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Biological Control of Seedling Damping – Off of Sugar Beet Plant

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ABSTRACT

Pythium ultimum causal of damping –off in sugar beet, which is one of the most destructive diseases in this crop worldwide..During the study, twelve bacterial isolates were isolated from rhizosphere soil of sugar beet crop. Three isolates of them showed antifungal activity against these phytopathogen. These isolates were identified as: *Bacillus amyloliquefaciens* , *Bacillus pseudomycodies* and *Bacillus* sp. by standard tests and the application of biolog system. Three species of fungi as *Trichoderma* spp.were successfully used by several investigators to control .*In vivo,* results of seeds soaking with tested *B. amyloliquefaciens* showed that the most effective in controlling damping –off disease(80%) followed by *B. pseudomycodies* (66.67%). While, *T. viride* recorded value of survival plants (60%), *T. hamatum* and *T.harzianum* (53.33%, 23.33%, respectively).At the same time seeds coating with *T.harzianum* was the most effective in controlling disease indicated that (86.67%), followed by *B. pseudomycodies* , *B. amyloliquefaciens* , *T. viride* , *Bacillus* sp. and *T. hamatum* (80.00%, 73.33%, 50.00%, 46.67%, 40.00 % respectively) in soil infested with *P. ultimum*.

Keywords: *Pythium ultimum*, *B. amyloliquefaciens*, *B. pseudomycodies*, *Trichoderma* spp.

INTRODUCTION

Sugar-beet (*Beta vulgaris*) is one of the most important sugar crops all over of the world. In Egypt, due to the great consumption of sugar, the production of sugar-beet must be increased to cover the requirement of sugar which depended sugar cane(Abo-Elnaga ,2014).

Seedling diseases can be caused by any of several common soil borne organisms, such as *Pythium, Fusarium* and *Rhizoctonia*. At least 14 species of *Pythium* have been previously identified that can cause seedling blight and root rot (Vincelli, 2008).

Bacillus spp. in particular are gaining recognition as safe biocontrol agents in a variety of crops, specifically as seed protectants and antifungal agents (Haggag, 2008).

Recent studies show that *Trichoderma* spp.they are not only parasites of fungal plant pathogens but also can produce antibiotics. Moreover, some strains may enhance plant growth and development(Anita *et al.* , 2012).In general, *Trichoderma* spp. are very effective biocontrol agents and controlling seedling disease in suger beet (Afify *et al.* , 2018).

The aim of the present study was planned to investigate the possibility of controlling sugar beet damping –off disease by using some bioagents (*Bacillus* spp. and *Trichoderma* spp.) .

MATERIALS AND METHODS

Soil samples

Soil samples were collected in sterilized pages from rhizosphere of sugar beet and then transfered to lab . for further studies .

Isolation and purification of Bacteria

Ten grams of soil samples was suspended in 90 ml of sterile tap water and serial dilutions were made. An one ml from each dilution was transferred to Petri-dishes. Nutrient agar (NA) medium was added thereafter and mixed thoroughly. Three replicates were prepared from each dilution. Colony units were obtained after two days of incubation at 30^0C. The bacteria were isolated and purified on nutrient agar (NA) medium.

Fungal strains as bioagents

Three fungal strains namely: *T.viride, T. harzianum* and *T. hamatum* were obtained from Plant Pathology Research Institute, Agric. Res. Center (A.R.C), Giza, Egypt.

Fungus pathogen strain

The pathogen was , *Pythium ultimum* was used in these experiment namely soil-borne fungi. The standard culture of this fungi was obtained from Agric. Res. Center (A.R.C), Plant Pathology Research Institute, Mycology Research& Plant Disease Survey Department, Giza, Egypt.

Host plant

Sugar beet (*Beta vulgaris* L.) cultivar Sultan provided by Sugar Crops Dis. Res. Dept., Plant Pathol. Res. Instit., Agric. Res. Center (A.R.C), Giza, Egypt.

***In vitro* experiment**

Antagonism between the isolated bacteria, *Trichoderma* spp. and the causal pathogen fungus

This experiment was carried out to study the relationship between the tested pathogenic fungs (*P. ultimum*) and bioagents according to (Ferreira *et al.*, 1991).

Identification of bacterial isolates

The isolates of bacteria were selected that gave comparable results *in vitro*. These bacterial isolates were identified by standard tests according to Bergy's Manual of Systematic Bacteriology (2005), and by the application of biolog system in the Cairo MIRCEN , Fac. of Agric. ASU. Egypt (Biolog ,2013).

Greenhouse experiment

Soil infestation technique

Glass bottles of 500 ml capacity containing 100 g barley grain and 100 ml water were autoclaved for 30 minutes at 1.5 atm, then inoculated with 7- day old pathogenic fungus culture and incubated at 28 + 1^oC for 15 days. Sandy-clay soil was prepared by mixing sand and clay (1: 2) and sterilizing by 5% formalin solution. The pots (35 cm diameter) supplied with 5 kg of the prepared soil were used. Infestation was carried out by fungs under the study at the rate of 2% of potted soil and the pots were moisted with water for one week before sowing.

Disease assessment

Readings of seedling and plant stands were taken at 15 and 45 days of planting**.** Disease assessment was carried out by record the percentage of pre, post-emergence damping-off after 15 and 45 days and survived plants after sowing**,** respectively as follow**:**

$$\text{Pre-emergence damping-off\%} = \frac{No.of\ non\ germinated\ seeds}{\text{Total cultivated seeds}} \times 100$$

$$\text{Post-emergence damping-off\%} = \frac{No.of\ dead\ seedling}{\text{Total cultivated seeds}} \times 100$$

$$\text{Survival plants\%} = \frac{No.of\ stand\ seedling}{\text{Total cultivated seeds}} \times 100$$

Seeds treatment and cultivation

Seeds of sugar beet was treated with bioagents by soaking. Bioagents bacterial or fungi antagonists ,were grown in shaking nutrient broth for three days for bacterial cultures or potato dextrose broth for five days for fungs cultures at at 28 + 1°C. After the incubation period, cultures were filtered through filter paper and centrifuged at 5000 rpm for twenty minutes. The supernatants were taken and used for soaking seeds. Soaking was done for overnight and seeds were immediately sown. Bacteria or fungs free media were incubated at the same conditions, the supernatant after centrifugation was used for soaking seeds as a control. While seeds coating were moistened with a volume of an aqueous solution of the bioagents sufficient to moist the seeds surface. Talc powder and few drops of solution from arabic gum assisted in coating seeds and air dried before planting. Seeds were cultivated in infested soil (10 seeds/pot). Three replicate pots (No. 35 cm diameter) were used and uninfested soil acted as a control (Singh and Mehrotra , 1980 & Kommedahl *et al.,*1981).

Detection of antagonistic compounds

1- Hydrogen Cyanide (HCN): Production of HCN was detected according to the method of Lorck (1948)

2- Indole Acetic Acid (IAA): Production of IAA was detected according to the method of Patten and Glick (2002).

3- Cellulase: Aerobic cellulose decomposition was determined using Dubos medium (Allen, 1959).

4- Chitinase: Colloidal chitin was prepared according modified method as described by Faramarzi *et al*., (2009).

Statistical analysis

The obtained data were subjected analysis of variance (ANOVA) (Steel and Terrie 1960). Duncan's multiple range test (MRT) was applied for comparing means under the study (Duncan, 1955).

RESULTS AND DISCUSSION

Antagonistic effect of different bacterial isolates against fungus pathogen under laboratory conditions

A twelve isolates of bacteria were tested *in vitro* antagonism against *P. ultimum* caused damping-off.

Table 1. Selecting of different bacterial isolates to antagonism against *Pythium ultimum* .

Bacterial isolates No.	*P. ultimum* Inhibition zone (mm)
1	0.0
2	0.0
3	0.0
4	0.0
5	0.0
6	0.0
7	0.0
8	1.2[d]
9	0.0
10	1.1[a]
11	0.43[b]
12	0.0
Control	0.0

Mean within a column with the same letter are not significantly different (P<0.05)

Three bacterial isolates (No. 8,10 & 11) (Table1) were gave better results for inhibition fungus pathogen (Sagahón *et al.,* 2011).

Data presented in Table (2) indicated that all *Trichoderma viride, T. harzianum* and *T. hamatum* were the most potent inhibitors to the growth of *P. ultimum* (Abo-Elnaga, 2014).

Table 2. Effect of *Trichoderma* spp. isolates on the growth *P. ultimum*

Trichoderma spp.	*P. ultimum*
T. viride	++
T.harzianum	++
T. hamatum	++

(++) inhibition of pathogen: by over growth

Identification of bacterial isolates

Data in Table (3) showed three isolates of bacteria were identified by morphological and biochemical characteristics tests. The isolates (No. 8,10 &11) belonging to *Bacillus* spp.

Table 3. Some morphological and biochemical characteristics of the effective biocontrol bacterial isolates

Tests	Bacterial isolates No.		
Morphological characters	**8**	**10**	**11**
Gram stain	+	+	+
Spore forming	+	+	+
Motility	+	+	-
Capsule formation	-	-	-
Measurement (µm)	(4 x 1.2)	(1.5x(3-4))	(4x1)
Biochemical characters			
Indole production	-	-	-
Voges- proskauer test	+	+	+
Methyl Red test	+	+	+
Citrate utilization	+	+	+
Catalase production	+	+	+
Starch hydrolysis	+	+	+
Casein hydrolysis	+	+	+
Gelatin liquefaction	+	+	+
Cellulase production	-	-	-
Sugar assimilation			
Glucose	+	+	+
Mannitol	-	-	+
Sucrose	+	+	+
Fructose	+	-	+
Lactose	-	-	-
Dextrin	-	-	-
Xylose	-	-	-
Glycerol	-	-	-

Identification of bacterial isolates by biolog system

After identification of the bacteria by morphological and biochemical methods according to Bergey's Manual of systematic Bacteriology (2005). Results in Table (4) shown the scientific name of three bacterial isolates (No. 8 ,10& 11) that the most effective towards fungal pathogen.

Table 4.Scientific name of bacterial isolates .

Bacterial isolates No.	Scientific name
8	*B. amyloliquefaciens*
10	*B. pseudomycodies*
11	*Bacillus* sp .

Greenhouse experiments

In greenhouse conditions, statistical analysis of data causes significant differences in pre-and post-emergence damping –off and also, survival plants for two methods of seed treatments. All of the tested bioagents for all methods applications are effective in reducing pre- and post-emergence damping-off; and increased survival plants caused by *Pythium ultimum*. A number of three bacterial bioagents shown in Table (5) which chosen for two seed treatments methods were effective in reducing pre- and post-emergence damping-off , and increased survival plants caused by *Pythium ultimum* of sugar beet. Also, data in (Table 5) indicated that seed soaking with *B. amyloliquefaciens* was the most effective in controlling disease, hence it gave the highest survival plants (80.00%), followed by *B.pseudomycodies*, *T. viride* and *T. hamatum* (66.67%, 60.00% and 53.33% % survival plants, respectively).On the other hand *Bacillus* sp. and *T. harzianum* were the lowest in controlling damping – off it recorded the lowest survival plants with the same percent (23.33%) compared with the control (16.67 %). As shown in Table (5) seed coating with tested by *T .harzianum* and *B.pseudomycodies* were the most effective in controlling damping- off hence gave the highest percentage of survival plants (86.67% and 80.00% respectively, followed *B. amyloliquefaciens* 73.33% survival plants). On the other hand *T. hamatum*, *Bacillus* sp. and *T. viride* were the lowest in controlling damping – off disease gave the lowest survival plants (40.00%, 46.67% and 50.00% respectively compared with the control (26.67% survival plants) in soil infested with *Pythium ultimum* .

Table 5. Effect of bioagents with two methods of seed application on controlling sugar beet damping – off disease caused by *Pythium ultimum* in greenhouse conditions .

Bioagents	Seed soaking			Seed coating		
	Damping- off %		Survival %	Damping- off %		Survival %
	Pre- emergence	Post- emergence		Pre- emergence	Post- emergence	
B. amyloliquefaciens	6.67^{d}	13.33^{b}	80.00^{a}	10.00^{c}	16.67^{abc}	73.33^{a}
B.pseudomycodies	13.33^{cd}	20.00^{ab}	66.67^{b}	6.67^{c}	13.33^{bc}	80.00^{a}
Bacillus sp.	43.33^{ab}	33.33^{a}	23.33^{a}	30.00^{ab}	26.67^{ab}	46.67^{b}
T. viride	20.00^{cd}	20.00^{ab}	60.00^{bc}	20.00^{bc}	30.00^{a}	50.00^{b}
T. harzianum	50.00^{a}	26.67^{ab}	23.33^{d}	3.33^{c}	10.00^{c}	86.67^{a}
T. hamatum	26.67^{bc}	23.33^{ab}	53.33^{c}	33.33^{ab}	26.67^{ab}	40.00^{bc}
Control	53.33^{a}	30.00^{ab}	16.67^{d}	43.33^{ab}	30.00^{a}	26.67^{c}

In the same column, means followed by the same letter are not.significantly different at 5% level.

The results in the greenhouse are in agreement with Jorjani *et al.(* 2012);Eid ,2014 & Naher*et al.*(2015).Beet root rot was found also, throughout the present investigation to be affected by bioagent treatments. It was reported that treatment seed with biocontrol agents is the most effective and economical method of introducing the bioagents against seed and soilborne pathogens. They prevent seed decay, seedling blight or pre-emergence damping off diseases. *Trichoderma* spp. and *Bacillus subtilis* were successfully used by several investigators to control some major diseases that affect field crops such as sugar beet by seed treatments Abo-Elnaga (2014).

Microbiological parameters for antagonistic

Production of HCN, IAA and enzymes by bioagents .

Data presented in Table (6) indicated that only two bacterial isolates which most effective were all negative for HCN, and cellulase. Similar results were obtained Singh *et al*. ,(2008). All bacterial isolates were positive for IAA and chitinase . In the case of *Trichoderma* as bioagents , reported that all isolates were positive for HCN and IAA. While negative for both enzymes cellulase and chitinase. The results are agreement with Ashour and Afify, (2017) ; Bayoumy *et al*., (2017) and Afify *et al.* ,(2017).

Table 6. Production of antagonistic properties from bacteria and fungi.

Microorganisms	HCN	IAA	Chitinase	Cellulase
B. amyloliquefaciens	-	+	+	-
B. pseudomycodies	-	+	+	-
T.viride	+	+	-	-
T. harzianum	+	+	-	-
T. hamatum	+	+	-	-

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المقاومة الحيوية لمرض موت البادرات فى نبات بنجر السكر

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يعتبر فطر بيثيم التيمم من الفطريات المرضيه الهامه التى تسبب موت البادرات فى بنجر السكر.وخلال هذه الدراسه تم عزل ١٢ عزله بكتيريه من منطقة الريزوسفير لنباتات بنجر السكر السليمه فى مصر بالإضافة الى ثلاث أنواع من فطر التريكودرما ، تم اجراء التضاد الحيوي فى المعمل للفطر المسبب لمرض موت البادرات لبنجر السكر مع العزلات البكتيريه وانواع فطر التريكودرما. وأشارت النتائج في المعمل أن جميع أنواع التريكودرما أعطت نتائج مماثله وأن ثلاث عزلات فقط من البكتريا أظهرت التضاد لفطر بيثيم التيمم .تم تعريف أكفأ عزلات البكتيريا بالإختبارات القياسيه وقد وجد ان هذه البكتريا تتبع جنس الباسيلس وأن عزلتان فقط تتبع النوعين باسيلس اميلوليكوفكشن و باسلس سيدوميكودس. وعند تطبيق إختبار المقاومه الحيويه فى الصوبه للسلالات البكتيريه والفطريه بطريقتى النقع والتغليف لبذور البنجرأظهرت السلالات نتائج متباينه فكانت كالتالى :أعطت بكتريا باسيلس اميلوليكوفكشن أعلي نسبه بطريقة النقع يليها باسلس سيدوميكودس ثم تريكودرما فيردي ثم تريكودرما هماتم و تريكودرما هرزيانم وكانت النسبه كالأتى (٨٠% - ٦٦.٦٧%- ٦٠% - ٥٣.٣٣%- ٢٣.٣٣%) علي التوالي. بينما بطريقة التغليف أعطت تريكودرما هرزيانم أعلي نسبه يليها باسلس سيدوميكودس وباسيلس اميلوليكوفكشن وتريكودرما فيردى و نوع باسلس و تريكودرما هماتم وكانت النسبه (٨٦.٦٧% -٨٠% - ٧٣.٣٣% -٥٠% - ٤٦.٦٧% - ٤٠%) على التوالى. وعند الكشف عن مواد التضاد البكتيريه والفطريه أظهرت النتائج أن البكتريا لها القدره علي إنتاج إندول حمض الخليك وإنزيم الكيتينيز بينما لاتستطيع إنتاج سيانيد الهيدروجين وإنزيم السيلوليز. اما بالنسبه لسلالات التريكودرما فإن لها القدره علي إنتاج سيانيد الهيدروجين وأندول حمض الخليك بينما لاتستطيع إنتاج إنزيمى الكيتينيز والسليلوليز.