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Improvement of Lytic Enzymes Producing *Pseudomonas fluorescens* and *Bacillus subtilis* Isolates for Enhancing their Biocontrol Potential Against Root Rot Disease in Tomato Plants.

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ABSTRACT

Among different isolates of bacteria there are two isolates *Pseudomonas fluorescens* and *Bacillus subtilis* which isolated from biocompost rice straw were used against pathogenic fungi causing root rot disease of tomato crop. Cultivation of *Bacillus subtilis* and *Pseudomonas fluorescens* by using agriculture waste as substrate for the management of root rot disease and growth promotion in tomato plants showed high effective against *Fusarium solani* and *Rhizoctonia solani* the causing agents of root rot disease in tomato plants. *Pseudomonas fluorescens* and *Bacillus subtilis* isolates have relatively strong lytic activities of chitinase, β-1,3 glucanase (laminarinase) and β-1,4 glucanase (cellulase) toward the tested fungi. Bacteria isolates play an important role in increasing yield and improving the growth and quality of the tomato fruits and offer as attractive way to replace chemicals fertilizers and fungicides. This study investigated lytic enzymes activities in the bacterial isolates, the results showed the presence of higher activities of chitinases, β-1,3 glucanases and β-1,4 glucanase in extracellular protein extract. Biocompost rice straw was effective bioagent and recorded highest reduction in root rot disease. These results were confirmed by the field and green house experiments.

Keywords: Lytic enzymes, Biocontrol agents, Root root disease, Bio-compost-rice straw.

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INTRODUCTION

Control of soil borne pathogens is very difficult because pathogens can survive for a long period as sclerotia in soil. Organic amendments play an important role as environmentally friendly and sustainable alternative approach to protect plants against pathogens [1].

Particularly useful for the biocontrol application are chitinolytic and glucanolytic enzymes because of their ability to efficiently degrade the cell wall of plant pathogenic fungi by hydrolyzing polymers not present in plant tissues. Each of these two classes of enzymes contains a number of proteins with different enzyme activity [2]. Many marketable PGPR (plant growth promoting rhizobacteria) that extend beneficial effects on plant development often related to the increments of nutrient avail ability to host plant [3]. However not all the PGPR showed exist their positive effect on plant growth via increasing nutrient status of host plants. PGPR seem to promote growth through suppression of plant disease, or through production of phytohormones and peptides acting as bio-stimulants [4].

Some rhizosphere microorganisms may be neutral or deleterious in regard to plant growth, whereas other microbes support their hosts [5,6]. Such plant growth-promoting bacteria (PGPB) [7] or plant growth-promoting rhizobacteria (PGPR) [8] can stimulate plant growth, increase yield, reduce pathogen infection, as well as reduce biotic or a biotic plant stress, without conferring pathogenicity [5,9]. Plant beneficial microorganisms are of interest for application in agriculture as bio-fertilizers or as pesticides as well as for phyto-remediation applications [10,11].

Soil amendments, using composted agricultural wastes fortified with biocontrol agents could be acceptable approaches in this regard. The use of organic agricultural wastes in this respect can be an advantageous both in soil fertility, recycling of agricultural residues and could provide a powerful tool for management of plant diseases. It has been reported that several composts and /or composts fortified with biocontrol agent used as soil amendments reduced pathogens propagates density and protected plants from soil borne plant pathogens [12].

The main objective of this study is to cultivate *Pseudomonas fluorescens* and *Bacillus subtilis* by using agriculture waste as substrate for the management of root rot disease and growth promotion in tomato plants. Due to the significant role of hydrolytic enzymes in the biocontrol activity of *Pseudomonas fluorescens* and *Bacillus subtilis* against pathogenic fungi, this study was undertaken to assess the activities of hydrolytic enzymes from the bacterial isolates. Furthermore, the efficiency of *Pseudomonas fluorescens* and *Bacillus subtilis* to produce lytic enzymes was demonstrated. The present investigation was designed to investigate the potential of manipulating soil with biocomposts made from composted agricultural wastes fortified with PGPR to reduce the tomato root rot diseases caused by *Fusarium solani* and *Rhizoctonia solani* under field conditions.

MATERIAL AND METHODS

In vitro antagonistic studies

Efficiacy of antagonistic bioagents against *Fusarium solani* and *Rhizoctonia solani*

Two isolates of *Pseudomonas fluorescens* and *Bacillus subtilis* isolated from compost were obtained from culture collection unit of Plant Pathology Department, National Research Centre, Giza, Egypt. These isolates proved their high antagonistic effect against several soil-borne plant pathogens causing root rot and wilt diseases to various vegetables and field crops. *In vitro* antagonistic ability of PGPR was tested against *Fusarium solani and Rhizoctonia solani* ,the causing pathogens of root rot disease of tomato crop using dual culture technique [13]. Two streaks of a bacterium were placed on the surface of a petri-dish containing PDA medium, arranged at the two sides of the central disc of the tested pathogenic fungus. Inoculated PDA medium with discs of the tested pathogenic fungi alone served as controls. The diameter of growth of the pathogenic fungi was measured when fungal growth in control covered completely the surface of the medium at 28°C. Inhibition percentage for pathogenic fungal growth was calculated, for each antagonistic test [13].

Enzymatic activity of bacterial biocontrol agents.

β- 1,3 glucanase (Laminarinase):

The enzyme-catalyzed reaction was measured with 2.5mg/ml soluble laminarin as a substrate in a 40mM potassium phosphate buffer, pH 7.0, at 30^{o}C, for 15 min to obtain the reaction-time course. The reducing power caused by the enzymatic reaction from the substrate was determined by Somogyi-Nelson a method of which absorbance at 600 nm was measured [14]. One unit of enzyme was defined as the amount of enzyme, which liberates 1µmol of reducing sugar equivalent to glucose per one min under the standard assay conditions.

β- 1,4 glucanase (cellulase):

Cellulase activity was determined by incubation of 900 µL of 1% (CMC) carboxy methyl cellulose in 20 mM phosphate buffer (pH 7.0) with 100 µL of appropriate concentration of enzyme at 50^{o}C. After 30 min reaction, 1 mL of dinitrosalicylic acid (DNS) was added and boiled in a water bath for 15 min to stop the reaction. The resulted samples were then cooled to room temperature and measured the absorbance at 540 nm [15]. One unit of cellulase activity was defined as the amount of enzyme that could hydrolyze carboxymethylcellulose and release 1µmol of glucose within 1 min reaction time at 50^{o}C.

Chitinase activity:

Colloidal chitin (1% in 50 mM acetate buffer) was used as the substrate for the measurement of chitinase activity. The mixture of enzyme solution (0.5 ml) and substrate (0.5 ml) was incubated at 40^{o}C for 60 min. After centrifugation, the amount of reducing sugar produced in the supernatant was determined by the method of Monreal and Reese with N-acetylglucosamine as a reference compound [16]**.** One unit of enzyme activity was defined as the amount of enzyme that produced 1 µmol of N-acetylglucosamine per min.

Pot Experiment

Preparation of antagonistic bacteria:

Bacillus subtilis and *Pseudomonas fluorescens* were prepared by grown on king's medium and counted by plate technique (10^{8} cfu /ml.) [17].

Pot experiment was designed under green house conditions using plastic pots 25cm, diameter containing sterilized loamy clay soil were infested with *Fusarium solani* and *Rhizoctonia solani* separately grown on barley grain at rate 5g/Kg soil before sowing. Infested pots were irrigated for 10 days before sowing bacterial suspension which used as bio-control agents. The bacterial inoculums of *B. subtilis* and *P. fluorescens* were added to the soil as cell suspension at the rate of 50 ml/pot. Also *B. subtilis* and *P. fluorescens* were formulated on the rice straw as a biocompost.

Ten tomato seeds were grown in each pot five replicate pots. The experiment included the following treatments: (non-infested soil control, soil treated with *F. solani* , *F. solani* + *B. subtilis, F. solani* + *P. fluorescens, F. solani* + rice straw + PGPR, *R. solani* alone, *R. solani* + *B. subtilis, R. solani* + *P. fluorescens and R. solani* + rice straw + PGPR. Pots were kept under green house conditions till the end of the experiment.

Disease assessment for incidence of pre and post emergence

Percentage of root rot incidence at the pre-emergence stage was calculated as the number of absent emerged seedlings in relative to the total number of sown seeds. Meanwhile, percentage of post- emergence root rot was calculated as the number of tomato plants showing disease symptoms in relative to the total number of emerged seedlings.

Field experiment

A field experiment was conducted at sahl el-hosenia el-Sharkia Government, Egypt, heavily naturally infested field with *Fusarium* and *Rhizoctonia* root rot diseases. Tomato (*Lycopersicon esculentum* Mill CV Rio Fuego) was grown from seeds in a mixture of peat and per liter (1:1, v/v), and seedlings were transplanted after 21 days separately into field. *Bacillus subtilis* and *Pseudomonas fluorescens* were grown on king's medium at 28^{o}C for 24 h. Bacterial suspension was adjusted to yield 1x10^{7} cfu/ml. Each isolate formulated on rice straw waste alone and combination to evaluate the percentage of infection with root rot , vegetative growth, plant height, shoot (leaves+stem) dry weight, root length, root dry weight, yield and fruit quality evaluation were determination after 60 days of transplant.

Shoot (leaves and stem) and root dry weights of four plants were determined after drying at 80^{o}C for 48 h. To constant weight. Each plant was a single replication and there were four replications per treatment (n=4).

Measurement of root rot disease incidence.

Root rot disease incidence in tomato plants in response to bio-control agent treatments under field conditions was recorded as a percentage of infected plants in relation to the total number of transplanted tomato seedling.

Plants and the percentage of disease reduction were also recorded up to 60 days from transplanting date. Five plants were chosen randomly from each replicate at 60 days after transplanting date.

Yield and quality evaluation.

All red fruits were harvested from four plants per treatment and the total yield per plant was obtained. A fruits were graded into three categories according to, economical grade (< 4cm in length), and marketable grade (4-7cm. in length). Size was determined by measuring fruit fresh weight, length and diameter of all red fruits from four plants per treatment. For texture determinations fruits were harvested at three different ripening stages: breaker (B), light red (LR) and red (USDA, 1991). Fruits was evaluated by a puncture test using a texture Analyzer TA.XT2.

Statistical analysis:

All experiments were set up in a complete randomized design. One-way ANOVA was used to analyze differences between antagonistic inhibitor effect and linear growth of pathogenic fungi *in vitro.* A general linear model option of the analysis system SAS (SAS Institute Inc. 1996) was used to perform the ANOVA. Duncan's multiple range tests at $P < 0.05$ level was used for means separation [18].

RESULTS AND DISCUSSIONS

Table (1) revealed that the isolates of bacteria proved their ability to antagonize pathogenic fungi *F. solani and R. solan*i. The highest reduction in the linear growth was found by treatment with *P. fluorescens* which isolated from compost against each pathogens, 81.1 and 77.8% respectively where the least reduction was recorded with treatment with *B. subtilis* against *F. solani* and *R. solani* (57.8 and 55.6 %). Similar results were also reported by many investigators inhibitory effect of antagonistic fungal and bacterial such as *Trichoderma* spp., *B. subtilis* and *P. fluorescen*s against growth reduction of *P. ultimum* and *R. solani* under *in vitro* conditions. The inhibition in the growth of the pathogen could be attributed to antibiosis, hyper-parasitism or production of chitinase, β-1,3 glucanase and β-1,4 glucanase enzymes which degrade the cell wall leading to lyses of mycelium of the pathogen [19,20].

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Table 1: *In vitro* antagonistic effect of different bio-agents isolated from soil and bio-compost of rice straw against *F. solani* and *R. solani*

Bioagents	*Fusarium solani*		*Rhizoctonia solani*	
	Linear Growth	% Reduction	Linear Growth	% Reduction
Bacillus subtilis	38.0 b	57.8	40.0 b	55.6
B. subtilis (c)	35.0 c	61.1	32.0 c	64.4
Pseudomonas fluorescens	21.0 d	67.7	28.0 d	68.9
P. fluorescens (c)	17.0 e	81.1	20.0 e	77.8
Control	90 a	------	90 a	-----

Figures with the same letter are not significantly different (p = 0.05)

Antibiosis activity of antagonistic biocontrol agents.

Biocontrol agents are known to produce various enzymes like β-1-3 glucanase, cellulase (β,1-4 glucanase) and chitinase which are involved in the antagonistic activity against phytopathogenic fungi which have the capability to degrade the cell wall of these fungi [21]. Data as shown in table 2, 3 and 4 indicated that the activities of lytic enzymes were high when the antagonistic microorganisms grown on compost tea medium as compared with growing on nutrient medium. Maximum levels of lytic enzymes production were observed in *Pseudomonas fluorescens* grown on compost medium after 72 h, then the activities decreased significantly (data not shown). Similar observations were recorded by Viterbo *et al*. [21]. Many reports on the production of lytic enzymes by microorganisms were achieved in soil inoculated with *Trichoderma harzianum* [22]. The major components of *R. solani* cell wall has been identified as β-glucan in addition to chitin [23], therefore, β-glucanases and chitinase can play an important role in antagonistic activity. In short, mechanisms of microbial antagonism toward plant pathogenic fungi include production of siderophores, hydrogen cyanide (HCN), antibiotics and production of fungal cell wall-degrading lytic enzymes.

Table 2: β-1,3 glucanase produced by *Pseudomonas fluorescens* and *Bacillus subtilis* grown on nutrient and compost media after 24 , 48 and 72 h of incubation at 25°C.

Bioagents	β-1,3 glucanase activity (IU/ml)		
	24 h	48 h	72 h
Pseudomonas fluorescens (N)	19.5	22.2	29.5
Pseudomonas fluorescens (C)	32.5	40.6	55.7
Bacillus subtilis (N)	15.1	17.5	20.9
Bacillus subtilis (C)	24.4	25.8	38.3

Note : N (Nutrient medium)- C (Compost tea medium).

Table 3: β-1,4 glucanase produced by Pseudomonas fluorescens and Bacillus subtilis grown on nutrient and compost media after 24, 48 and 72 h of incubation at 25○C.

Bioagents	β-1,4 glucanase activity (IU/ml)		
	24 h	48 h	72 h
Pseudomonas fluorescens (N)	25.5	32.2	40.1
Pseudomonas fluorescens (C)	39.5	50.6	65.7
Bacillus subtilis (N)	15.1	25.5	32.9
Bacillus subtilis (C)	27.4	35.8	55.3

Note : N (Nutrient medium)- C (Compost tea medium).

Table 4: Chitinase produced by Pseudomonas fluorescens and Bacillus subtilis grown on nutrient and compost media after 24, 48 and 72 h of incubation at 25○C.

Bioagents	Chitinase activity (IU/ml)		
	24 h	48 h	72 h
Pseudomonas fluorescens (N)	11.3	20.2	29.1
Pseudomonas fluorescens (C)	18.5	30.6	45.7
Bacillus subtilis (N)	7.1	15.5	25.9
Bacillus subtilis (C)	11.4	22.8	33.3

Note : N (Nutrient medium)- C (Compost tea medium).

Green house experiments:

In vitro experiments were carried out to evaluate the effect of different bio composts as soil treatment on *Fusarium solani* and *Rhizoctonia solani* root rot incidence of tomato at (NRC). Data in Table (5) show that all soil treatments reduced the percentage of disease incidence .The highest reduction in disease incidence with the treatment, of *R. solani* + rice straw + PGPR and *F. solani*+ rice straw + PGPR (69.2 and 57.1) respectively. Meanwhile, soil treatments with biocompost showed considerable effect in reducing disease comparing with control treatment. In this regards, several researchers have been recorded that biocompost application as soil amendment could suppress diseases caused by *R. solani* and *Fusarium* spp. on many economic crops [24-26]. Such means comprise elimination of pathogens density in the soil and maintaining soil condition, favorable for root development and enhancement the competitive ability of bioagents against pathogens. Therefore, these methods introduced efficient disease control and increasing yield of many crops [27].

Control of root rot pathogens through amended soil with organic materials formulated with biocontrol agents may be attributed to : i) increasing the activity of indigenous micro-flora resulting in suppression of pathogens population through competition or specific inhibition, ii) releasing degradation compounds such carbon dioxides, ammonia, nitrites, saponin or enzymes which are generally toxic to the pathogens, iii) inducing plant defense mechanisms, iv) cellulose and glucan are prevalent to high concentration in soil as a result of biodegradation of cellulose and lignin [28,29].

Table 5: Incidence of tomato root rot diseases in response to soil treatment with different bioagents formulated on the rice straw under greenhouse condition

Treatments	Pre-emergency		Post-emergency	
	% Disease incidence	% Reduction	% Disease incidence	% Reduction
F.solani	42.0 a	----------	37.9 a	------
F. solani +B. subtilis	28.0 b	33.3	16.6 b	56.2
F. solani + P. fluorescens	20.0 b	52.3	15.0 b	60.4
F. solani + Rice straw + PGPR	18.0 c	57.1	9.8 d	74.1
R. solani	52.0 a	--------	41.6 a	--------
R. solani + B. subtilis	24.0 b	53.8	18.4 b	55.8
R. solani+P. fluorescens	22.0 b	57.7	17.9 b	57.0
R. solani + Rice straw + PGPR	16.0 c	69.2	11.1 d	73.3

Figures with the same letter are not significantly different (p = 0.05)

Field experiments:

Effect of soil treatments with different bioagents formulated of rice straw waste on tomato plant.

Treatment soil with different biocompost before sowing Tomato plants resulted in reducing root rot disease under field conditions. Results in table 6 showed that all applied soil treatments reduced the incidence of root rot caused by *F. solani* and *R. solani*. The highest reduction in disease incidence was observed with the treatment by PGPR formulated on rice straw reduced the pre-emergence by 72.3 % and 83.5% at post-emergence. Also data showed that treatment with rice straw +*Bacillus subtilis* or with *Pseudomonas fluorescens* were significantly reduced pre and post –emergence compared with control.

Table 6: Tomato root rot disease incidence in response to applied treatments different bio-agents and rice straw PGPR under field conditions.

Treatments	Pre- emergency		Post- emergency	
	% Disease incidence	% Reduction	% Disease incidence	% Reduction
Rice straw + *B. subtilis*	25.0 b	39.8	5.3 b	68.8
Rice straw + *P . fluorescens*	21.5c	48.2	3.2 c	81.2
Rice straw + PGPR	11.5d	72.3	2.8 d	83.5
Control	41.5a	-----	17.0 a	-----

Figures with the same letter are not significantly different (p = 0.05)

PGPR is known to exert its beneficial effect on plant through several mechanisms. Plant growth-promoting mechanisms include root colonization, nitrogen fixation, solubility of phosphate, production of IAA, and other phyto-hormones, siderophore, and volatiles. Suppression of biotic stress is through competition for space, food and nutrition with phyto-pathogens, production of antibiotic, siderophores, volatiles, and by inducing systemic resistance (ISR).

Effect of PGPR on root growth and yield in tomato plants under field conditions.

Data in Table (7) showed that the average plant height of 52 cm. in plants treated with biocontrol PGPR formulated on rice straw which was followed by plants treated with *Pseudomonas fluorescens* or *Bacillus subtilis* formulated on rice straw (48 cm and 46 cm.).

However plant height was 45cm in the case of control. Even thought plant height was highest also root dry weight was highest in treatment with PGPR formulated with rice straw (8.2g) followed by plants treated with *Pseudomonas fluorescens* or *Bacillus subtilis* formulated on rice straw were 7.2 and 6.8g respectively.

Table 7: Effect of PGPR on root growth and yield in Tomato plants under field conditions.

Treatments	Root length (cm)	Root dry weight (g)	Yield (plant/g)
Rice straw + *B. subtilis*	46.0 b	6.8 c	720 c
Rice straw + *P. fluorescens*	48.0 b	7.2 b	796 b
Rice straw + PGPR	52.0 a	8.2 a	816 a
Control	45.0 b	6.3 c	659 d

Figures with the same letter are not significantly different (p = 0.05)

However dry weight was 6.3 g in case of control .The yield take the same trend the most significant increases in yield with treatment by PGPR formulated on rice straw 816g. Followed by treatment with *Pseudomonas fluorescens* or *Bacillus subtilis* formulated on rice straw were 796 and 720g compared with control 659g. *Pseudomonas fluorescens* was the effective in this respect than other PGPR. It is attributing for positive root colonization also exhibits strong antifungal activity against Rhizoctonia and Fusarium found in rice and sugarcane rhizosphere, mainly through the production of antifungal metabolites [30].

Data in table (8) observed that treatment by PGPR formulated with rice straw the most effective in marketable grade, fruit weight, light red and red comparing with all treatments and control (70.2, 12.8 and 10.0) respectively but in case of length cm. diameter cm. there is no significance different between all treatments.

Table 8: Effect of PGPR on some marketable grade on Tomato fruits.

Treatments	Marketable grade	Fruit weight (g)	Length (cm)	Diameter (cm)	Light red	Red
Rice straw + *B. subtilis*	66.5	54.5	5.1	4.2	12.3	8.9
Rice straw + *P. fluorescens*	69.5	60.0	5.2	4.1	12.5	9.5
Rice straw + PGPR	70.2	61.5	5.3	4.3	12.8	10.0
Control	58.1	52.5	5.1	4.0	12.0	8.5

The use of plant growth promoting rhizobacteria (PGPR) as biocontrol is beneficial for tomato in saline soil.

Using agricultural wastes, domestic food wastes or some grains as substrates for *T. harzianum* growth formulation and directly delivery in soil for controlling soil borne pathogens on some crops were recorded [31-33].

CONCLUSIONS

Rice straw is agriculture waste produced in large amounts from rice cultivation and consists of different biopolymers such as cellulose, hemicellulose and lignin. The polysaccharides in straw (bio-straw extract) many serve as substrate for complex microbial communities. The cell wall degrading enzymes from

Pseudomonas fluorescens and *Bacillus subtilis* have great potential in agriculture as active components in new fungicidal formulation. Utilization of PGPR in order to increase the productivity may be a viable alternative to organic fertilizers which also helps in reducing the pollution and preserving the environment in spirit of an ecological agriculture. Thus rhizospheric bacteria can be a promising source for plant growth promoting agent in agriculture inoculants for improving the growth and yield of agricultural crops.

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