Biological and Physiological Control of *Pythium ultimum* Preemergence Damping-off Disease in Sweet Sorghum

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ABSTRACT

Of 32 *Pseudomonas fluorescens* isolates were isolated from the rhizosphere and seedlings of sweet sorghum cultivated in Nubaria soils. All isolates were tested for their ability to inhibit mycelial growth of *Pythium ultimum*, in laboratory and to suppress damping-off in the field. In the field experiments, the isolates NU25, NU28, NU16, NU22, NU14 and NU26 were effective in reducing *Pythium* damping-off and increasing seedling survival compared with untreated seeds. The isolate NU 25 of *P. fluorescens* provided superior seed protection from *Pythium* damping-off in naturally infested soils, and significantly increased the yields of all traits above the other five isolates. In the first greenhouse trial, when applied to dry seed 5.1 $\log_{10}$ cfu seed^{-1} of *P. fluoresens* NU 25 increased the emergence of sorghum over that of the control. In the second greenhouse trial, when seed hydration was combined with bacterial seed inoculation, seedling emergence in cold greenhouse soil was greatly increased over that of the control. Application of *P. fluorescens* NU 25 to dry seed was considerably less effective than seed bacterization with hydration treatment. This treatment provided protection against damping-off as good as seed treatment with captan when seeds were planted in cold soil. This process may be interest and value to growers who wish to planting sweet sorghum or other temperature sensitive crops into cold soil where damping-off is a problem and the use of chemical seed treatment is not desired.

INTRODUCTION

Sweet sorghum (*Sorghum bicolor* L. Moench) had been succefully used as a syrup crop. In Egypt, it is subjected to study the possibility to replace sugar cane for syrup production and this will spare a large area of sugar cane for sugar production. Since the 1980's, Sugar Crops Research Institute began to build up sorghum genotype collection and evaluating them for evolving commercial cultivars suitable to the agro-climatic conditions of the country. The genotype S 301 is characterized by poor stand and low seedling vigor in comparison with standard sweet sorghum.

Seed of sweet sorghum, like that of several other warm-season crops such as soybeans (*Glycine max* (L.) Merr.) and cotton (*Gossypium hirsutum* L.) is subjected to imbibitional chilling injury, a physiological disorder that occurrs under water uptake begins under low temperature (Cohn and Obendrof, 1976). Stand failure in sweet sorghum has been associated with susceptibility to pre-emergence damping-off caused by *Pythium* spp., especially when seeds are planted in cold soil (Guzman *et al.*, 1983). The most practical methods of controlling *Pythium* damping-off is by chemical, physiological, or biological seed

treatment. To overcome imbibitional injury of sweet sorghum, hydrating presown seed (Bennett and Waters, 1987) and osmotic conditioning or osmopriming (Bradford, 1986) have been recommended.

The aim of this work are to isolate one or more naturally occurring rhizobacteria that could be applied to sweet sorghum seeds for protection from *Pythium* pre-emergence damping-off and to delivery system that would provide consistent control of this disease in cold soil.

MATERIALS AND METHODS

Isolation and characterization of potential seed bioprotectants:

Isolates of *Pythium ultimum* were obtained from infected sweet sorghum seeds planted at the Agricultural Research Station Farm in Nubaria. Cultures were maintaned on commeal agar (CMA). Flats of soil were sown with seeds of sweet sorghum (S 301) and incubated at 25° C for 96 hr. After removal of the larger soil particles and the coleoptile, each seedling with adhering seed remnant was placed in a 5 ml sterile water blank. Plugs of *P. ultimum* (2 mm diam.) were incubated for 48 hr at 25° C in small culture dishes containing 4 ml of commeal broth (CMB) according to (Dhingra and Sinclair, 1985). The resultant mycelial mats were then transferred individually to the tubes containing seedlings, shaken thoroughly, and incubated at 25° C for 16 hr. Mycelial mats were removed, rinsed in strile water, and sonicated for 5 min in 100 ml of sterile water to release any attched bacteria. Serial dilutions were placed on CMA, potato dextrose agar (PDA) or one-tenth-strength tryptic soy agar (TSA) and incubated at 25° C for 96 hr, when individual colonies were selected. The selected isolates were streaked, six per plate, around the edge of PDA plates, and a 2 mm plug of *P. ultimum* was placed in the center of each plate. After 36 hr, plates were examined for a zone of inhibition around the bacterial colony or for apparent lysis of hyphae in the area of contact. Selected isolates were grown for 24 hr in 3 ml of King's medium, after which 2 ml of 80 % glycerol was added and cultures were stored at -5° C until further testing.

Greenhouse tests:

Two greenhouse trials involving 32 bacterial isolates were used to further delimit potential bioprotectans for field testing. Bacterial isolates were grown on PDA medium for 48 hr at 25° C. One plate of each isolate was scraped into 4 ml of 1.5 % methylcellulose (MC), medium viscosity. Sixty seeds of the genotypt S301, previously surface disinfested for 5 min in 0.25 % NaOCl, were coated with the bacterial suspension and allowed to dry for 20 hr (Juhnke *et al.*, 1987). Twelve seeds were planted 2 cm deep in one row of a 72 cell flat in a randomizd complete block design with four replications. Flats contained clay loam soil naturally infested with 1,000 propagules g^{-1} of *Pythium ultimum*. Populations of *P. ultimum* were dtermined by the method of Ali-Shtayeh *et al.*, (1986). The trial

included surface disinfested, non-treated seeds and seeds treated with methylcellulose and captan (1.0 g a.i. Kg^{-1} seed). Soil temperature was 25/10 (max/min).

Field testing of bacterial isolates:

The six most consistently performing isolates were selected from 32 bacterial isolates for further testing in the field in the Libean farm at Al-Nasser village, Nubaria during summer season of the 2002. Four PDA plates were scraped into 15 ml of MC and applied to 300 sorghum seeds of the genotype S 405. The plot area was 15 m (5 m^2 long and 3 m wide) and each plot had six rows. Seed was planted by hand, 3 cm deep and 20 cm apart, in a randomized complete block design with three replications. Planting dates were 15 May, 15 June, and 15 July. Treatments were included surface disinfested seed treated with captan, or MC alone and surface disinfested non-treated seeds. Soil temperature at 5 cm deep was measured. At dough stage, the plants were harvested using the four middle rows of each plot for each planting date. Stripped stalks were weighed to estimate the fresh yield per Kg and squeezed for determining juice yield. Sugar yield determined using the methods described in A.O.A.C. (1960). Analysis of varianc was conducted on data combined over the three dates of planting.

Levels of seed bacterization at planting were determined by dilution plating. Three replications of five seeds each were washed in 5 ml of 0.1 M phosphate buffer, pH 6.8 for 30 min with vortex agitation at 10 min intervals (Juhnke *et al.*, 1987). Then, 0.1 ml of the appropriate dilution was plated on one-tenth-strength TSA, and bacterial colonies were counted after two days.

Bacterization of dry seed:

Seeds of the sorghum genotype S 301 were treated with isolate NU 25 of *Pseudomonas fluorescens* suspended in MC to achive approximately $\log_{10}$ 3, 5, 7, 9 cfu $seed^{-1}$. Thirty six seeds were planted in 3 rows of a 72 cell flat of naturally infested clay loam soil in a randomized complete block design with 6 replications. Treatments were included surface disinfested seed treated with captan or MC, and surface disinfested non-treated seed. Soil temperature was 18/13 (max/min). Emergence was tabulated daily.

Seed bacterization with hydration:

Three hundred and fourty of S 301 sweet sorghum seeds (50 g) were coated with *P. fluorescens* NU 25 in MC as described above and either allowed to dry overnight or, after drying 2 hr, placed in 25 g of talc powder as a carrier and 50 ml of sterile water was added, all in plastic bag. Seeds were incubated for 20 hr at 22 C. At the end of this period, the seed moisture content was 30 %.

Seeds were planted in a 72 cell flats of naturally infested clay loam soil (800-1000 g^{-1} propagules of *P. ultimum*) as described above. Seeds treated with captan, or MC alone, MC-treated and non-treated hydrated seed, and surface disinfested hydrated seed were also included. Greenhouse temperature was 18/12 (max/min).

RESULTS AND DISCUSSION

Of 32 isolates of *Pseudomonas fluorescens* were isolated from the rhizosphere of field grown with sweet sorghun to select candidate strains for biological control of preemergence damping-off disease. Data in Table 1 show the efficiency percentage of 32 isolates of *P. fluorescens* against hyphal growth of *Pythium ultimum*. There were six isolates gave highly efficiency namley, NU25, NU28, NU16, NU22, NU14, and NU26. *P. fluorescens* NU 25 was identified as the most effective and consistently performing isolate.

Table 1. A list, code and efficiency percentage of the isolates *Pseudomonas fluorescens* in the laboratory.

No. of isolate	Isolate code	% efficiency	No. of isolate	Isolate code	% efficiency
1	NU 1	22	17	NU 17	46
2	NU 2	31	18	NU 18	35
3	NU 3	51	19	NU 19	43
4	NU 4	36	20	NU 20	35
5	NU 5	48	21	NU 21	54
6	NU 6	42	22	NU 22	78
7	NU 7	15	23	NU 23	47
8	NU 8	33	24	NU 24	36
9	NU 9	42	25	NU 25	85
10	NU 10	25	26	NU 26	76
11	NU 11	38	27	NU 27	26
12	NU 12	51	28	NU 28	81
13	NU 13	35	29	NU 29	45
14	NU 14	78	30	NU 30	55
15	NU 15	52	31	NU 31	33
16	NU 16	80	32	NU 32	56

In field experiment, soil temperature measured at 5 cm averaged 22.5/9.3° C (max/min) during the 7 days followed the 15th May planting, 25.4/13.6° C following the 15th June planting, and 30.2/18.9° C following the 15th July planting. When the six most effective isolates were applied to field planted seed, *P. fluorescens* NU 25 provided significant seed protection when compared with the surface disinfested control and the other five isolates (Table 2). At the

first planting date (15 May), which was conducted in cold soil, *P. fluorescens* NU 25 provided high protection than did the captan treatment. Various isolates of *P. fluorescens* have been found to be effective seed protectants against *Pythium* spp. In a number of crop species (Suslow and Schroth, 1982 and Loper, 1998). The isolate of *P. fluorescens* NU 25 was effective in protecting S 405 sweet sorghun genotype against *Pythium ultimum* preemergence damping-off under a range of soil conditions and temperature, as evidenced by the control it provided during various times of the year. Nelson, *et al.*, (1986) indicated that biological seed treatment may not provide adequate seed protection under all conditions. The applied bioprotectant may fail to establish on the seed protection under all conditions. The applied bioprotectant may fail to establish on the seed or in the rhizosphere at sufficient levels for disease control because of unfavorable soil temperature, pH, or moisture. Furthermore, biological control may be difficult to achieve with seeds releasing high level of exudates during germination.

Table 2. Biological of preemergence damping-off, caused by *Pythium ultimum* of sweet sorghum by treatment of dry seed with six isolates of *Pseudomonas fluorescens* under natural field conditions.

Treatment	Emergence (%)			Average
	15 May	15 June	15 July	
NU 25	76.6	84.2	93.0	84.60 a
NU 28	32.8	79.5	86.4	66.23 b
NU 16	18.6	75.9	78.2	57.57 c
NU 22	11.7	73.6	74.8	53.37 c
NU 14	10.5	71.5	72.9	51.63 c
NU 26	7.2	70.5	71.5	49.73 cd
Captan	5.4	63.8	64.6	44.60 cd
Carrier alone	3.6	56.5	57.0	39.00 de
Disinfested control	2.9	49.8	53.5	35.40 e

Data presented in Table 3 show the effect of seed bacterization of sweet sorghum with different isolates of *P. fluorescens* on yields (Kg/plot) of fresh stalks, juice and sugar at three planting dates. *P. fluorescens* NU 25 treatment significantly increased the yields of all traits above the other five isolates. This isolates gave high yields than did captan treatment.

Table 3. Effect of dry seed bacterization with six isolates of *Pseudomonas fluorescens* on yield traits (Kg/plot) of sweet sorghum at three planting dates during summer season of the 2002.

Treatment	Yield of								
	Stalks			Juice			Sugar		
	May	June	July	May	June	July	May	June	July
NU 25	32.51	36.68	42.55	8.16	10.56	11.58	1.08	1.33	1.74
NU 28	28.60	34.34	39.24	8.02	9.35	10.75	0.93	1.12	1.52
NU 16	21.81	30.16	32.10	7.11	7.30	8.52	0.86	0.89	1.12
NU 22	20.59	30.00	30.46	6.22	7.31	7.53	0.73	0.88	1.08
NU 14	21.37	28.25	30.96	6.50	7.72	8.18	0.80	0.96	1.16
NU 26	22.30	28.38	33.52	6.71	8.44	9.25	0.90	1.01	1.22
Captan	29.02	29.41	32.71	5.81	6.92	8.81	0.81	1.08	1.26
Carrier	19.05	20.22	21.93	3.55	4.18	5.85	0.63	0.75	0.89
Control	18.51	19.41	20.66	3.28	4.06	4.61	0.55	0.61	0.66
L.S.D0.05	5.36	3.18	4.26	0.84	2.14	1.68	0.18	0.23	0.28

In the first greenhouse trial, when applied to dry seed 5.1 $\log_{10}$ cfu seed^{-1} of *Pseudomonas fluoresens* NU 25 increased the emergence of sorghum over that of the control, but at least 7.4 $\log_{10}$ cfu seed^{-1} were needed for control of damping-off supperior to that provided by captan under the conditions of this greenhouse experiment (Fig. 1).

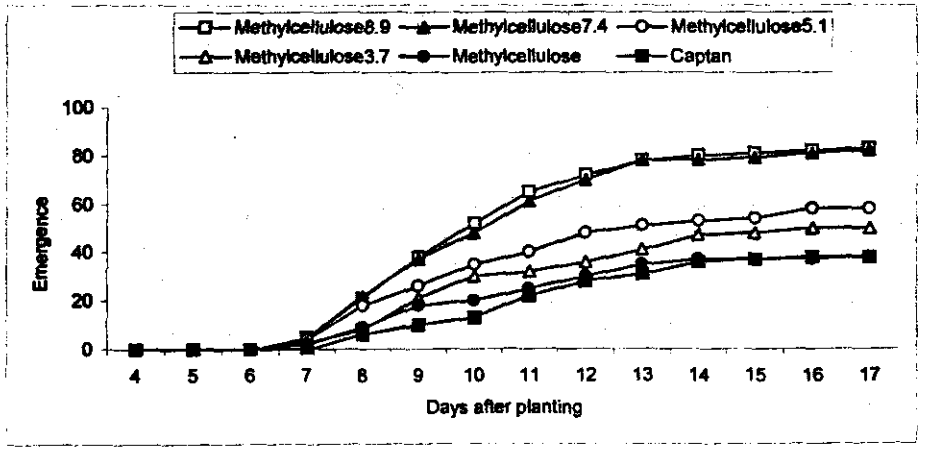

Fig.1. Biological control of preemergence damping-off, caused by *Pythium ultimum* of sweet sorghum by application of *Pseudomonas fluorescens* NU 25 to dry seed.

In the second greenhouse trial, when seed hydration was combined with bacterial seed inoculation, seedling emergence in cold greenhouse soil was greatly increased over that of the control (Table 4). Application of *P. fluorescens* NU 25 to dry seed was considerably less effective than seed bacterization with hydration treatment. At low temperature, imbibition increased by the poor emergence of captan treated seed and the increase in emergence after seed hydration alone. Bennett and Waters (1987) indicated that in seed bacterization with hydration treatment, temperature and moisture conditions during seed imbibition are optimized to eliminate imbibitional chilling injury, while a bacterial bioprotectant becomes established on the seed.

Table 4. Seed bacterization with hydration of sweet sorghum with *Pseudomonas fluoresens* NU 25 for control of damping-off, caused by *Pythium ultimum*, in naturally infested soil under grennhouse conditions.

Treatment	Emergence (%)
NU 25 seed bacterization with hydration	42.5 a
Hydrated only	21.9 b
NU 25 seed bacterization	9.7 c
Captan	2.6 c
Disinfested control	1.0 c
Methylcellulose (MC)	1.0 d

Means within a column followed by different letters are significantly different. ($P < 0.05$) according to Student-Neuman-Keuls multiple range test.

Seed bacterization with hydration treatment emerged at a more rapid rate than did captan-treated seed (Fig. 2). Seed hydration with no additional treatment improved emergence compared with that of MC-treated or nontreated seed, but to a lesser extent than did seed bacterization with hydration or captan. Seed hydrated after treatment with MC emerged at a higher rate than seed hydrated after only surface disinfestation. The high levels of nutrient exudation by sorghum seeds during imbibition provide a challenge to biocontrol of seed-rotting pathogens (Nelson *et al.*, 1986). *P. fluorescens* NU 25 may be able to defend sweet sorghum spermoplane against attack by *Pythium* spp. by antibiotic production (Howell and Stipanovic, 1980) or by competing for iron through siderophore release (Loper, 1988). The relative effectiveness of hydration alone in seed protection may depend on crop species, soil temperature, and level of disease pressure.

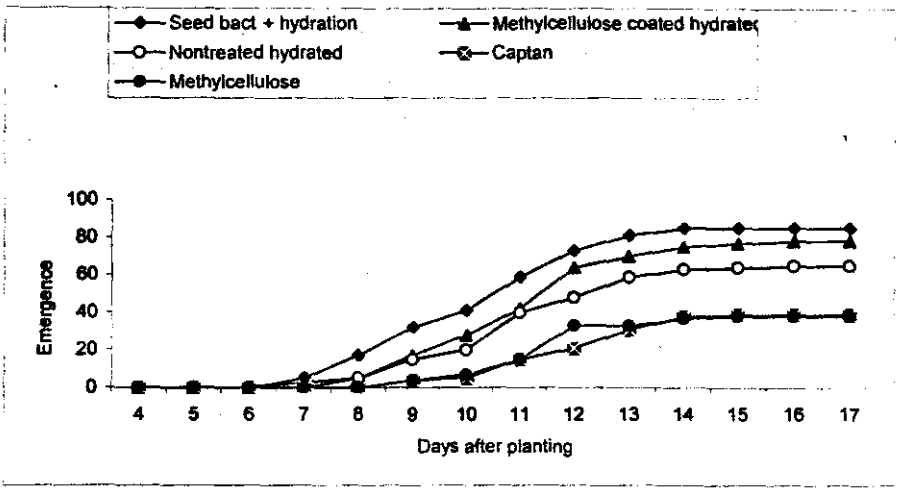

Fig. 2. Control of preemergence damping-off, caused by *Pythium ultimum* of sweet sorghum by seed bacterization with hydration treatment with *Pseudomonas fluorcesens* NU 25.

During seed bacterization with hydration, the initial populations of bacteria on seed were 2.9, 4.8, 6.8, 7.6, and 8.9 $\log_{10}$ cfu seed^{-1} then increased to 6.8, 7.1, 7.6, 8.4, and 9.2 $\log_{10}$ cfu seed^{-1}, respectively, after seed bacterization with hydration treatment (Table 5). This indicates that relatively low seed treatment bioprotectant levels may be relied on to increase to effective populations during the treatment of seed bacterization with hydration. Nelson *et al.*, (1986) reported that a biological seed protection system that adresses problems of seed physiology, such as imbibitional chilling, would appear to have a greater potenial for seed protection under adverse germination conditions.

Table 5. Bacterial populations on seed of sweet sorghum

Levels of seed bacterization ($\log_{10}$ cfu seed^{-1})	
Initial population	**After seed bacterization**
2.9	6.8
4.8	7.1
6.8	7.6
7.6	8.4
8.9	9.2

Ingeneral, an isolate of *Pseudomonas fluorescens* NU 25, provided superior seed protection from *Pythium ultimum* damping-off in naturally infested soils. Moreover, seed bacterization with hydration treatment can provide a high level of protection against preemergence damping-off of sweet sorghum seed caused by *P. ultimum*. This process appears to be especially effective when seed is planted in cold soil. Protection by this treatment was generally superior to the control provided by captan. As such, it may be of interest and value to growers who want to grow good sweet sorghum in areas where planting conditions involve cold soils and to growers who wish to avoid chemical treatment of their seeds.

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الملخص العـربى

مقاومة فطر بيثيم ألتيمم المسبب لمرض موت البادرات فى الذرة السكرية حيويا وفسيولوجيا

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تـــم عـــزل ٣٢ عـــزلة من بكتيريا سيدوموناس فلوروسنس معمليا لتقييم مدى تأثيرها كعامل مضاد حـــيوى لـــنمو فطر بيثيم ألتيمم المسبب لمرض موت البادرات لأختيار أفضلها وتقييمها تحت ظروف العدوى الطبيعـــية فى الحقل وأثر ذلك على المحصول – كما أجريت تجربتان فى الصوبة تحت ظروف العدوى بأفضل العـــزلات لدراسة تأثيرها على الفطر عند تلقيح بذور الذرة السكرية بها سواء البذرة الجافة أو البذرة المجففة بعد البلل مقارنة بالمبيد الفطرى كابتان وأوضحت النتائج مايلى :-

معمليا كان أفضل العزلات البكتيرية هى NU26, NU14, NU22, NU16, NU28, NU25 حيث أعطت أعلى نسب تثبيط (٨٥ و ٨١ و ٨٠ و ٧٨ و ٧٨ و ٧٦ %) على التوالى. وكانت العزلة البكتيرية NU 25أكثر العزلات كفاءة كمقاوم حيوى لفطر بيثيم التيمم . تحت ظروف الصوبة وجد أن تلقيح بذور الذرة السكرية المجففة بعد البلل ببكتيريا سيدوموناس فلوروسنس العزلة NU 25 أدى الى زيادة نسبة أنبات البذور عـــن معاملـــة تلقيح البذرة الجافة مقارنة بالكنترول فى التربة المنخفضة فى درجة الحرارة . أستخدام العزلة البكتـــيرية NU 25 لبكتـــيريا سيدوموناس فلوروسنس كانت أقل كفاءة عند معاملتها للبذرة الجافة بينما زادت كفاءة العزلة عند معاملة البذرة المجففة بعد البلل . أدت معاملة تجفيف البذرة بعد البلل الى زيادة تعداد البكتيريا

عن مستوى العدوى الابتدائى . وكانت هذه المعاملة أكثر كفاءة من الكابتان فى مقاومة فطر بيثيم التيمم المسبب لمرض موت البادرات .

فـــى الحقـــل تـــم دراسة تأثير العزلات الستة السابقة على مدى حدوث المرض والمحصول وجد أن العزلات تتباين فى تأثيرها حيث أدت معاملة بذور الذرة السكرية بالعزلة البكتيرية NU 25 الى زيادة محصول الســـبقان الطازجة والعصير والسكر عن باقى العزلات المستخدمة بينما قل تأثير الكابتان عن العزلات البكتيرية وذلك تحت ظروف الحقل.

معاملـــة تجفـــيف البذرة بعد البلل يمكن أن تكون مهمة وذات قيمة للمزارعين الذين يفضلون زراعة الـــذرة الســـكرية أو المحاصـــيل الحساسة لدرجة الحرارة فى التربة الباردة عندما يسبب مرض موت البادرات مشكلة وتكون معاملة البذرة كيماويا غير مرغوب فيه .