presented as different letters in table.

RESULTS

The results of this study are shown in tables 1 and 2. Table 1 notes the biofrmulations developed and prepared in this study. The ingredients used for preparation of different bioformulations are shown in this table.

In table 2, results of the effectiveness of various bioformulations for controlling sugar beet seedling mortality disease are shown as the number of healthy seedlings in different treatments, in first and second time interval (15, 30, 45 and 60 days after sowing). According to table 2, all bioformulations significantly increased the number of healthy seedlings by decreasing the disease incidence (compared to the untreated control). The most effective bioformulations 15 days after sowing, included Peat-B2 and Talc-B1 which were more effective than chemical fungicide (carboxin-thiram) in reducing the disease incidence significantly (Table 2). Other bioformulations although reduced the disease, but were as effective or less effective than carboxin-thiram.

Results of second evaluation of different treatments at 30 days after sowing are also shown in table 2. As the Table shows, the results in this time interval are very different than the previous evaluation time as the effectiveness of carboxin-thiram has been reduced. As a result of this reduction, six out of eight bioformulations, have performed more efficiently than carboxin thiram in disease reduction (Table 2). According to this table, 2 bioformulations that showed less effectiveness are Bent.-B2 and R.B.-B1.

The results of the third and fourth evaluation are indicated in table 2 as well. According to these data, 45 days after sowing the results are slightly different compared to the previous ones. In this time interval, six bioformu-

Table 1. Bioformulations prepared and used in the study

No.	Formulations/Treatments	Ingredients/Methods
1	BENT-B1	suspension of *P. fluorescens* (40 ml) containing 10^9 CFU/ml mixed with fine grade bentonite (100 g) and CMC (1 g)
2	BENT-B2	suspension of *B. coagulans* (40 ml) containing 10° CFU/ml mixed with fine grade bentonite (100 g) and CMC (1 g)
3	TAL-B1	suspension of *P. fluorescens* (40 ml) containing 10^9 CFU/ml mixed with fine grade talc (100) and CMC (1 g)
4	TAL-B2	suspension of *B. coagulans* (40 ml) containing 10^9 CFU/ml mixed with fine grade talc (100 g) and CMC (1 g)
5	PT-B1	suspension of *P. fluorescens* (40 ml) containing 10^9 CFU/ml mixed with peat powder (100g) and CMC (1 g)
6	PT-B2	suspension of *B. coagulans* (40 ml) containing 10^9 CFU/ml mixed with peat powder (100 g) and CMC (1 g)
7	RB-B1	suspension of *P. fluorescens* (40 ml) containing 10^9 CFU/ml mixed with rice bran powder (100 g) and CMC (1 g)
8	RB-B2	suspension of *B. coagulans* (40 ml) containing 10^9 CFU/ml mixed with rice bran powder (100 g) and CMC (1 g)

Table 2. Evaluation of the efficacy of different bioformulations in controlling sugar beet seedling mortality disease 15, 30, 45 and 60 days after sowing in the greenhouse

No.	Treatments	Days after sowing							
		15		30		45		60	
		A*	D*	A	D	A	D	A	D
1	The control	5.25 d	–	3.25	–	3.25 c	–	2.75 c	–
2	Peat-b1	11.75 ab	55.3	9.50 a	65.8	9.25 a	64.9	9.25 a	70.3
3	Peat-b2	13.25 a	60.4	8.75 a	62.9	8.50 a	61.7	8.50 a	67.6
4	R.B.-b1	10.0 b	47.5	4.0 c	18.7	4.0 c	18.7	4.0 c	31.2
5	R.B.-b2	10.75 b	51.2	7.25 ab	55.2	6.75 b	51.9	6.75 ab	59.9
6	Bent-b1	9.50 b	44.8	6.25 b	48.0	6.25 b	48.0	6.25 b	56.0
7	Bent-b2	7.50 c	30.0	3.50 c	7.1	3.50 c	7.1	3.50 c	21.4
8	Talc-b1	13.0 a	59.4	7.25 ab	55.2	7.25 ab	55.2	7.0 b	61.0
9	Talc-b2	11.50 ab	54.4	6.50 b	50.0	6.0 b	45.8	5.50 b	50.0
10	Car-Tir	11.25 ab	53.4	4.0 c	18.8	4.0 c	18.7	4.0 c	31.2

A*– average number of healthy seedlings

D* – % disease reduction (compared to the control)

Each value is the average of four replicates. In each column, values marked with the same letters are not statistically different according to Duncan's multiple range test (p = 0.05)

lations performed more effectively than carboxin-thiram whereas the other two (Bent.-B2 and R.B.-B1) showed the same effectiveness as chemical fungicide (Table 2).

Table 2 also shows the results of the fourth and final evaluations which were obtained 60 days after sowing. The results, 60 days after sowing, are very similar to those of the previous time interval (45 days after sowing). In the 60 days after sowing time interval, six bioformulations also showed more effectiveness than carboxin-thiram for controlling sugar beet seedling mortality disease (Table 2). However, the remaining bioformulations (Bent.-B2 and R.B.-B1) did not performed very well and showed less effectiveness than carboxin-thiram, the commonly used chemical fungicide (Table 2). According to the results of the final evaluation, six out of eight prepared bioformulations have performed very effective in reducing the incidence of sugar beet seedling mortality disease (compared with the control and chemical fungicide). These biofrrmulations could be good candidates for the future studies in the field (Table 2).

DISCUSSION

The overall results of this study show that it may be possible to control sugar beet seedling mortality disease by development and application of new bioformulations. This is probably because these products may increase, establish, and maintain antagonistic microorganisms in soil and possibly produce antibiotics, siderophores, hydrolytic enzymes, phytohormones and/or other volatile extracellular metabolites (Weller 1998; Shahraki *et al.* 2009).

A successful biocontrol agent must survive formulation and storage, and must be a competitive and aggressive colonizer after inoculation (Beatty and Jensen 2002; Selim *et al.* 2005). Lee and Kobayashi (1989) observed deformation in *R. solani* hyphae due to the action of the antifungal metabolites produced by *Burkholderia cepacia*. Successful formulations of *B. subtilis* and *P. fluorescens* are commercially available (Jayaraj *et al.* 2005). Those of *Bacillus* are very stable due to the ability of this bacterium to form spores that are long-lived, and are resistant to heat and desiccation that are long-lived, and are resistant to heat and desiccation (Jayaraj *et al.* 2005). *B. subtilis* and *P. fluorescens* are being marketed as dry formulations with talc or peat as carriers, and are also used for mixing with potting soil or with compost for incorporation into nursery beds or field soil (Sridhar *et al.* 1993; Kannan and Jayaraj 1998).

In our study, *P. fluorescens* and *Bacillus coagulans* strains performed more effectively than the common seed dressing fungicides (Carboxin/Thiram) for controlling sugar beet damping off (mortality) disease under greenhouse conditions. Such results are in line with the notion that these bacteria are allegedly effective biocontrol agents, as repeatedly reported in the literature (Weller 1998; Amer and Utkhede 2000; Collins and Jacobson 2003; Shahraki *et al.* 2008, 2009). In addition to the above researchers, Saravanakumar *et al.* (2007) tested the efficacy of PGPR formulations against *Exobasidium vexans* in *Camellia sinensis*. They found that foliar applications of *P. fluorescens* pf1 at 7-day intervals reduced the incidence of blister blight, almost as much as fungicide treatments, and increased the yield significantly. Moreover, Bharathi *et al.* (2004) observed while evaluating the efficacy of 13 bacterial strains against chilli fruit rot and dieback incited by *Colletotrichum capsici*, that *P. fluorescens* pf_1 and *B. subtilis* were effective in increasing seed germination and seedling vigor, and that a mixed bioformulation (pf1 + *B. subtilis* + neem + chitin) was the best for reducing fruit rot incidence while increasing plant growth and yield.

In another study, Khodakaramian *et al.* (2008) reported that *P. fluorescens* strains pf16 and pf19 were strong antagonists of *Xanthomonas citri*. Howie and Suslow (1986) and Sivan and Chet (1986), though, showed that some *P. fluorescens* isolates produce antifungal compounds that protected cotton from infection by *Fusarium* sp. and *Pythium ultimum*. A comparable antagonistic action was displayed by several isolates of *P. fluorescens* against *R. solani*, agent of cotton and cabbage seedling damping-off (Howell *et al.* 1997; Chung *et al.* 2005).

Results of the above studies indicate that development of stable formulations of antagonistic bacteria and other biocontrol agents are of great importance to many countries. This is especially true in countries where subsistence agriculture is prominent, soil-borne diseases are the main problem, and fungicides are unaffordable. In our study, the *P. fluorescens* bacterial strain performed relatively better than *B. coagulans*, probably due to the variation in its antagonistic mechanisms (root colonization, antibiosis, siderophore production, *etc.*). From among carriers, peat showed the most effectiveness and this could possibly be due to its organic nature and carbon sources. However, inorganic carriers also performed well in for increasing the effectiveness of bacterial strains. Another important point that was observed in the green house experiment was the low number of healthy seedlings in almost all treatments. This happened because we purposely selected an isolate of *R. solani* which was the most pathogenic among our isolates to evaluate the bioformulations in the highest pressure of the disease.

The results of the present study may have practical applications in disease management strategies. As already noted, the formulation and establishment of biocontrol agents are very important for the effectiveness of the strategies. The formulations we have developed and tested can be used for controlling sugar beet mortality and seedling damping-off and possibly other plant-pathogen combinations. These formulations have the potential to replace chemical fungicides and to be utilized as an important component of Integrated Pest Management (IPM) which is a promising approach for use in sustainable agriculture.

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