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PREPARATION OF NEW BIOFUNGICIDES USING ANTAGONISTIC BACTERIA AND MINERAL COMPOUNDS FOR CONTROLLING COTTON SEEDLING DAMPING-OFF DISEASE

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Abstract: The overuse of chemical pesticides in agriculture has caused serious environmental problems and thus the demand for safer pesticides is increasing. One alternative is microbial pesticides that suppress plant pathogens via their microbial activities. As microbial pesticides are eco-friendly products, in this study we prepared four biological fungicides using two isolates of *Pseudomonas fluorescens* that included a talc-based powder and bentonite-based powder as mineral carriers. Then we evaluated the efficacy of these products in controlling cotton seedlings, damping-off, a fungal disease caused by *Rhizoctonia solani* at four intervals of 15, 30, 45 and 60 days after sowing the cotton seeds under greenhouse conditions. The results of greenhouse experiment on application of biofungicides showed that the efficacy of Bentonite-B_1 treatment to control *R. solani* was promising as it increased the number of healthy seedlings 3.42 to 3.57 – fold and was much more effective than the carboxin/thiram fungicide in all stages.

Key words: damping-off, eco-friendly, biological control, environmental contamination, *Pseudomonas fluorescens*, *Rhizoctonia solani*

INTRODUCTION

Plant diseases are mostly controlled by chemical pesticides and in some cases by cultural practices. However, the widespread use of chemicals in agriculture has been a subject of public concern and scrutiny due to the potential harmful effects on the environment, their undesirable effects on non-target organisms and possible carcinogenicity of some chemicals (Cook and Baker 1983). Other problems include development of resistant races of pathogens, a gradual elimination and phasing out of some available pesticides and the reluctance of some chemical companies to test new pesticides due to the problems with registration process and cost. The need for the development of non-chemical alternative methods to control plant diseases is therefore clear (Cook and Baker 1983). Biological control of plant diseases has been considered a viable alternative method to manage plant diseases (Atkinson *et al.* 1994; Borowicz 2001). Biocontrol is environmentally safe and in some cases is the only option available to protect plants against pathogens (Atkinson *et al.* 1994).

Cotton is an important cash crop which is being cultivated in many countries around the world including Iran (Naraghi *et al.* 2007). Like other crop plants, cotton is also susceptible to several plant pathogenic agents including fungi. *Verticillium* wilt and seedling damping-off are considered the most important diseases of cotton in Iran (Mansoori and Hamdollahzadeh 1995).

Damping-off caused by *Pythium* spp., *Fusarium* spp., nematodes and especially *Rhizoctonia solani* is one of the most important diseases of cotton causing high mortality of cotton seedlings grown in the field in many countries. For controlling cotton seedling damping-off cultural practices and the use of fungicides as seed treatment are the most common strategies which are either not available or effective. Biological control methods using antagonistic microorganisms offer a powerful and eco-friendly alternative to the use of synthetic chemicals that have been applied to control the plant diseases (Aziz *et al.* 1997; Emmert and Handelsman 1999; Heydari and Gharedaghli 2007; Heydari and Misaghi 1997, 1998, 2003; Spinks and Rowe 1989).

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Among the biocontrol agents, plant growth promoting rhizobacteria (PGPR) viz., *Pseudomonas* spp. and *Bacillus* spp. have shown the activity in suppressing the fungal infection (Chen *et al.* 2000). It means that in addition to direct antagonistic activity against several soil-borne fungal pathogens, these bacteria are known to promote plant growth and yield (Krebs *et al.* 1998; Schmiedeknecht *et al.* 1998; Ryder *et al.* 1999). They activate systemically the plant's latent defense mechanisms called induced systemic resistance against pathogens. This mechanism operates through the activation of multiple defense compounds at sites distant from the point of pathogen's attack (Bharathi *et al.* 2004). When PGPR are mixed with some other strains, or other bacteria or fungal antagonists the biocontrol efficacy is increased (Duffy *et al.* 1996). Mixing of talc and bentonite with the PGPR has also been found to increase the biological efficacy (Jayaraj *et al.* 2005). *Bacillus subtilis* and *Pseudomonas fluorescens* have been successfully used to control disease caused by *Pythium*, *Rhizoctonia*, *Gaeumannomyces*, *Sclerotinia*, *Fusarium* and others (Zhang 1996; Schmiedeknecht 1998; Bacon *et al.* 2001). The antagonistic activities of these bacteria are mainly attributed to the production of antibiotic substances, most of which are dipeptides or cyclic peptides (Loeffler *et al.* 1990). These bacteria are also capable of producing certain volatile extra cellular metabolites that have antifungal activity (Loeffler 1990; Podile and Prakash 1996; Cameco *et al.* 2001). *B. subtilis* is also a well known producer of hydrolytic enzymes, including cellulase, chitinase and β-1,3-glucanase (Marten *et al*. 2000), which cause lysis of fungal cell walls and membranes. There is also emerging evidence indicating that these bacteria could be involved in induction of systemic acquired resistance in plants (Podile and Laxmi 1998; Ghonim 1999; Collins and Jacobson 2003).

The success of biocontrol eventually depends upon development of suitable formulations in which the antagonistic microorganisms can survive for extended periods of time. The objectives of this study were to develop some biofungicides using bacterial antagonists and mineral compounds and possible replacement of chemical fungicides with these products in controlling cotton seedling damping-off disease.

MATERIALS AND METHODS

Materials

Chemicals, microbial growth media and ingredient used for the preparation of various formulations were of laboratory chemical-reagent grade and were purchased from Tehran's chemical market, Iran. Seeds of cotton (*Gossypium hirsutum* L.) were obtained from the Department of Plant Pathology, Iranian Research Institute of Plant Protection, Tehran, Iran.

Microbial cultures

R. solani used in this study was obtained from the Microbial Culture Collection, Beneficial Microorganisms Research Laboratory, Iranian Research Institute of Plant Protection, Tehran, Iran. The fungus was maintained on Potato Dextrose Agar medium (PDA). The bacterium, *P. fluorescens* Q_{18} and CKK-3, which effectively inhibited the growth of *R. solani* in previous *in vitro* experiments, was used in the present study. These isolates of the bacterium exhibited a high level of resistance to ampicillin and erythromycin, which was not observed in many native *P. fluorescens* isolates. The bacterial culture was maintained on King's B (KB) medium. These isolates were sub-cultured once a month and maintained until the end of the experiment.

Preparation of bioformulations

Preparation of mineral carriers

The two powdered minerals of talc and bentonite in the silica class of mines were chosen as carriers in this study. The carrier materials were steam sterilized at 140 kPa for 30 min, and dried aseptically in glass trays for 12 h at 50°C before using.

Preparation of bacterial suspension

The *P. fluorescens* cells were harvested and centrifuged at 6000 rpm for 15 min and resuspended in phosphate buffer (0.01 M, pH 7.0). The concentration was adjusted using a spectrophotometer to approximately 10^8 cfu/ml (OD_{595}= 0.3), and used as bacterial inoculum (Thompson 1996). These isolates were kept at –80°C in 44% glycerol and cells from stocks were first grown in KB medium. The inoculum was produced by transferring one loopful from that culture to 100 ml of KB broth in a 250 ml Erlenmeyer flask and incubating at room temperature (28±2°C) on a shaker at 150 rpm for 48 h.

Development of talc-based and bentonite-based formulations of *Pseudomonas* strains

A loopful of individual *Pseudomonas* isolates were inoculated into the KB broth and incubated on a rotary shaker at 150 rpm for 48 h at room temperature (28± 2°C). After 48 h of incubation, the broth containing 9×10^8 cfu/ml was used for the preparation of talc-based and bentonite-based formulations. To the 400 ml of bacterial suspension, 1 kg of a purified talc or bentonite powder, calcium carbonate 15 g (adjusted to neutral pH) and carboxymethyl cellulose (CMC) 10 g (adhesive) were mixed under sterile conditions, following the method described by Vidhyasekaran and Muthuamilan (1995). The product was shade dried to reduce the moisture content (less than 20%) and then packed in polypropylene bags and sealed.

Efficacy of bioformulations on plant growth promotion

The cotton seeds were surface sterilized with 1% sodium hypochlorite and coated with the bioformulations prepared with *P. fluorescens* isolates. A slurry of the formulations was prepared using sterile distilled water (at 4 ml/g of seeds) and the seeds were kept in the slurry for 24 h. Seeds soaked in sterile distilled water served as control (Bharathi *et al.* 2004)

Efficacy of bioformulations against cotton seedling damping-off under greenhouse conditions

Seed treatment/coating

For seed treatment, the seeds were initially surface sterilized with 1% sodium hypochlorite and soaked in

double volume of sterile distilled water containing talc-based and bentonite-based formulations (10 g/kg of seed) and carboxin/thiram (5 g/kg). After 12 h, the bacterial suspension was drained off and the seeds were dried under shade for 30 min and sown (Vidhyasekaran *et al.* 1997).

Preparation of fungal inoculum

Glass bottles containing granulated maize-sand medium were steam sterilized at 140 kPa for 45 min, and then inoculated with mycelial discs (1 cm diameter) cut out from a 5-day-old PDA culture of *R. solani* (1 disc/bottle). The bottles were incubated for 15 days at 28°C and stirred frequently.

Greenhouse studies

Various formulations were assessed for their efficacy in controlling *R. solani* incidence in greenhouse conditions. A pot culture study was undertaken with the following treatments by using completely randomized design (CRD) with four replications. The treatments are shown in table 1. Soil collected from a Varamin's cotton fields in Tehran province of Iran was air-dried, homogenized using a revolving jar mill and sterilized using a steam sterilizer for 3 h at 85°C, and mixed with 1% (w/w) of pooled inoculum of *R. solani*. Pots (20 cm diameter) were filled with *R. solani* infested soil (3.5 kg). 20 treated cotton seeds with *P. fluorescens* formulations or carboxin/thiram were sown (depth 2 cm; spacing 2×3 cm) in each pot containing infested soil. 6 pots/treatment were inoculated. Infested control (infested soil+ untreated seeds) was also included. The number of emerged seedlings was recorded 15, 30, 45 and 60 days after sowing the cotton seeds (Table 2–5).

Statistical Analyses

This experiment was performed in 4 replications, inducing an appropriate control. All data were analyzed for significant differences by analysis of variance (ANOVA). Comparison of means was performed using least significant differences (LSD) (p=0.05), by the SPSS statistical package, 15 evaluation version (Table 6).

RESULTS

The results of this study are shown in included below tables. They indicate that 15 days after sowing the cotton seeds treated with Bentonite-B_1, cotton seedling damping-off caused by *R. solani* was 81.75% as compared with the control (76.25%), and this indicated the greatest efficacy. Furthermore, Talc-B_1 treatment, Bentonite-B_2 treatment, carboxin/thiram treatment and Talc-B_2 treatment showed the efficacy of 47.5%, 45%, 41.25% and 38.75%, respectively, in controlling the cotton seedling damping-off disease in this period of time (Table 2).

In a period of 30 days after sowing, the Bentonite-B_1 treatment showed 66.25% of control and was the most effective. In addition, Bentonite-B_2 treatment showing 38.75% of control, Talc-B_1 treatment showing 37.5% of control and carboxin/thiram treatment with 32.5% and Talc-B_2 treatment with 22.5% of control, respectively, also were capable of reducing damping-off during this period of time (Table 3).

In a period of 45 days after sowing, also the Bentonite-B_1 treatment showed 62.50% of control having the greatest efficacy. In this time interval, Talc-B_1 treatment with 37.5%, Bentonite-B_2 treatment with 35%, carboxin/thiram treatment with 32.5% and Talc-B_2 treatment with 20% of control were ranked next (Table 4).

Finally, 60 days after sowing, the Bentonite-B_1 treatment with 62.50% control of cotton seedling damping-off disease was the most effective and was followed by Talc-B_1 treatment, Bentonite-B_2 treatment, carboxin/thiram treatment and Talc-B_2 treatment (Table 5).

Table 1. Description of different treatments in the greenhouse experiment

Formulations/Treatments	Ingredients/Method
Bentonite-based powder (B_1)	Q_{18} isolate suspension of *P. fluorescens* (400 ml) containing 9×10^8 cfu/ml was mixed with fine grade bentonite (1 kg) and carboxymethyl cellulose (10 g)
Bentonite-based powder (B_2)	CKK-3 isolate suspension of *P. fluorescens* (400 ml) containing 9×10^8 cfu/ml was mixed with fine grade bentonite (1 kg) and carboxymethyl cellulose (10 g)
Talc-based powder (B_1)	Q_{18} isolate suspension of *P. fluorescens* (400 ml) containing 9×10^8 cfu/ml was mixed with fine grade talc (magnesium trisilicate) (1 kg) and carboxymethyl cellulose (10 g)
Talc-based powder (B_2)	CKK-3 isolate suspension of *P. fluorescens* (400 ml) containing 9×10^8 cfu/ml was mixed with fine grade talc (magnesium trisilicate) (1 kg) and carboxymethyl cellulose (10 g)
Control	Homogenized soil mixed with 10 disk of pooled inoculum of *R. solani* and 20 untreated cotton seeds
Carboxin/thiram	Homogenized soil mixed with 10 disk of pooled inoculum of *R. solani* and 20 treated cotton seeds with carboxin/thiram fungicide at the rates of 4 g per kg seeds

Table 2. Assessment of efficacy of different treatments against cotton seedling damping-off disease by counting the number of emerged seedlings 15 days after sowing

Treatments	Total number of emerged seedlings	Average No of emerged seedlings	Ratio of increase in the number of healthy seedlings (compared with control) as folds	Disease control (per cent)
Control	19	4.75 [a]	1.00	–
Bentonite-B_1	65	16.25	3.42	81.25
Bentonite-B_2	36	9.00	1.89	45.00
Talc-B_1	38	9.50	2.00	47.50
Talc-B_2	31	7.75	1.63	38.75
Carboxin/thiram	33	8.25	1.74	41.25

[a] the average number of emerged seedlings in 4 replicates

Table 3. Assessment of efficacy of different treatments against cotton seedling damping-off disease by counting the number of emerged seedlings 30 days after sowing

Treatments	Total number of emerged seedlings	Average No. of emerged seedlings	Ratio of increase in the number of healthy seedlings (compared with control) as folds	Disease control (per cent)
Control	15	3.75 [a]	1.00	–
Bentonite-B_1	53	13.25	3.53	66.25
Bentonite-B_2	31	7.75	2.06	38.75
Talc-B_1	30	7.50	2.00	37.50
Talc-B_2	18	4.50	1.20	22.50
Carboxin/thiram	26	6.50	1.73	32.50

[a] the average number of emerged seedlings in 4 replicates

Table 4. Assessment of efficacy of different treatments against cotton seedling damping-off disease by counting the number of emerged seedlings 45 days after sowing

Treatments	Total number of emerged seedlings	Average No. of emerged seedlings	Ratio of increase in the number of healthy seedlings (compared with control) as folds	Disease control (per cent)
Control	14	3.50 [a]	1.00	–
Bentonite-B_1	50	12.50	3.57	62.50
Bentonite-B_2	28	7.00	2.00	35.00
Talc-B_1	30	7.50	2.14	37.50
Talc-B_2	16	4.00	1.14	20.00
Carboxin/thiram	26	6.50	1.86	32.50

[a] the average number of emerged seedlings in 4 replicates

Table 5. Assessment of efficacy of different treatments against cotton seedling damping-off disease by counting the number of emerged seedlings 60 days after sowing

Treatments	Total number of emerged seedlings	Average No. of emerged seedlings	Ratio of increase in the number of healthy seedlings (compared with control) as folds	Disease control (per cent)
Control	14	3.50 [a]	1.00	–
Bentonite-B_1	50	12.50	3.57	62.50
Bentonite-B_2	28	7.00	2.00	35.00
Talc-B_1	30	7.50	2.14	37.50
Talc-B_2	16	4.00	1.14	20.00
Carboxin/thiram	25	6.25	1.79	31.25

[a] the average number of emerged seedlings in 4 replicates

Table 6. Statistical grouping of different treatments in controlling cotton seedling damping-off disease in the greenhouse study

Treatments	Statistical grouping
Control	D
Bentonite-B_1	A
Bentonite-B_2	B
Talc-B_1	B
Talc-B_2	CD
Carboxin/thiram	BC

LSD (p = 0.05)
A, B, C… – the letters represent the statistical differences among different treatment

DISCUSSION

The overall results of this study show that efficacy of bentonite-B_1 treatment in controlling cotton seedling damping-off during 4 time intervals of 15, 30, 45 and 60 days after sowing under greenhouse conditions were 3.42, 3.53, 3.57 and 3.57 fold compared with the control and were more effective than the carboxin/thiram treatment in this experiment. This is probably because new bioformulations may play an effective role in increasing durability and establishment of antagonistic microorganisms in soil and also producing more effective antibiotics, siderophores, hydrolytic enzymes and other volatile extra-cellular metabolites. In other words, plant pathogenic agents were more resistant to synthetic chemicals. Thus, we must develop these eco-friendly products and replace synthetic fungicides with biofungicides in controlling and management of plant diseases including cotton seedling damping-off.

This promising strategy can in turn result in reduction of environmental contamination, reduction of yield loss and establish a sustainable agricultural system. Also it seems that antagonistic action of Q_{18} isolate of *P. flourescens* is more effective than CKK-3 isolate of *P. flourescens*. This could be due to the differences in biochemical and genetic characteristics of this isolate.

The success of biological control of plant diseases depends on the availability of effective formulations of biocontrol agents, their survival during storage, and rapid multiplication and colonization after inoculation (Becker and Schwinn 1993). *B. subtilis* and *P. fluorescens* have been successfully formulated, and some formulations are available commercially for biological control of crop diseases (Jayaraj *et al*. 2005). These formulations are very stable due to the ability of Bacillus bacterium to from spores (Emmert and Handelsman 1999). The spores are long-lived, and are resistant to heat and desiccation (Kloepper 1991). These bacteria are also being marketed as a dry formulation with talc or peat as carriers. These formulations are often used for seed treatment, and also used to a limited extent 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 the bioformulations prepared were more effective than common seed treatment fungicide, carboxin/thiram, in terms of disease control under greenhouse conditions.

Bharathi *et al.* (2004) evaluated the efficacy of 13 plant growth promoting antagonistic rhizobacterial strains, they observed among these formulations *P. fluorescens* (pf_1) and *B. subtilis* to be effective in increasing the seed germination and seedling vigor. They also found that the PGPR mixed bioformulation (pf_1+*B. subtilis*+neem+chitin) was the best for reducing the fruit rot incidence beside increasing the plant growth and yield parameters under both greenhouse and field conditions. Khodakaramian *et al.* (2008) reported that two strains of *P. fluorescens* (pf-16 and pf-19) showed the highest antagonistic activity against the pathogenic bacterium and significant reduction of the leaf spots in controlling citrus canker disease.

Several *P. fluorescens* isolates produce antifungal compounds and were shown to inhibit the infection by fungal pathogens including *Fusarium* sp. and *Pythium ultimum* in cotton (Howie and Suslow 1986; Sivan and Chet 1986). Several isolates of *P. fluorescens*, which showed antagonistic effects on *R. solani* by producing antibiotics such as pyrolnitrin and inhibited the fungus which causes cotton and cabbage seedling damping-off (Harris *et al.* 1994; Howell *et al.* 1997; Chung *et al.* 2005). Nandakumar *et al.* (2001) also reported that the application of *P. fluorescens* strains increased the yield of rice in the field.

Results of the above-mentioned studies clearly indicate that development of stable formulations of antagonistic bacteria and other biocontrol agents is of a great importance to many countries including developing countries where subsistence agriculture is prominent, and soil-borne diseases are the main problems in crops grown with limited rotation, and where fungicide treatments are unaffordable. Thus biological control of plant diseases using antagonistic microorganisms offers a powerful and eco-friendly alternative to the use of synthetic chemicals and is also important to other agro-systems because it minimizes the dependence on pesticides.

The results of this study may have practical application in establishing of plant disease management strategies. As was mentioned previously establishment and formulation of biocontrol agents play very important role in their activity and effectiveness. The bioformulations developed and prepared in this study may be used in controlling cotton seedling damping-off and possibly in other plant-pathogen combinations. They have the potential of replacement of harmful chemical fungicides and could be used as an important component of Integrated Pest Management (IPM) which is a promising approach to sustainable agriculture.

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POLISH SUMMARY

PRZYGOTOWANIE NOWYCH BIOFUNGICYDÓW DO ZWALCZANIA ZGORZELI SIEWEK BAWEŁNY PRZY WYKORZYSTANIU ANTAGONISTYCZNYCH BAKTERII I MINERALNYCH SKŁADNIKÓW

Nadmierne stosowanie chemicznych pestycydów rolnictwie było przyczyną poważnych problemów środowiskowych, co spowodowało wzrost zapotrzebowania na bezpieczniejsze pestycydy. Jedną z alternatyw są pestycydy mikrobiologiczne wykazujące supresyjne działanie wobec patogenów roślin poprzez ich aktywność mikrobiologiczną. Ponieważ mikrobiologiczne pestycydy są przyjazne środowisku, w toku przeprowadzonych badań przygotowano cztery biologiczne fungicydy, wykorzystując dwa izobaty *Pseudomonas fluorescens*, zawierające proszek oparty na talku i bentonicie, które były nośnikami mineralnymi. Następnie oceniano skuteczność tych produktów w zwalczaniu zgorzeli siewek bawełny wywołanej przez *Rhizoctonia solani* zachowując 15, 30, 45 i 60-dniowe przerwy po wysiewie w warunkach szklarniowych. Uzyskane wyniki doświadczenia szklarniowego wykazały, że skuteczność zastosowania Bentonitu-B_1 w celu zwalczania *R. solani* była obiecująca, gdyż zabieg ten powodował wzrost liczby zdrowych siewek o 3,42 do 3,57 razy i był skuteczniejszy niż użycie we wszystkich stadiach fungicydu zawierającego karboksynę i tiuram.