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Bacillus Subtilits and *Trichoderma Harzianum* as Wheat Inoculants for Biocontrol of *Rhizoctonia* Solani

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Abstract: This study evaluated the effectiveness of applying the fungal isolate *Trichoderma harzianum* and *Bacillus subtilis* either alone or in combination to control wheat root rot pathogen *Rhizoctonia solani in vitro* and in greenhouse. *T. harzianum* and *B. subtilis* were selected for their strong antagonists to *R. solani* in *in vitro* experiments.*B. subtilis* and *T. harzianum* were applied to wheat grains and sown in soil infested with *R. solani* and tested in greenhouse experiments. The treatments were compared with a synthetic fungicide such as carbomar. Parameters observed were seed germination, plant height, fresh and dry weight of seedlings. Photosynthetic pigments, carbohydrates, total amino acids and total soluble sugars decreased due to infection. Application of *T. harzianum*, *B. subtilis* either alone or in combination or the application of carbomar, improved the biochemical parameters in the treated seedlings.A combination of *B. subtilis* and *T. harzianum* was most effective than using each alone in controlling the pathogen in greenhouse. Conclusion and application of findings: Chemical and biological control were efficient against root rot pathogen *in vitro* and in greenhouse. Application of *T. harzianum* with the coating of wheat grains with *B. subtilis* is a promising approach.

Key words: *Bacillus subtilis, Trichoderma harzianum,Rhizoctonia solani,* Biocontrol,*Wheat,Carbom*

INTRODUCTION

Although synthetic fungicides showed effectiveness, there are potential harmful effects on human health and the environment (Demoz and Korsten, 2006). There is then a need to examine possible non-synthetic chemical approaches for disease management.

Despite the many definitions accorded biological control, it is a strategy for reducing disease incidence or severity by direct or indirect manipulation of microorganisms. The principle may be eradication or protection, depending on specific tactics involved in promoting biological control. Antagonists that produce antibiotics kill pathogens and eradicate them from a substrate. Some microorganisms occupy niches and exclude pathogens from becoming established, thereby protecting plants from infection (Maloy, 1993; Shoda, 2000).

The soilborne pathogen *Rhizoctonia solani* is ubiquitous and, under the appropriate environmental conditions, can severely damage more than 200 crops of economic importance. The destruction includes seed and seedling rot, root and hypocotyls damage, aerial blight and fruit rots. Rhizoctonia root rot, caused by the fungus *R. solani* AG-8, has been identified as an important, yield-limiting disease in direct-seed systems (MacNish, 1985, Rovira, 1986; Smiley and Uddin, 1993; Demoz and Korsten, 2006 and Turchetti, 2007). Rhizoctonia root rot causes brown, sunken lesions in the root cortex, and serious infection leads to severance of the root, creating what is known as the spear tip symptom in roots. Under acute disease pressure, plants are stunted, creating bare patches in the field that can severely limit grain yield (Pumphrey *et al*., 1987; Cotterill, 1990; Smiley, 1996 and Smith *et al*. 2003).

Several non-plant pathogenic oomycetes and fungi, including some chitrdiomycetes and hyphomycetes, and some pseudomonads and actinomycetous bacteria infect the resting spores of several plant pathogenic fungi.

Among the most common mycoparasitic fungi are *Trichoderma* sp., mainly *T. harzianum*. The latter fungus has been shown to parasitize mycelia of *Rhizoctonia* and *Sclerotium*.

Trichoderma spp. are common inhabitants of the rhizosphere and are well recognized as biocontrol agents of soilborne plant pathogens (Chet, 1987; Harman and Lumsden, 1990 and Harman, 2000).

The application of *Trichoderma* to the soil as a biocontrol agent, in the greenhouse or under field conditions, not only resulted in reduced disease severity but also enhanced plant growth (Chang *et al*., 1986; Harman and Bojorkman, 1998 and Kildea *et al*., 2008).

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T. atroviride C52 was first identified as a biological control agent for onion white rot in 1994 (McLean *et al.* 2005).

In addition to fungi, bacteria of the genera *Bacillus*, *Enterobacter*, *Pseudomonas* and *Pantoea* have been shown to parasitize and/or inhibit *Rhizoctonia* and many other pathogenic fungi.

Bacillus species occur mainly in the soil, and because of spore forming the bacteria have the ability for survival in soil environment. Bacteria produce a number of antimicrobial compounds. Loeffler and Co-Workers (1986), Mizumoto *et al*., (2007) and Turchetti (2007), found that *Bacillus subtilis* gave good control of *Rhizoctonia solani* in many crops.

Bacillus species produce many kinds of antibiotics which share a full range of antimicrobial activity such as bacitracin, pumulin and gramicidin (Todar, 2005). *B.subtilis* is one of the most widely distributed bacterial species in agricultural systems. This rhizosphere isolate is used as biocontrol of soilborne root diseases, well established commercial applications.

The aim objective of this study is to evaluate an antagonistic strain of *T. harzianum* and *B. subtilis* and their performance either on their own or in combination, for integrated control of *R. solani* causing root rot of wheat seedlings *in vitro* and in the greenhouse. In addition we investigated the significance between biological control and that of carbomar as a fungicide (chemical control).

MATERIALS AND METHODS

Strains and Grains Used:

Grains of wheat cultivar, *Triticum aestivum* L. used in this investigation was obtained from the Agriculture Research Center, Giza, Egypt.

The pathogen *Rhizoctonia solani* Kühn (Ag-8) was obtained from the Agriculture Research Center, Giza, Egypt.

Trichoderma harzianum Rifai EMCC 540 was obtained from Cairo MIRCEN Faculty of Agriculture - ASU.

Synthetic medium for the two fungi consisted of, g/L: dextrose, 30; KNO_3, 2; KH_2PO_4, 1; $MgSO_4 \times 7\ H_2O$, 0.5; KCl, 0.5; and 1 ml of each stock solution (1g L^{-1}) of $FeSO_4 \times 7\ H_2O$, $MnSO_4 \times 7\ H_2O$, $ZnSO_4 \times 7\ H_2O$; thiamine and 20 gm agar (Johnson and Curl, 1972).

Bacillus subtilis was kindly submitted from Faculty of Agriculture, Zagazig University. It was isolated from the rhizosphere of wheat roots.

T. aestivum grains used in this work were surface - disinfected with 70 % ethanol for 1-2 min and then with 1 % sodum hypochlorite for 30 min and rinsed in sterile distilled water three times. The seeds were then soaked in sterilized distilled water for 3-4 hours in dark at 25 °C.

Production of Rhizoctonia solani:

Inoculum was produced on sterilized barley grains in 2-L wide mouth flasks. Five hundred milliliters of barley grains were imbibed overnight with 300 mL of distilled water. The barleys were subsequently autoclaved for 1 h at 120 °C [twice at a 24 h interval (Ogoshi *et al*., 1990)]. Inoculum from a single potato dextrose agar (PDA) plate colonized with *R. solani* AG-8 was placed in a sterilized blender and suspended in 500 mL of sterile water. A third of the mixture was poured into each of three flasks containing autoclaved barley grains. The fungal mycelium was allowed to grow for 2 to 3 wk, and the flasks were shaken manually 1 or 2 times each week to facilitate colonization of the grains (Weller *et al*., 1986). The barleys were air-dried overnight in a Laminar airflow hood, and then ground into a course meal using a standard coffee-bean grinder.

Pathogenicity Study of R. solani:

The inoculum of *R. solani* as previously prepared was added to 100 g of sterilized vermiculite in different concentrations in sterilized pots of 20 cm diameter.

Three replicates were included per treatment. Control pots were treated with an equal quantity of autoclaved barley vermiculite preparation.

Pots were left for two weeks at room temperature in a greenhouse and watered every 3 days by dist. Water. Wheat grains (15 per pot) were surface disinfected as described above and sown. The pots were watered every three days by Knop's solution.

Grain Treatments (Schmidt et al., 2004):

B. subtilis was cultured (in 500 ml batches at 30 °C, 200 rpm on a rotary shaker) in Saburote medium for 4 days. The cultures were centrifuged at 2, 556 g for 20 min at 4 °C. Seeds were pelleted in a rotating vessel at 45 rpm. 4 days old *B. subtilis* cultures were suspended in the adhesive solution (1 % Carboxymethyl cellulose. Trichoderma harzianum was added when required as 3% w/w. The treatment resulted in a bacterial inoculum of 10^6 to 10^7 CFU per grain pellet.

T. Harzianum Filtrate Preparation:

T. harzianum was also cultured in Saburote medium for 7 days and was filtered under aseptic conditions. Different concentrations of the filtrate were used.

B. Subtilis Filtrate Preparation:

Cell free culture filtrate of *B. subtilis* was obtained as descried in *T. harzianum* filtrate treatments.

Fungicide Treatment:

The allowed dose of the fungicide carbomar was 50 g / 100 L.

Effect of Different Fungicide Concentrations on R. solani:

Carbomar was applied to Saburote media at rates ranging from none to 5.0 /ml. *R. solani* was cultured in three replicates for each treatment in 250 ml flasks and was incubated for 7 days.

Fungicide in Greenhouse Treatments:

Carbomar in the ratio of 50 g / 100 L was used once to water the infested or uninfested pots before sowing the grains.

Control pots and grain disinfection were treated as previously mentioned.

Analytical Techniques:

After 3 weeks of sowing, samples from the seedling leaves af the different treatments were taken at random to record their morphological data as percentage of healthy seedlings, length, fresh and dry weight. Pigment content was determined by the method of Lichtenthaler (1987). Total soluble sugars was determined by Cooper and McDaniell method (1970). Total amino acids was determined by Mutting and Kaiser (1963) and total nitrogen was detected by the method of Naguib (1969).

RESULT AND DISCUSSION

It appears from Table 1 that as the percentage of *R. solani* inoculum increased, the percent of unhealthy seedlings significantly increased. This is due to that the fungus produces hydrolyzing enzymes cellulose, polygalacturonase and pectin methyl esterase, responsible for the lysis of the host cell wall (Vazquez and Martinez, 1993 and Madhosingh, 1995).

Table 1: Effect of different inoculum concentrations g% (w/w) of *R. solani* on the incidence of root – rot disease in *T. aestivum* L. (means of replicates ± SE).

% of inoculum w/w	% of healthy Seedlings	% of unhealthy seedlings
0.0 (control)	97.50 ± 0.17	97.50 ± 0.17
1.0	88.35 ± 0.81**	11.65 ± 0.21**
2.0	72.50 ± 0.17**	27.50 ± 0.17**
3.0	55.58 ± 1.86**	44.42 ± 1.86**
4.0	41.92 ± 0.65**	59.08 ± 0.65**
5.0	32.43 ± 1.47	67.57 ± 1.47**

The results obtained in Table 2 reveal that the used different concentrations of carbomar, significantly inhibited the biomass of *R. solani* in relation to control treatments (free from fungicide).

Table 2: Effect of different concentrations of carbomar (fungicide) on the dry weight of *R. solani* after 7 days of incubation (means of replicates ± SE).

Fungicide carbomar conc (µ/ml)	Dry wt mg/biomass
0.0 (control)	629.33 ± 28.48
1.0	426.67 ± 17.64**
2.0	276.06 ± 23.33*
3.0	170.33 ± 15.77**
4.0	113.67 ± 13.33**
5.0	63.33 ± 14.53

Moreover, different concentrations of cell free culture filterate of *T. harzianum* or *B. subtilis* added to the pathogen, in *in vitro* experiments decreased its dry weight significantly as filtrate concentrations increased (table 3).

Table 3: Effect of cell free culture filtrate of *T. harzianum* or *B. subtilis* on the dry weight of *R. solani* after 7 days of inculation (means of replicates ± SE).

Filtrate concentration %	Dry weight mg / biomass	
	1	2
0.0 (control)	629.33 ± 28.48	629.33 ± 28.48
10.0	450.33 ± 62.03*	493.33 ± 11.55*
20.0	293.67 ± 23.33**	311.67 ± 8.82**
30.0	163.33 ± 17.64**	252.00 ± 9.31*
40.0	99.12 ± 14.53	143.67 ± 14.53**
50.0	54.33 ± 5.78**	89.15 ± 6.03*

1 : Culture filtrate of *Trichoderma harzianum*.
2 : Culture filtrate of *Bacillus subtilis*.
* , ** : Significance.

Previous reports showed that *B. subtilis* NB 22 and *B. subtilis* RB 14 produced antifungal peptide antibiotic iturin A and surfactin in solid state fermentation using soybean curd residue (Akihiro *et al*., 1993). Iturin A is a small molecules yet displays strong antifungal activity. The other lipopeptide, surfactin, in contrast has weak antibiotic activity.

In the greenhouse seedling tests the performance of the *B. subtilis* was comparable to that of *T. harzianum* and carbomar treatments. The results obtained in table 4 showed that coating the grains with *B. subtilis,* or treatment of the soil with 3 % *T. harzianum*, or soil treatment with carbomar showed slight differences in the estimated parameters: percentage of healthy seedlings, length of seedlings and its fresh and dry weight.

Table 4: Effect of biocontrol or chemical control of root rot disease caused by *R. solani* on some growth parameters of *T. aestivum* seedlings (means of replicates ± SE).

Inoculum of *R. solani* %	Healthy seedlings %			Length of healthy seedlings %		
	1	2	3	1	2	3
0.0	95.00 ± 0.56	95.00 ± 0.56	95.00 ± 056	55.78 ± 0.59	55.78 ± 0.59	55.78 ± 0.59
1 %	92.50 ± 0.86**	91.65 ± 0.12**	91.11 ± 2.22*	50.23 ± 0.47*	41.97 ± 0.34**	41.56 ± 3.01*
2 %	90.00 ± 0.90**	85.00 ± 0.33**	88.89 ± 2.22*	44.15 ± 0.31**	38.46 ± 0.51**	39.96 ± 1.67**
3 %	81.65 ± 0.50**	76.65 ± 0.57**	80.00 ± 6.67*	40.06 ± 0.60**	35.23 ± 0.48	36.43 ± 0.96*
4 %	72.25 ± 0.28*	68.35 ± 0.33*	75.56 ± 8.01*	34.26 ± 0.62*	31.33 ± 0.22**	32.03 ± 1.00
5 %	65.50 ± 0.76	60.55 ± 0.78*	73.33 ± 3.85*	30.19 ± 0.13*	27.23 ± 0.26*	29.34 ± 0.73*

Table 4: Continue

Inoculum of *R. solani* %	Fresh wt of healthy seedlings (mg)			Dry wt of healthy seedlings (mg)		
	1	2	3	1	2	3
0.0	293.75 ± 4.85	293.75 ± 4.85	293.75 ± 4.85	91.30 ± 0.29	91.30 ± 0.29	91.30 ± 0.29
1%	257.16 ± 2.94**	210.03 ± 3.34*	219.67 ± 3.18*	82.95 ± 1.04**	72.27 ± 2.27**	72.95 ± 5.52*
2%	228.73 ± 2.23**	194.82 ± 4.94**	198.33 ± 2.96*	78.06 ± 0.81**	70.26 ± 3.24*	71.15 ± 3.12
3%	196.45 ± 2.02*	121.23 ± 3.74**	182.00 ± 4.03*	70.23 ± 0.19*	63.45 ± 2.13**	69.33 ± 3.56**
4%	179.33 ± 3.44**	160.09 ± 3.36*	165.15 ± 3.06**	62.11 ± 2.25**	53.41 ± 2.80**	60.67 ± 4.13
5%	158.18 ± 2.26**	146.35 ± 4.12**	159.67 ± 3.38	58.29 ± 1.67**	43.21 ± 0.19**	53.57 ± 2.96**

1 : Soil treated by 3 % *T. harzianum*.
2 : Grains coated by *B. subtilis*.
3 : soil treated by Carbomar.
* , ** : Significance.

The results in Table 5 suggest that the combination of *T. harzianum* and *B. subtilis* in the substrate, significantly reduces the root rot caused by *R. solani* in wheat plants. This is in agreement with Duffy and Weller (1995); Pierson and Weller (1994) and Muis and Quimio (2006). They stated that a combination of biocontrol strains have been shown to be superior to single inocula in a number of cases.

The results also in table 5 show that the combined treatment resulted in significant increased amounts of total soluble carbohydrates, total soluble nitrogen and amino acids compared with control and/or the presence of carbomar. Table 5 reveals that, soil infested with *R. solani* significantly decreased the biosynthesis of amino acids, total nitrogen and total soluble carbohydrates of the seedlings.

The root rot pathogen decreased photosynthetic pigments (chlorophyll a and b and carotenoids) whereas the control agents (sterile soil) increased them in leaves of healthy seedlings.

Table 5: Effect of biological control of root rot disease in *T. aestivum* seedlings by *T. harzianum* and/or *B. subtilis* or chemical control treatment (carbomar) on some metabolic aspects (means of replicates ± SE).

Treatment	Pigment content (mg / gm fresh leaves)			Total soluble sugars mg/g D.wt.	Total amino Acids mg/g D.wt.	Total nitrogen mg/g D.wt.
	Chlorophyll a	Chlorophyll b	Caroteniodes			
Sterile soil	6.41 ± 0.41	4.33 ± 0.10	2.65 ± 0.11	33.78 ± 2.01	12.61 ± 0.78	189.88 ± 21.11
Soil infested with 3% *R. solani*	3.83 ± 0.22**	2.77 ± 0.45**	1.46 ± 0.45**	28.45 ± 2.39*	10.06 ± 0.85**	146.70 ± 18.33*
Sterile soil treated with Carbomar	6.39 ± 0.75*	4.12 ± 0.08**	2.57 ± 0.11*	31.83 ± 3.05**	11.32 ± 0.76**	175.77 ± 15.03**
Soil infested by 3% *R. solani* and treated by Carbomar	4.89 ± 0.30*	3.10 ± 0.17*	1.88 ± 0.62*	29.76 ± 1.19*	10.38 ± 0.63**	171.52 ± 19.21*
Sterile soil + 3% *T. harzianum*	6.63 ± 0.92*	4.79 ± 0.12**	2.83 ± 0.05*	38.03 ± 1.17**	14.07 ± 0.15*	191.73 ± 14.63
Soil infested with 3% *R. solani* + 3% *T. harzianum*	5.36 ± 0.66*	3.51 ± 0.37*	1.88 ± 0.17*	33.51 ± 3.76**	13.11 ± 0.32**	189.78 ± 12.25**
Sterile soil+grain coated with *B. subtilis*	5.91 ± 1.08**	4.54 ± 0.83**	2.67 ± 0.16	35.81 ± 0.87*	13.69 ± 0.21*	193.01 ± 16.42**
Soil infested with 3% *R. solani*+grains coated with *B. subtilis*	4.31 ± 0.81*	3.13 ± 0.08*	1.69 ± 0.26*	32.84 ± 2.59*	12.50 ± 0.66**	188.54 ± 27.10**
Infested soil by 3% *R. solani* + grains coated with *B. subtilis* + 3% *T. harzianum*	7.01 ± 0.36**	5.16 ± 0.19*	3.11 ± 0.21*	38.86 ± 1.26**	14.93 ± 0.71*	194.03 ± 13.51**

* , ** : Significance.

B. subtilis and *T. harzianum* were the most efficient agents in increasing photosynthetic pigments. This means that the efficacy of photosynthetic apparatus was closely associated with the production of carbohydrates, amino acids, and total nitrogen.

Mixtures of strains may exert biological control under a broader range of conditions, because strains with different ecological optima may complement each other. This is thought to be the reason for the improved biocontrol efficacy of mixed strains in the field (El-Meleigi *et al*., 2007 and Kildea *et al*., 2008).

Two groups of antibiotics were produced by *B. subtilis* one group consisting of several antibacterial compounds, and the other consisting of only two antifungal compounds. One of these antifungal compounds is known to be bacillomycin (Landy *et al*., 1948). The other antibiotic produced by *B. subtilis* is fengymycine and proved to be different from the other compounds. It was less toxic to the test plants and protected them better from Rhizoctonia disease (Loefler *et al*., 1986).

Trichoderma spp. are among the most studied fungi for biocontrol of plant diseases. Chitinolytic enzymes have been implicated as factors contributing to the ability of *Trichoderma* spp. to act as biocontrol agents (Cherif and Benhamou, 1990; and Ulhoa and Peberdy 1991 and others).

T. harzianum is known to produce relatively high concentrations of cell-wall degrading enzymes as beta - 1, 3 - glucanases and different chitinolytic enzymes. Several enzymes have been purified and characterized, and their ability to inhibit the germination of spores and elongation of the hyphae belonging to the pathogenic fungi has been shown *in vitro* (Lorito *et al*., 1993).

The use of fungicides, besides being expensive and involving risks to the environment associated with the application of chemicals, is not totally effective and may lead to the appearance of new, resistant strains of pathogens (Bruin and Edgington, 1980). It is therefore necessary to develop alternative ways of control. One such alternative is biological control, in which micro-organisms are selected for their ability to antagonize pathogens. Seeds treated with bioagents are safe to handle by users and could be fed to poultry or livestock with minimum risk than if they were chemically treated.

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