

Biological Control of Wheat Take-All Disease: II - Rapid Screening for Selection of Bacteria Suppressive to *Gaeumannomyces graminis* var. *tritici* in Laboratory with Greenhouse and Field Confirmation Trials

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ABSTRACT

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A quick test tube assay was developed to rapidly screen selected bacterial isolates for their ability to suppress take-all disease of wheat, caused by *Gaeumannomyces graminis* var. *tritici* (Ggt). The assay involved the use of test tubes (18 mm in diameter X 95 mm long) in which either vermiculite or soil was infested with Ggt. Seeds of bread wheat (*Triticum aestivum*) cv. 'Madsen' inoculated with a bacterial isolate were planted in vermiculite or soil containing Ggt growing medium plugs. After 7-10 days, the amount of the take-all symptoms on the roots was determined. Among 23 isolates studied, 3 were selected based on their effectiveness in suppressing the take-all disease symptoms on wheat roots. A greenhouse assay using soils from Tunisia and Missouri with various histories of wheat production and a field trial in Mexico silt loam soil confirmed significant inhibition of take-all symptoms by two bacterial strains: *Pseudomonas aureofaciens* 6 from Missouri and *Bacillus subtilis* 177 from Tunisia. Based on results of our screening procedures, these two bacterial strains were selected for their effectiveness as potential biocontrol agents against wheat take-all to use in specific soils.

Keywords: Biological control, *Bacillus* spp., *Pseudomonas* spp., rhizobacteria, *Gaeumannomyces graminis* var. *tritici*, soilborne plant pathogens, wheat

Take-all caused by the soilborne fungal pathogen *Gaeumannomyces graminis* var. *tritici* (Ggt) is one of the most destructive root diseases in wheat and other cereal grain crops in the world.

Very little progress has been made in breeding wheat varieties resistant to root pathogens and in developing effective systemic fungicides against root diseases (2). Current best management practices for controlling root diseases include crop rotation, crop residue manipulation, tillage, type of nitrogen fertilizer used, and addition of organic soil amendments. Under these cultural conditions severity of take-all is often suppressed sometimes leading to a condition known as "take-all

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decline”, which is strongly associated with the development of antagonistic microorganisms in the wheat rhizosphere (2, 8). The most prominent of these antagonistic microorganisms are bacteria of the genus *Pseudomonas* which are able to suppress Ggt in both saprophytic and parasitic stages (3, 5, 7, 8, 22). Considerable subsequent research has demonstrated that antagonistic pseudomonads suppress Ggt growth through different mechanisms such as production of antibiotics and competition for iron and others (1, 10, 11, 13, 15, 17, 21, 23). Therefore, the current approach for take-all management integrates cultural practices in order to optimize pseudomonad activity in the wheat rhizosphere. This approach may also lead to establishment of “suppressive soils” in which antagonistic pseudomonads and other microorganisms naturally control Ggt (25).

An additional non-chemical strategy of biological control for managing Ggt has been pursued by several research groups since the 1970's (7, 8, 18, 20, 25). The general approach to selection of biological control agents for suppression of Ggt has relied on culturing individual bacterial antagonists from wheat seedlings growing in take-all suppressive soils (25, 26). Some reports suggest that pseudomonads selected for potential biological control of Ggt should be developed for specific wheat-producing regions (18, 19) due to influence of soil properties and characteristics of the microbial communities. Our previous survey for antagonistic bacteria in soils from cereal-producing fields in Missouri and Tunisia revealed a variety of

rhizobacteria with suppressive activity to Ggt regardless of cropping system or soil characteristics (16). These results suggested that effective biological control of Ggt might be achieved by integrating different mechanisms of action by using multiple antagonistic bacterial cultures from both areas (Tunisia and Missouri) in an inundative biocontrol (biofungicide) approach. In order to obtain the most effective strains for this approach, large numbers of bacterial isolates need to be assessed. The objective of this research was to develop a method to rapidly screen bacterial isolates for their ability to suppress Ggt causing take-all disease of wheat and then to test selected isolates under greenhouse and field conditions.

MATERIALS AND METHODS

Biological material. *Gaeumannomyces graminis* var. *tritici* (Ggt) strain Ggt R3-IIIa-1 and the reference bacterial strain *Pseudomonas fluorescens* strain 2-79, both kindly donated by L. S. Tomashow, USDA-ARS, Pullman, Washington U.S.A., were used in this study. Bread wheat (*Triticum aestivum*) cv. ‘Madsen’ was used as the indicator species. At the beginning of the study, 23 bacterial isolates were tested in planta against the fungus. These bacteria were isolated from different soils and identified as described previously (16) (Table 1).

Soils. Soil samples used in bioassays were collected from Missouri (Sanborn Field) and Tunisia (Sub-Humid and Semi-Arid areas). Soil characteristics are summarized in Table 2 (16).

Table 1. Wheat rhizobacteria isolated from Tunisian and Missourian soils and used in this study (See Nasraoui *et al.* (16) for more details)

Bacterial identification	Rhizobacteria accession code	Soil code	Soil origin
<i>Burkholderia glathei</i>	35	SB2	Missouri
<i>Pseudomonas huttiensis</i>	69	SB2	
<i>Pseudomonas</i> sp.	79	SB3	
<i>Pseudomonas aureofaciens</i>	6	SB5	
<i>Burkholderia cepacia</i>	73	SB9	
<i>Burkholderia glathei</i>	153	SB9	
<i>Burkholderia cepacia</i>	250	SB9	
<i>Pseudomonas aureofaciens</i>	314	SB20	
<i>Bacillus pumilus</i>	420	SB31	
<i>Burkholderia cepacia</i>	310	SB34	
<i>Pseudomonas aureofaciens</i>	373	BJ	Tunisia
<i>Pseudomonas aureofaciens</i>	148	KR	
<i>Pseudomonas syringae syringae</i>	401	NB	
<i>Pseudomonas aureofaciens</i>	499	J2	
<i>Pseudomonas aureofaciens</i>	505	J2	
<i>Bacillus subtilis</i>	177	J3	
<i>Xanthomonas</i> sp.	128	J5	
<i>Bacillus subtilis</i>	263	J5	
<i>Pseudomonas syringae</i> pv. <i>syringae</i>	295	J5	
<i>Pseudomonas putida</i>	227	K1	
<i>Bacillus subtilis</i>	299	K1	
<i>Pseudomonas aureofaciens</i>	31	PK1	
<i>Bacillus subtilis</i>	58	PK1	

Table 2. Characteristics and cropping histories of soils collected from Missouri and Tunisia and used in laboratory test tube and greenhouse pot assays (See Nasraoui *et al.* (16) for more details)

Soil origin	Soil code	Cropping system (time in place)	Soil pH	Organic matter (%)
Missouri	SB2	Continuous wheat, full fertility ^b (112 yr)	5.9	2.7
	SB5	Continuous wheat, manure ^c + N fertilizer (112 yr)	5.9	2.7
	SB9	Continuous wheat, no fertility (112 yr)	4.9	2.1
	SB20	Grain sorghum-soybean-wheat, full fertility (12 yr)	5.6	2.6
	SB34	Corn-soybean-wheat, manure (12 yr)	6.0	3.0
Tunisia	KF	Wheat-barley-barley-barley (4 yr)	8.0	1.3
	MT	Wheat-oat/hairy vetch-wheat-barley (4 yr)	7.9	3.9
	NB	Wheat-wheat-fallow-wheat (4 yr)	7.9	2.7
	J3	Wheat-wheat (2 yr)	7.3	2.8
	J5	Wheat(1 yr)	7.4	2.5

Media. The fungus and the bacterial isolates were routinely cultured on Potato Dextrose Agar (PDA) (6) and King's Medium B (KMB) (12), respectively. To verify the presence of Ggt, infected wheat seedling roots were checked on Ggt selective medium SM-GGT 3 [39 g Difco PDA, 10 mg dicloran, 10 mg metalaxyl, 25 mg 1-(3,5-dichlorophenyl)-3-methoxymethylpyrrolidin-2-dion (Hoe 00703, kindly donated by Dr. D. E. Mathre, Montana State University), 100 mg streptomycin sulfate and 500mg L-β-

3,4-dihydroxyphenylalanine (L-DOPA) in 1 l of deionized water] (9). All media were autoclaved for 20 min at 121°C.

Vermiculite test tube assay. A 1-liter beaker was filled with vermiculite up to 500 ml, moistened with 250 ml of water and autoclaved for 1 h at 121°C. Sterilized vermiculite was mixed with 4-mm (diameter) plugs of PDA medium and 4-mm plugs from the fungal culture and then homogenized 30 sec in a Stomacher Lab-Blender 80 (Tekmar,

Cincinnati, OH)¹. The recovered product was placed in 18 mm x 95 mm test tubes (5 cm/tube) at a final concentration of 10 PDA plugs + 10 fungal plugs per tube. One cm of vermiculite only was added to each tube and wheat seeds were sown in the higher layer. Seeds were previously sterilized in 0.6 % sodium hypochlorite for 4 min, rinsed in sterile deionized water, and soaked in a bacterial suspension (10⁸ cells/ml) mixed with autoclaved 1% carboxymethyl cellulose (Sigma, St. Louis, MO). Sterilized deionized water (1.0 ml) was added and the tubes were capped. The assay was performed at room temperature (22 to 25 C) under fluorescent light of 12-hour photoperiod.

Soil test tube assays. To test bacteria in original or different soils, each test tube was first filled to the 2 cm level with autoclaved vermiculite on which 1 g of dry soil sample was placed. Ten PDA plugs + 10 fungal plugs (as in vermiculite assay) were scattered on the soil surface. One more gram of dry soil was added, followed by 1 cm of autoclaved vermiculite. Surface-sterilized seeds of wheat treated with test bacteria as in the vermiculite assay were sown in the higher layer of vermiculite. Sterilized deionized water (2 ml) was added and the tubes were capped. The temperature and the light conditions were the same as in the vermiculite assay.

Greenhouse pot assay. The greenhouse assay was performed in 50 mm x 80mm pots ('Nu Pots', Hummerts, St. Louis, MO) which were first filled to a 3-cm depth with autoclaved vermiculite followed by 3 g of dry soil. Ten 7-mm PDA plugs + ten 7-mm fungal plugs were scattered on the soil surface. Two more grams of dry soil and 1-cm level of autoclaved vermiculite were added to

each pot. Surface-sterilized seeds of wheat treated with the selected bacteria as in the vermiculite assay were sown in the upper layer of vermiculite. Pots were then placed on a large tray containing tap water to accommodate capillary irrigation. The assay was performed at 21-28 °C under natural sunlight.

Field trial. The field trial was performed in Bradford Farm of the University of Missouri-Columbia (MO) in Mexico silt loam soil, using 'Madsen' wheat variety. Experimental design was Randomized Complete Block Design with 4 replicate blocks and 1-meter row experimental unit. Surface-sterilized wheat seeds, treated with the selected bacteria as in the vermiculite assay, were mixed (50/50) with Ggt-inoculated autoclaved grains of oats and then planted (April 12, 2002). Two months later (June 17, 2002), 5 individual-plant-samples from each experimental unit were carefully harvested and adhering soil removed by washing. Fungal infection of roots was evaluated and fresh weight and dry weight (105 °C for 48 h) were determined.

Assessment of fungal infection. To evaluate the severity of wheat root infection, the following widely used scale (14) was adopted: 0 = no blackened tissue in the root, 1 = 1-25 % blackened tissue, 2 = 26-50 % blackened tissue, 3 = 51-75 % blackened tissue, 4 = 76-100 % blackened tissue. Evaluation was performed at 1 week for the assay with vermiculite in test tubes, 10 days with soil in test tubes, 3 weeks with greenhouse soil in pots, and around 9 weeks in field. Results were subjected to an analysis of variance and, when the F-test was significant ($P < 0.05$), Fisher's protected least significant difference (LSD) test was used for mean separation.

RESULTS

Infection inhibition in vermiculite. Wheat seedlings inoculated with the

* Trade names are used for clarity and do not represent endorsement by USDA-ARS or the University of Missouri.

fungus (F) alone developed the highest root infection rate (rating > 3) (Fig. 1). With bacterial treatment, a range of root infection was observed with an important disease inhibition (rating 1.0-1.5) for

many isolates including 6, 73, 153, 177 and 310. In contrast, isolates 69, 79, 148, 420, and 505 were less effective in reducing root infection with ratings of 2.0-2.5.

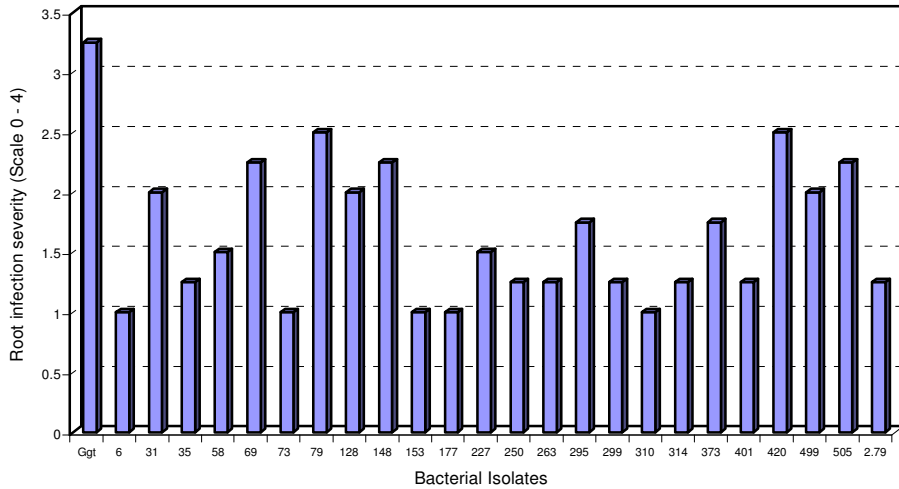


Fig. 1. Effect of bacterial seed treatment on the severity infection of wheat roots by Ggt in vermiculite test tube assay, one week after inoculation. LSD (0.05) = 0.82.

Infection inhibition in original soil. Based on the results of vermiculite assays (Fig. 1), the most effective bacterial species were selected to include at least two isolates from each different soil. Those selected isolates were 6, 35, 73, 153, 177, 250, 263, 295, 310, 314 and 401. These 11 isolates were then tested in their original soils. Results in Fig. 2 show a high degree of root infection (> 3) in all soils inoculated with the fungus alone

(Control). With bacterial treatment, 3 of the 11 isolates exhibited strong inhibition of root infection. Those isolates were 6, 153 and 177 which are, respectively, *Pseudomonas aureofaciens* and *Burkholderia glathei* from Missouri soils SB5 and SB9, and *Bacillus subtilis* from Tunisian soil J3. These 3 isolates were selected for the following experiment examining activity across a range of soils.

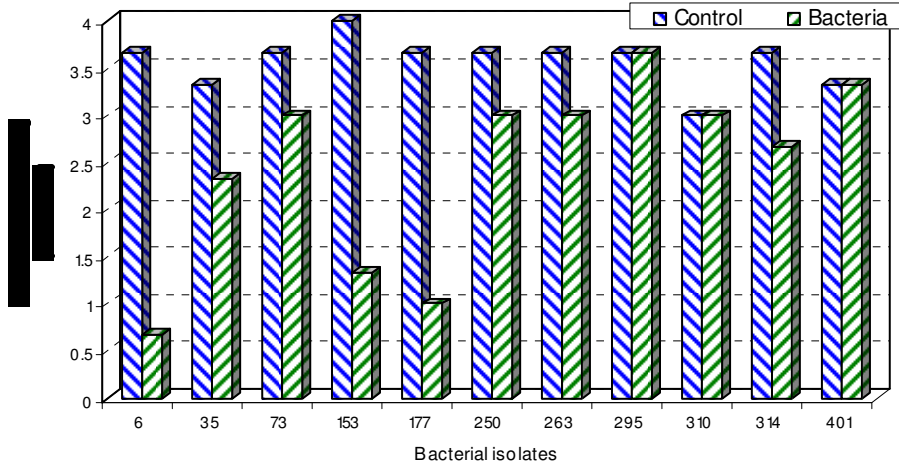


Fig. 2. Effect of bacterial seed treatment on the severity infection of wheat roots by Ggt in soil test tube assay (each bacterial isolate tested in its original soil), 10 days after inoculation. LSD (0.05) = 0.76.

Infection inhibition in different soils: Results in Fig. 3 show that inoculation of all soils with Ggt resulted in high levels of wheat root infection (infection degree 3-3.5). In the presence of bacterial isolates, wheat root infection

was highly inhibited by isolate 6 in SB2 soil and isolate 177 in KF soil (infection degree 1.0-1.5). For all the other bacterial isolates/soil combinations, no significant inhibition of root infection was observed (infection degree 3.0-3.5).

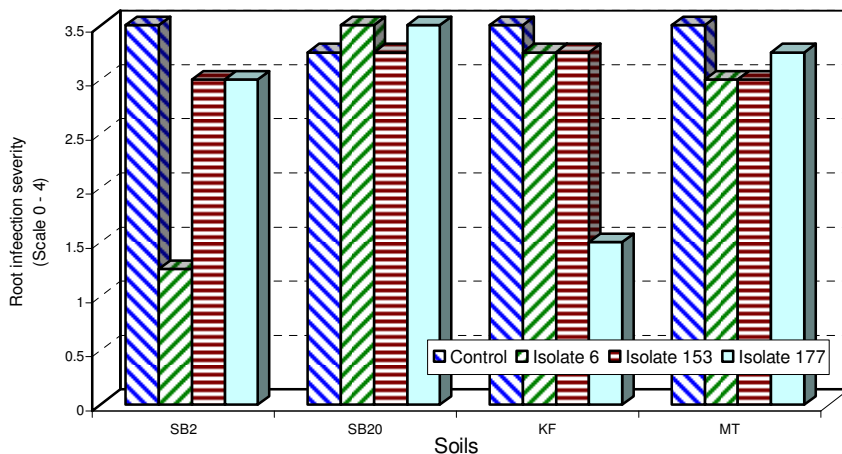


Fig. 3. Effect of bacterial seed treatment on the severity infection of wheat roots by Ggt in soil test tube assay (each bacterial isolate tested in different soils), 10 days after inoculation. LSD (0.05) = 0.85.

Greenhouse infection inhibition in original and different soils. When soils were inoculated with Ggt (Control), the infection degree on wheat roots was very high (3.0-3.5) (Fig. 4). Infection was strongly inhibited by isolate 6 in its original soil (SB5) and in soil SB2

(infection degree around 1.0-1.5). Isolate 177 inhibited root infection about 30 and 50%, respectively, in its original soil (J3) and in KF soil compared to the control. All the other bacterial treatments caused no significant reduction in root infection (Fig. 4).

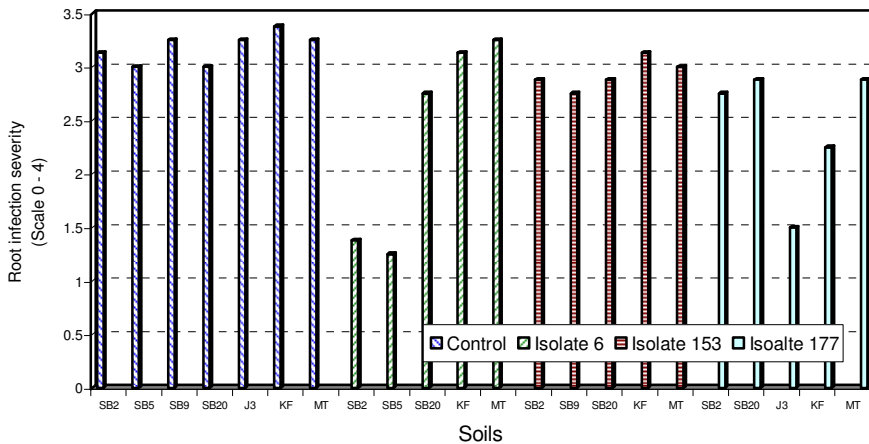


Fig. 4. Effect of bacterial seed treatment on the severity infection of wheat roots by Ggt in greenhouse different soil pot assay, three weeks after inoculation. LSD (0.05) = 0.68.

Field trial. Application of bacterial isolates 6 and 177 (separately or in mixture) as seed treatment before planting wheat seeds inoculated with Ggt reduced significantly the infection degree of wheat by Ggt (infection degree < 0.25) as compared to untreated seeds (infection degree > 2.5) (Fig. 5). Based on fresh and dry weights data, the disease-suppressive effect induced by the two-tested bacterial isolates was consistent (Fig. 6). Therefore, it appears from Fig. 6 that inoculation of wheat seeds with Ggt

highly reduced plant fresh and dry weights as compared to the un-inoculated controls. Bacterization of seeds by the selected isolates 6 and 177 before challenge with the fungal pathogen, protected effectively the plants from fungal attacks and yielded plant fresh and dry weights similar to those obtained by the non-infected controls. Despite its effectiveness, association of the two tested bacteria in the same treatment has not improved the results of the individual bacterial treatments.

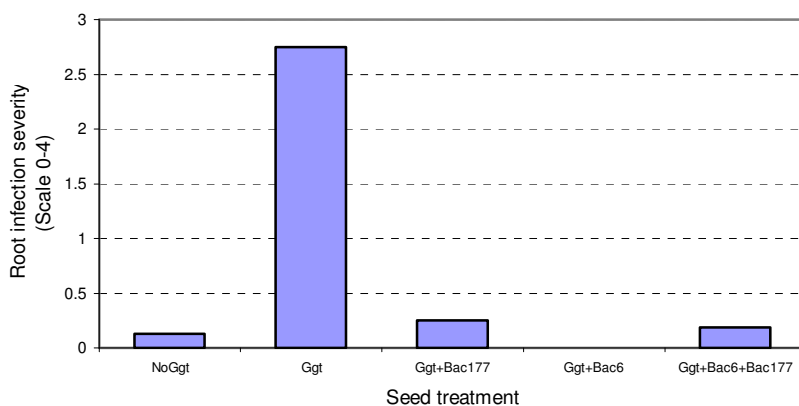


Fig. 5. Effect of bacterial seed treatment on the severity infection of wheat roots by Ggt in Mexico silt loam soil field experiment, 9 weeks after inoculation. LSD (0.05) = 0.34.

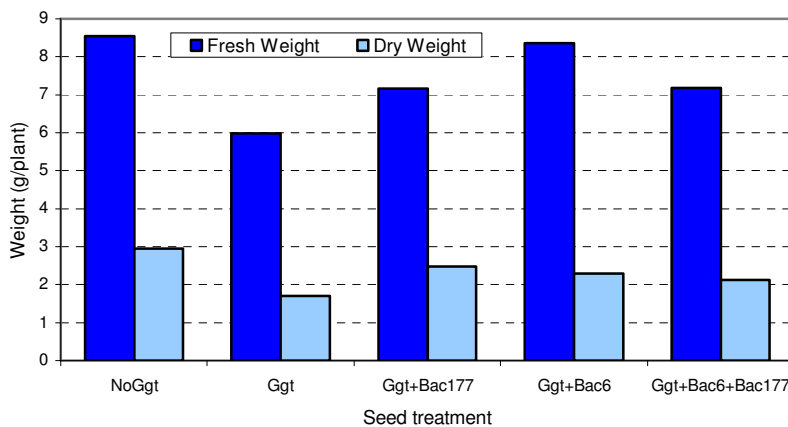


Fig. 6. Effect of bacterial seed treatment on fresh and dry weights of wheat plants inoculated by Ggt in Mexico silt loam soil field experiment, 9 weeks after inoculation. LSD (0.05) = 1.12 and 0.67, respectively.

DISCUSSION

In the vermiculite test tube assay, a one-week duration was sufficient to select bacterial species able to significantly reduce the infection of wheat roots with Ggt (Fig. 1). These results indicate that this brief assay may be very useful in bacterial screening aiming to eliminate bacteria ineffective in planta despite their apparent effectiveness in vitro on culture medium against Ggt (16). A further confirmation of the efficiency of our assay to detect antagonistic bacteria is similarity in activity of our most effective isolates with *Pseudomonas fluorescens* 2-79, which was reported to be very successful in inhibiting Ggt in the Pacific Northwest (USA) soils (24). Our soil assay in test tubes was more rapid when compared to the widely used technique of soil-applied Ggt-infected oat particles (27). The use of a layer of infested Ggt agar plugs in soil, instead of infected oat grains, shortened the duration of the assay from 3-4 weeks to only 10 days. With this method, we were able to select three strong inhibitors, all effective in their original soils (Fig. 2). The use of these bacterial isolates in different soils can be possible since two of these isolates were effective in soils different from their original soil (Fig. 3). These observations open the possibilities for the reintroduction of bacteria into their original soil and also for their introduction into additional different soils without loss of effectiveness against Ggt. This approach would promote any additive or synergistic inhibitory effects resulting from use of multiple bacterial strains, as suggested by Pierson and Weller (18).

The greenhouse pot assay was performed to better simulate natural field conditions. This assay, using higher volumes of soil as compared to test tube assays, showed that a period of three weeks was sufficient for symptom

development. Greenhouse and field experiments confirmed the effectiveness of two bacterial species among the three previously selected, based on their effectiveness in reducing the infection of wheat roots by Ggt. These two effective species were *Pseudomonas aureofaciens* (isolate 6 from Missouri) and *Bacillus subtilis* (isolate 177 from Tunisia). While *P. aureofaciens* was effective in both, its original soil and an additional soil from Sanborn Field (Missouri), *B. subtilis* was mainly effective in its original soil (Figs. 4, 5, 6).

Our studies allowed selection of bacteria that inhibited Ggt both in vitro in dual cultures (16) as well as in vivo in test tubes, in the greenhouse and under field conditions. Despite the fact that our in vitro investigations seem to indicate that these antagonistic rhizobacteria inhibit the growth of the pathogen through antibiosis, different other modes of action, such as competition for nutrients, direct parasitism and induction of plant defense mechanisms could not be ruled out (1, 10, 13, 15, 17, 23, 25, 26).

Overall, the assays reported here seem to be very effective in selecting bacteria highly suppressive to Ggt and allowed the elimination of bacteria strains which showed no or poor effectiveness under the rigorous soil environment. The selected bacterial species might be formulated as biofungicide seed treatment. These biofungicides may be readily integrated within a disease management program for the control of the take-all disease of wheat caused by Ggt.

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RESUME

Nasraoui B., Hajlaoui M. R., Gargouri S. et Kremer R. J. 2007. Lutte biologique contre le piétin-échaudage du blé: II - Criblage rapide pour la sélection de bactéries suppressives de *Gaeumannomyces graminis* var. *tritici* au laboratoire avec des essais de confirmation sous serre et au champ. *Tunisian Journal of Plant protection* 2: 35-46.

Un essai rapide en tubes à essai a été développé pour cribler et sélectionner des isolats bactériens pour leur capacité à supprimer le piétin-échaudage du blé causé par *Gaeumannomyces graminis* var. *tritici* (Ggt). L'essai implique l'utilisation de tubes à essai (18 mm de diamètre x 95 mm de longueur) dans lesquels la vermiculite ou le sol est infesté avec Ggt. Des semences de blé tendre (*Triticum aestivum*) cv. 'Madsen', inoculées avec un isolat de bactérie, sont semées dans la vermiculite ou le sol renfermant des morceaux de milieu de culture contenant Ggt. Après 7-10 jours, l'importance des symptômes du piétin-échaudage sur les racines est déterminée. Parmi 23 isolats étudiés, 3 ont été sélectionnés sur la base de leur efficacité dans la suppression des symptômes de la maladie du piétin-échaudage sur les racines du blé. Un essai sous serre utilisant des sols de la Tunisie et du Missouri avec diverses histoires de production de blé et un essai au champ dans un sol à texture équilibrée (*Mexico Silt loam*) ont confirmé une inhibition significative des symptômes du piétin-échaudage par deux souches bactériennes: *Pseudomonas aureofaciens* 6 du Missouri et *Bacillus subtilis* 177 de la Tunisie. Sur la base des résultats de nos procédures de criblage, ces deux souches bactériennes ont été sélectionnées pour leur efficacité comme agents potentiels de lutte biologique contre le piétin-échaudage du blé à utiliser dans des sols spécifiques.

Mots clés: Lutte biologique, *Bacillus* spp, *Pseudomonas* spp, rhizobactéries, *Gaeumannomyces graminis* var. *tritici*, phytopathogènes transmis par le sol, blé

ملخص

نصراوي، بوزيد ومحمد رايح الحجاوي وسامية القرقوري وروبرت ج. كريم. 2007. مكافحة البيولوجية/الأحيائية لمرض التعفن الكلي (أو الساق الرنوعي) للقمح: II - غربلة سريعة لاصطفاء بكتريات مثبطة للفطر *Gaeumannomyces graminis* var. *tritici* في المخبر مع تجارب للتثبيت تحت ظروف الدفيئة والحقل. *Tunisian Journal of Plant protection* 2: 35-46.

وقع تطوير تجربة سريعة في أنابيب الاختبار لغربلة عزلات بكتيرية مصطفاة لقدرتها على تثبيط مرض التعفن الكلي (أو الساق الرنوعي) للقمح الذي يحدثه الفطر *Gaeumannomyces graminis* var. *tritici* (Ggt). استعملت في هذه التجربة أنابيب اختبار (18 مم للقطر X 95 مم للطول) تحتوي على الفرميكوليت أو التربة ملوثة بالفطر Ggt. بعد إقحاح بذور القمح اللين/الطري (*Triticum aestivum*) صنف 'مادسن' بالعزلة البكتيرية، زرعت في الفرميكوليت أو التربة المحتوية على أجزاء من المستنبت الحامل للفطر Ggt. وبعد 7-10 أيام، تم تقدير مستوى أعراض التعفن الكلي على الجذور. من بين 23 عزلة مدروسة، أمكن اصطفاء 3 عزلات بالاعتماد على مقدرتها في تثبيط أعراض التعفن الكلي على جذور القمح. أثبتت تجربة نفذت تحت الدفيئة على أترية من تونس وميزوري ذات تواريخ إنتاج قمح مختلفة وكذلك تجربة في حقل تربته سلتية لومية، فاعلية سلالتين بكتيريتين في تثبيط أعراض التعفن الكلي وهما: *Pseudomonas aureofaciens* 6 من ميزوري و *Bacillus subtilis* 177 من تونس. بالاعتماد على نتائج طرق الغربلة، أمكن لنا اصطفاء سلالتين بكتيريتين اثنتين لفاعليتهما كعوامل مكافحة بيولوجية/أحيائية يمكن استعمالهما ضد التعفن الكلي للقمح في أنواع معينة من الأترية.

كلمات مفتاحية: مكافحة بيولوجية/أحيائية، *Bacillus* spp، *Pseudomonas* spp، البكتيريا المحيطة جو الجذور، *Gaeumannomyces graminis* var. *tritici*، الممرضات النباتية المنقولة مع التربة، القمح

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