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Bacillus subtilis as a Biocontrol Agent for Controlling Sugar Beet Damping-off Disease

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Pythium ultimum is an important soil borne pathogen causing damping-off of sugar beet. Ten fungal isolates were obtained from naturally infected roots of sugar beet. All isolates proved to be pathogenic on Demapoly cultivar of sugar beet. The fungus usually attacks the germinated seeds and seedlings causing pre-and post-emergence damping-off. *Bacillus subtilis* isolated from rhizosphere of sugar beet plants could partially protect the plants against the infection with the isolated pathogens. Antibiosis tests against *Pythium ultimum* showed that the two bacterial strains of *Bacillus subtilis* examined restricted the mycelial growth of five isolates of *Pythium ultimum in vitro*. The usage of *Bacillus subtilis* as seed treatment reduced the percentage of damping-off incidence of sugar beet under greenhouse conditions.

Key words: *Bacillus subtilis*, biological control, damping-off, *Pythium ultimum* and sugar beet.

Sugar beet (*Beta vulgaris L.*) is the second important crop for sugar production in Egypt. *Pythium ultimum* is one of the most soil-borne pathogens which attacks sugar beet crop in Egypt as well as other countries causing pre and post-emergence damping-off (Baker and Rush, 1988; Osburn and Schroth, 1988; Charles, 1991; Abada, 1995; Brantner and Carol, 1998; Nielsen *et al.*, 1998 and Georgakopoulos *et al.*, 2002). The use of biological control in plant disease became promising instead of chemicals for avoiding environmental pollution.

Several investigators reported the use of biological control against soilborne diseases (Rath and Wolf, 1992; Bowers and Park 1993; Harris *et al.*, 1994; Saleh, 1997 and Weller, 1998). Biocontrol of damping-off diseases has been successfully applied using *B. subtilis* (Berger *et al.*, 1996; Harris and Adkins, 1999; Georgakopoulos *et al.*, 2002 and Schmidt *et al.*, 2004).

The aim of this work was to screen some isolates of *B. subtilis* from the rhizosphere of sugar beet plants as a bioagents for controlling damping-off disease.

Materials and methods

Isolation and identification trials:

Naturally infected sugar beet seedlings showing damping-off symptoms were collected from several fields at Assiut Governorate. Isolation of the causal pathogens were carried out using small pieces of infected roots which were previously washed in running tap water, surface sterilized with 1% sodium hypochlorite solution for two minutes washed three times with sterilized water and dried between sterilized

filter papers. The surface sterilized root pieces were then placed into Petri dishes containing PDA medium, then incubated at 20°C for seven days. Preliminary identification of the growing fungi were carried out according to (Domsch *et al.*, 1980 and Moubasher *et al.*, 1988) and then confirmed by Assiut University Mycological Centre (AUMC).

Pathogenicity test:

Ten pure fungal isolates of *Pythium ultimum* were tested for their pathogenic capabilities. Inocula of the isolated fungi were prepared in Roux bottles (1000 ml), each containing 100 ml of PDA medium, were seeded with mycelial disks from a five days old culture of *Pythium ultimum* and incubated for 14 days at 20°C, then suspended in sterile water and homogenized in a blender for 5 min. Earthen pots (25cm-diameter) filled with loamy sand soil (84% sand : 14.5% clay : 1.5% silt) were used. Soil infestation was carried out 7 days before sowing by adding ten ml of the homogenized culture containing 5×10^6/ ml of fungal propagules to 1kg of soil and mixed thoroughly. Each pot was filled with 3kg sterilized autoclaved soil. Five pots were used for each tested isolate. Seeds of the tested Demapoly cultivar were sown after disinfection with 2% sodium hypochlorite solution for two minutes at the rate of 10 seeds/pot. Irrigation was performed when necessary. The percentage of pre- and post-emergence damping-off was calculated after 15 and 30 days, respectively. Uninoculated soil was served as control.

Re-isolation of the pathogenic fungal isolates from diseased plants was carried out to meet Koch postulates.

Isolation and identification of Antagonist:

Bacillus subtilis isolates which isolated from the rhizosphere of sugar beet plants cultivar Demapoly were tested for their antagonistic activity against pathogenic fungi according to the method described by Skinner *et al.* (1952); Breed *et al.* (1957) and Louw and Webely (1959).

The method described by Anonymous (1984) was used to identify the isolated bacteria using the following tests, shape of cells, motility, Gram's staining, aerobiosis, starch hydrolysis, gelatine liquefaction, nitrate reduction, acetyl methyl carbinol production (VP test) fermentation, reactions with mannitol, glucose, sucrose, arabinose, xylose and lactose and pigment production.

Antagonism between the isolated bacteria and the causal pathogen:

A- In vitro:

The antagonism between the isolated bioagent *Bacillus subtilis* and the causal pathogen was tested *in vitro* using three different growth media, *i.e.* tryptic soybean agar medium (TSA); Potato Dextrose Agar medium (PDA) and Soil Extract Agar medium (SEA). Five isolates of *P. ultimum* and two isolates of *B. subtilis* were used in this test. Petri dishes containing 10 ml of the tested medium were inoculated with equal disks (7-mm-diam.) of *Pythium ultimum* obtained from 5 days old culture which placed at the periphery of the plate. The antagonistic bacterium (*B. subtilis*) was streaked at the centre of each plate by a loop loaded with 48 hr old bacterial culture grown at 27°C on nutrient broth. Three replicates were used for each

particular treatment. Antagonistic effect was determined by measuring the longest and shortest free growth zone between the antagonistic bacteria and the tested fungi. Petri dishes inoculated with *Pythium ultimum* only were served as control check.

B- In vivo:

Earthen pots (25cm diameter) were filled with soil preinfested with the causal pathogen as mentioned before. At the same day of sowing, surface sterilized seeds were soaked for one hour before sowing in a 3-day-old culture of *Bacillus subtilis* (10^8-10^9cfu/ml) grown in liquid TS medium.

Ten seeds were sown in each pot and five replicates were used for each treatment. Surface sterilized un-soaked seeds were served as a control check. Assessment of disease incidence was calculated as a percentage of pre-, and post-emergence damping-off after 15 and 30 days of sowing. These experiments were carried out under greenhouse conditions for two successive growing seasons 2003/2004 and 2004/2005.

Statistical analysis:

The obtained results were statistically analyzed according to Gomez and Gomez 1984.

Results and Discussion

Isolation and identification trials:

Ten different isolates of *Pythium ultimum* were isolated from diseased sugar beet plants collected from different locations at Assiut Governorate, *i.e.* Assiut, Manfulate and Ibrahim Sons. Isolates were identified as *Pythium ultimum* according to (Domsch *et al.*, 1980 and Moubasher *et al.*, 1988). Identification was confirmed by Assiut University Mycological Centre (AUMC).

Pathogenicity tests:

Isolates of *Pythium ultimum* were tested for their pathogenic capability against sugar beet cv. Demapoly. Data presented in Table (1) indicate that all tested fungal isolates were able to cause symptoms of pre-emergence and post-emergence damping-off of sugar beet seedlings.

Data also indicate that isolates Nos. 1, 3 and 4 showed high virulence, while isolates Nos. 6,7,8 and 10 was moderately virulent to sugar beet seedlings. Isolates Nos. 2, 5 and 9 was consider as weak virulent.

Moreover, the tested isolates varied regarding their virulence. Variation in virulence of the pathogenic isolates on sugar beet plants may be due to the presence of genetic differences among the fungal isolates. These results are also in harmony with the results of William and Asher, 1996.

The antagonistic effect of Bacillus subtilis against the growth of Pythium ultimum on different media:

Pythium ultimum isolates Nos. 1, 3 and 4 which previously proved to be high pathogenic were used in this test.

Table 1. Pathogenicity test of different isolates of *Pythium ultimum* on sugar beet cv. Demapoly

Fungal isolate	Damping-off (%)		Healthy survivals
	Pre-emergence	Post-emergence	
1	58	20	22
2	30	18	52
3	56	20	24
4	64	20	16
5	30	14	56
6	30	20	50
7	46	10	44
8	50	20	30
9	30	14	56
10	40	10	50
Control	0	0	100
L.S.D 5%	5.41	4.39	6.54
1%	7.24	5.88	8.74

Data in Table (2) reveal that the inhibition zone area fluctuated significantly in response to either antagonists strains or growth media. As for media, it is clear that TSA medium enhanced antagonistic effect of bacterial isolates against fungal isolates, that the largest inhibition zone area was recorded between 10-12 mm. The observed effect was also recorded for PDA and SEA media in descending order. Data also show that isolates of *P. ultimum* varied in their response against the antagonistic effect of *B. subtilis* isolates B_1 and B_2. The variation in the inhibitory effect of the bioagent against the tested pathogen with the use of different media may be due to that the fungal and/or bacterial growth depends on its growth on nutrients available on the characteristic media (Maurhofer *et al.*, 1995).

Biological control of sugar beet damping-off under greenhouse conditions:

The efficacy of using two *B. subtilis* isolates as biocontrol agent against damping-off incidence of sugar beet was evaluated under greenhouse conditions. Application of bioagents was carried out as seed dressing at the same sowing date. Three isolates of *P. ultimum* were used individually for soil infestation one week before sowing. At the first cultivation season 2003/2004, data in Table (3) show that the introduced bacterial isolates could significantly reduced the percentage of damping-off incidence for the three tested pathogenic fungal isolates. *P. ultimum* isolates Nos. 1 and 3 showed more sensitive response than isolate No. 4 to the introduced isolates of bacteria. Data also showed that *B. subtilis* isolate No. 1 showed superior effect comparing with isolate No. 2 on the pathogenic fungal isolates which reflected on damping-off incidence.

Table 2. Effect of different media on the antagonistic effect of *B. subtilis* against the growth of *P. ultimum in vitro*

Fungal isolate	Tested bioagent	Average inhibition zone (mm)			
		Media			Mean
		T.S.A	P.D.A	S.E.A	
1	B_1	11	6	3	6.3
	B_2	12	8	2	7.3
	Mean	11.5	7	2.5	6.8
3	B_1	10	7	1	6
	B_2	10	5	1	5.3
	Mean	10	6	1	5.6
4	B1	11	7	2	6.3
	B2	10	7	2	6.3
	Mean	10.5	7	2	6.3
5	B_1	10.6	6.6	2	6.2
	B_2	10.6	6.6	1.6	6.3
	Mean	10.6	6.6	1.8	6.2

LSD at:	5%
Isolate	0.276
Media	0.370
Bioagent	0.370
IXM	0.478
IXB	0.478
MXB	0.585
IMB	0.828

Table 3. Effect of *B. subtilis* as a seed treatment on sugar beet damping-off caused by *P. ultimum* during the growing seasons 2003/2004

Bacillus subtilis isolates	Damping off %							
	Pythium ultimum isolates							
	Pre- emergence				Post- emergence			
	Isolate No.1	Isolate No.3	Isolate No. 4	Mean	Isolate No.1	Isolate No.3	Isolate No. 4	Mean
B_1	12	12	40	21.3	16	4	2	7.3
B_2	30	26	24	26.6	0	0	20	6.6
Control	56	34	60	50	18	14	22	18

LSD 5%	Isolate	Bioagent	Isolate× Bioagent	Isolate	Bioagent	Isolate×Bioagent
	3.3	3.2	5.6	4.1	4.8	7.0

Similar trend was also observed during the second growing season 2004/2005 (Table 4). In this regard, Saleh (1997) stated that the addition of antagonists to the contaminated soil could influence the growth of pathogenic fungi.

Biological control of plant pathogens aims to minimize the inoculum density of the pathogen and reduce the disease incidence (Berger *et al.*, 1996 and Harris *et al.*, 1994). The antagonistic effects of *Bacillus subtilis* may be attributed to the competition which occurs between the two organisms require the same nutrients and the use of these nutrients by one reduce the amount available to the other.

Also the antagonistic effect may be attributed to toxins or antibiotics secreted in the growth medium as well as the production of antifungal volatiles (Lifshitz *et al.*, 1986; Elad and Chet, 1987; Fiddaman and Rossal, 1993; Lefiert *et al.*, 1995; Saleh, 1997 and Walker *et al.*, 1998).

Table 4. Effect of *B. subtilis* as a seed treatment on sugar beet damping-off caused by *P. ultimum* during the growing seasons 2004/2005

Bacillus subtilis isolates	Damping off (%)							
	Pythium ultimum isolates							
	Pre-emergence				Post-emergence			
	Isolate 1	Isolate 3	Isolate 4	Mean	Isolate 1	Isolate 3	Isolate 4	Mean
B_1	10	10	38	19.3	14	4	4	7.3
B_2	26	20	22	22.6	0	0	18	6.6
Control	52	30	48	43.3	16	12	16	14.7
LSD	Isolate	Bioagent	Isolate× Bioagent		Isolate	Bioagent	Isolate×Bioagent	
5%	4.2	4.1	7.2		5.9	5.8	10.1	
1%	5.6	5.6	9.7		7.8	7.8	13.6	

Reference

Abada, K.A. 1995. Chemical control of sugar beet damping-off and root-rot diseases. *Egypt. J. Phytopathol.*, 33: 1-8.

Baker, E.M., and Rush, C.M. 1988. Reaction of selected sugar beet varieties exposed to two pathogenic *Pythium* spp. *Phytopathology*, 78: 1566. (Abstr.).

Berger, F.; Hong, Li.; White, D.; Frazer, R. and Lieifert, C. 1996. Effect of pathogen inoculum, antagonist density, and plant species on biological control of Phytophthora and Pythium damping-off by *Bacillus subtilis* cot 1 in High-humidity fogging glasshouses. *Phytopathology*, 86: 428-433.

Anonymous, 1984. *Berggey's Manual of Systematic Bacteriology*. Amer. Soc. J. Met. The Will and Wilk Co., Baltimore, USA.

Bowers, J.H. and Park, J.L. 1993. Epidemiology of *Pythium* damping-off and *Aphanomyces* root-rot of peas after seed treatment with bacterial agents for biocontrol. *Phytopathology*, **83**: 1466-1473.

Brantner J.R. and Carol, E.W. 1998. Variability in sensitivity to metalaxyl *in vitro* pathogenicity and control of *Pythium* spp. on sugar beet. *Plant Dis.*, **82**: 896-899.

Breed, R.S.; Murray, E.G.D. and Smith, N.R. 1957. *Bergey's Manual of Determinative Bacteriology*. 7th Ed. Williams Wilkins Company, 1094 pp.

Charles, M.R. 1991. Comparison of seed priming techniques with regard to seedling emergence and Pythium damping-off in sugar beet. *Phytopathology*, **81**: 878-882.

Domsch, K.H.; Gams, W. and Traute-Heidi A. 1980. *Compendium of Soil Fungi*. Academic Press. A Subsidiary of Harcourt Brace Jovanovich Publishers, London. 859pp.

Elad, Y. and Chet, I. 1987. Possible role of competition for nutrients in biocontrol of *Pythium* damping-off by bacteria. *Phytopathology*, 77: 190-195.

Fiddaman, P.J. and Rossal, S. 1993. The production of antifungal volatiles by *Bacillus subtilis*. *J. Appl. Bacteriol.*, **74**: 119-126.

Georgakopoulos, D.G.; Fiddaman, P.; Leifert, C. and Malathrakis, N.E. 2002. Biological control of cucumber and sugar beet caused by *Pythium ultimum* with bacterial and antagonists. *J. Appl. Microbiol.*, **92**: 1078-1086.

Gomez, K.A. and Gomez, A.A. 1984. *Statistical Procedures for Agricultural Research*. John Wiley & Sons, Inc. New York, U.S.A. 680pp.

Harris, A.R. and Adkins, P.G. 1999. Versatility of fungal and bacterial isolates for fungal and bacterial isolates for control of damping-off disease caused by *Rhizoctonia solani* and *Pythium* spp. Biological control: theory and applications in pest management. *Biol. Control*, **15**: 10-18.

Harris, A.R.; Schisler, D.A.; Ryder, M.H. and Adkins, P.G. 1994. Bacteria suppress damping-off caused by *Pythium* var. *sporangiiferum* and promote growth. *Soil biol. Biochem.*, **26**: 1437.

Lefiert, C.; Li. H.; Chidburee, S.; Hampson, S.; Workman, S.; Digel, D.; Epton, H.A.S. and Harbour, A. 1995. Antibiotic production and biocontrol activity by *Bacillus subtilis* CL 27 and *Bacillus pumilus* CLAS. *J. Appl. Bacteriol.*, **78**: 97-108.

Lifshitz, A.R.; Windham, R.M.T. and Beker, R. 1986. Mechanism of biological control of pre-emergence damping-off of pea. *Phytopathology*, **76**: 720-725.

Louw, H.A. and Webely, D.W. 1959. The bacteriology of root region of the oat plant grown under controlled pot culture conditions. *J. Appl. Bacteriol.*, **22**: 216-226.

Moubasher, A.H.; Mazen, M.B. and El-Sharouny, H.M. 1988. Studies on the genus *Pythium* in Egypt. II. Records of *Pythium* and other root infecting fungi in Egyptian soil. *Bull. Fac. Sci. Assiut Univ.*, 17: 19-27.

Nielsen, M.N.; Sensen, J.; Fels, J. and Pedersen, H.C. 1998. Secondary metabolite and endochitinase-dependent antagonism toward plant pathogenic Microfungi of *Pseudomonas fluorescens* isolates from sugar beet rhizosphere. *Appl. Environ. Microbiol.*, 64: 3563-3569.

Osburn, R.M. and Schroth, M.N. 1988. Effect of sugar beet seed on germination rate and incidence of *Pythium* damping-off. *Plant Dis.*, 73: 21-24.

Rath, M. and Wolf, G. 1992. Biological control of seed rot and damping-off of sugar beet with microbial antagonists. *IOBC/WPRS Bulletin*, 1: 113-115.

Saleh, O.I. 1997. Wilt, root rot and seed diseases of groundnut in El-Minia Governorate, Egypt. *Egypt. J. Phytopathol.*, 25: 1-18.

Schmidt, C.S.; Agostini, F. Leifert, C. Killham, K. and Mullins, C.E. 2004. Influence of soil temperature and metric potential on sugar beet seedling colonization and suppression of *Pythium* damping-off by the antagonistic bacteria *Pseudomonas fluorescens* and *Bacillus subtilis*. *Phytopathology*, 94: 351-363.

Skinner, F.A.; Jones, P.G. and Molison, J.E. 1952. A comparison of a direct and a plate counting technique for quantitative counts of soil microorganism. *J. Gen. Microbiol.*, 6: 261-271.

Walker, R.; Powell, A.A. and Seddon, B. 1998. *Bacillus* isolates from the spermosphere of peas and French beans with antifungal activity against *Botrytis cinerea* and *Pythium* species. *J. Appl. Microbiol.*, 84: 791-801.

Weller, M. 1998. Biological control of soil borne plant pathogens in the rhizosphere with bacteria. *Ann. Rev. Phytopathol.*, 26: 379-407.

William, G.E. and Asher, M.J.C. 1996. Selection of rhizobacteria for the control of *Pythium ultimum* and *Aphanomyces cochlioides* on sugar beet seedlings. Crop Production. *Crop Protect.*. 15: (5) p. 479-486

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استخـــــدام بكتريـــــا باسيليـــــس ساتيليـــــس كمبيـد حيـــــوى لمقاومة مرض سقوط البادرات فى بنجر السكر

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يعتبر فطر البيثيوم التيمم من الفطريات الممرضة الهامة الموجودة فى التربة. وقد تم عزل الفطر من نباتات بنجر مصابة طبيعياً. وعند إجراء اختبار القدرة المرضية لعدد عشرة عزلات وجد أن هذه العزلات لها القدرة على إحداث الإصابة لبذور وبادرات البنجر السليمة. وقد اختلفت العزلات فى قدرتها على إحداث الإصابة وتراوحت بين عزلات ضعيفة، متوسطة وعالية الإصابة. وتم عزل البكتريا من ريزوسفير نباتات مصابة والتى تم تعريفها على أنها باسيليس ساتيليس وتم استخدام عزلتين منها فى المقاومة البيولوجية وذلك بعد تقييمها معمليا ضد ثلاث عزلات فطرية. وأثبتت قدرتها على احداث تثبيط لنمو العزلات الفطرية. أعطت بيئة مستخلص فول الصويا TSA فى وجود بكتريا باسيليس ستيليس أعلى تثبيط لنمو فطر البيثيوم التيمم بينما كانت بيئة مستخلص دكستروز البطاطس PDA متوسطة التأثير بينما أظهرت بيئة مستخلص التربة SEA أقل فاعلية.

وعند إجراء اختبار المقاومة البيولوجية تحت ظروف الصوبة وجد أن بكتريا باسليس ساتيليس قد خفضت فى نسبة موت البادرات قبل وبعد ظهورها فوق سطح التربة وذلك عند استخدامها فى صورة معاملة بذور قبل الزراعة.