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Biological control of *Rhizoctonia solani* of *Phaseolus vulgaris* by *Bacillus subtilis*

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FOUR growth culture media were used for assay of antagonistic potential of *B. subtilis* against *R. solani*. Both cell suspension and culture filtrate of *B. subtilis* had an antagonistic potential towards *R. solani*; the culture filtrate was the more active. The application of the formulated *B. subtilis* was effective against *R. solani* under pot experiment conditions, the highest disease suppression being with seed treatment. There was no significant difference between the antagonistic potential of formulated *B. subtilis* compared with Mancozeb fungicide, especially when applied as seed treatment.

Keywords: *Bacillus subtilis*, Biological control, *Rhizoctonia solani*.

Rhizoctonia solani Kühn is a major soil and seedborne pathogen that attacks agricultural crops worldwide (Thakur *et al.*, 1991) causing huge economic losses to growers. Control of the pathogen is currently limited to the use of long rotations (Specht & Leach, 1987), soil solarization (DeVay & Stapleton, 1997) and prophylactic fungicide treatment (Baider & Cohen, 2003).

Among the alternative methods that have been pursued for control of *R. solani* are biological control (Fravel & Lewis, 2004). The application of microorganisms as agents for bio-control of plant diseases in agriculture is now considered as an important alternative to use chemical fungicides (Sivan & Chet, 1992) due to the frequent development of *R. solani* isolates that are resistant to common fungicides and the desire to reduce pesticide use have led to efforts to develop alternatives methods (Stephens *et al.*, 2001).

Selected microbial biocontrol agents are active bio-protectors and have been widely used in bio-control of *R. solani* in different agricultural crops, such as soyabean (Datta *et al.*, 2000), cotton (Izhar *et al.*,1999), oilseed rape (Berg *et al.*, 1996), potato (Haggag & Nofal, 2000) and chickpea (Prasad & Rangeshwaran, 2000).

Bacillus subtilis is registered in controlling *R. solani* in rice (Kanjanamaneesathian *et al.*, 2001). Antifungal production (Abd-Allah, 1995)

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plant growth-promoting (Abd-Allah *et al.*, 1997) and induction of systemic resistance (Abd-Allah & Ezzat, 2004) are involved in the control of plant pathogenic fungi by *B. subtilis*, although their role in promoting plant growth is less documented and understood .

We undertook this study to test the antagonistic potential of the bio-control agent *B. subtilis* on *R. solani* (*in vitro*) and on the development of a disease index on the bean plant.

Material and Methods

The plant pathogen *Rhizoctonia solani* Kühn that was isolated from bean seedling collected from a greenhouse in Ismailia, Egypt had the typical symptoms of root rot disease , and its pathogenisty was tested following Tuite (1969).

The biocontrol agent , *Bacillus subtilis* was isolated from the rhizosphere of tomato seedling (cv. Peto 86) grown in Sharkia Governorate, Egypt (Abd-Allah, 1995). Kidney bean seeds (*Phaseolus vulgaris* L. cv. Brittle Wax) with 98% germination percent were used. Soil used in this study was a sandy loam with the following properties: organic carbon, 0.68 %; total nitrogen, 0.031 %; total soluble salts, 0.25 %; maximum moisture holding capacity, 14.2 % and pH, 7.3.

The antagonism of *B. subtilis* against *R. solani* was carried out on four growth media, *e.g.*, nutrient agar (Stanisich & Holloway, 1972), potato dextrose agar (Booth, 1977), yeast extract glucose calcium carbonate agar (Mondal *et al.*, 1999) and King's agar (King *et al.*, 1954). The antagonism was tested on agar plates at 28 ^{0}C for 8 days according to Rangeshwaran & Prasad (2000). Both washed cells suspension and culture filtrate (free cells) were screened for *in vitro* inhibition of *Rhizoctonia solani*. Sclerotia of *R. solani* were soaked in a washed bacterial suspension (2.8 x 10^8 cfu / ml), 1.0% (w/v) carboxy methyl cellulose was add as adhesive (Abd-Allah *et al.*, 1997) and their cell free culture filtrate (filter sterilized) at 100 sclerotia per test tube.

The tubes were then incubated at 28 ^{0}C for 1 hr, one day, three days and five days. Three replications were mainted for each treatment. Sterile saline solution (8.5g NaCl / liter distilled water) was used in control tubes. Sclerotial viability was tested by transferring surface sterilized sclerotia (0.1 % w/v $HgCl_2$ was used for seeds washing for 30 sec, seeds were then rinsed four times in sterile water and finally blotted dry) onto fresh potato dextrose agar plates. Mycelial growth was checked after incubation at 28 ^{0}C for 5 days according to Vasantha *et al.* (1989).

Talc powder was chosen as a carrier to prepare formulation of *B. subtilis* . Its pH was adjusted to 7.0 using calcium-carbonate . Carboxy methyl cellulose (10 g / kg of carrier) was used as adhesive. *B. subtilis* was grown in King's broth

medium for 72 hr to obtain 9 x 10^8 cfu /ml. The bacterial suspension was mixed with sterile carrier (400 ml / kg) and air dried according to Vidhyasekaran *et al.* (1996). Bean seeds were coated with thin film of 1.0 % (w/v) carboxy methyl cellulose and mixed with the respective formulation at 4 g / kg seed.

For soil treatment, 2 g / kg soil was used. Mass culture of *R. solani* was raised on corn : sand : meal (Dohroo, 1988). The medium was inoculated with mycelial bit of *R. solani* taken from the margin of an active growing culture and incubated at 25°C for 14 days (Mathew & Gupta, 1998) and added as 10 g / pot (circa 2.9 g sclerotia / 10 g inoculum).

Seeds of bean were sown after two days at 5 seeds / pot (Plastic pots containing about 350 g sterilized sandy loam soil). Mancozeb at 2.5 g seed, was used for comparative analysis. Each treatment was replicated four times. The experiment was conducted in a randomized block design and percent of disease index (disease-free plants) were calculated 20 days after sowing.

For each experiment, the data were statistically analyzed using the Analysis of Variance for completely randomized design. Treatments means were compared using the protected Least Significant Difference (LSD) values according to Daniel (1987).

Results and Discussion

There were significant differences among the culture media tested (Table 1). *B. subtilis* had moderate to good inhibition activity on all growth media except potato dextrose agar media, which was lowest one. Evidence supporting the performance of King's medium for antifungal production by *B. subtilis* against *R. solani* (Wei *et al.*, 1991), *F. oxysporum* f.sp. *lycopersici* (Abd-Allah, 1995) and *Colletotrichum gossypii* (Freitas *et al.*, 1997) is presented in the literature. Suppresion of fungal growth by bacteria on other ferric iron-supplemented media has been reported by Mishagi *et al.* (1982), some of rhizobacteria showed antifungal activity under iron stress conditions (Laha *et al.*, 1996).

TABLE 1. *In vitro* evaluation of a biological antagonist (*B. subtilis*) against *R. solani* in dual cultures.

Dual culture growth media		Mycelial growth inhibition (%)
Nutrient agar		44.0
Potato dextrose agar		23.3
Yeast extract glucose calcium carbonate agar		46.6
King's agar		57.6
L S D at :	0.05 %	3.10
	0.01 %	4.57

Washed bacterial cells as well as culture filtrates were assayed against the germination percentage of sclerotia of *R. solani* (Table 2). Only a marginal decrease in germination was noticed after 1 hr by culture filtrate. However, bacterial cells had no inhibitory effect after such time. Overmuch the incubation time decreased germination percent caused by both bacterial cell suspension and bacterial culture filtrate which showed more active at all times examined. The higher inhibitory effect of bacterial culture filtrate than bacterial cells suspension indicated that the antimicrobial compounds are relased in growth medium. Such observation was reported by Rangeshwaran & Prasad (2000). The extra-cellular production of antifungal substances by *B. subtilis* was confirmed by Grau *et al.* (2001), who reported that *B. subtilis* produced an extracellular lipo-peptide antifungal compounds.

TABLE 2. Effect of cell suspension and bacterial culture filtrate of *B. subtilis* on the germination of sclerotia of *R. solani*.

Time(hr)	Germination percentage (%)*	
	Cell suspension	Bacterial culture filtrate
1	100.0	92.3
24	84.0	23.6
72	41.0	12.3
120	21.6	6.7
L. S. D. at :		
0.05%	5.8	6.5
0.01%	10.7	12.6

*Germination percentage for control treatment was 100 at all incubation periods.

The potential of formulated *B. subtilis* for control of plant pathogenic fungi under greenhouse conditions has been demonstrated by Grau *et al.* (2001). In the present study , the number of stand plants was higher in seed treatments than soil treatments with *B. subtilis* (Table 3). The finding that most of biocontrol agents were used as seed treatment (in case of soil or seed borne diseases control), especially in large scale application (Vidhyasekaran & Muthamilan, 1995) might be due to that the seed tretments lead to survival of biocontrol agents up to 45 days (Amer & Utkhede, 2000) as a secondary development of these biocontrol agents along the root (Harman *et al.*,1989), which may prevent infection by root-rot and wilt pathogens (Sivan & Chet, 1989).

Moreover, soil treatment was not as effective as seed treatment and the disease was not effectively suppressed (Rangeshwaran & Prasad, 2000). Also, in our study we observed that the number of stand plants was higher in Mancozeb treatments than that both seed and soil treatments of *B. subtilis*, the difference in case of seed treatment was not significant (Table 3).

Furthermore, the deregistration of many fungicides for control of plant diseases has resulted in an increased interest in alternatives and also safe from the public health and environmental points of view.

TABLE 3. Effect of seed and soil application of *B. subtilis* in comparison with mancozeb fungicide on disease index (%) of kidney bean caused by *R. solani*.

Treatment		Disease Index (% of plant stand)	
		Seed Treatment	Seed Treatment
Plant + *R. solani*		23	14
Plant + *B. subtilis* + *R. solani*		72	55
Plant + Mancozeb + *R. solani*		82	82
Plant + *B. subtilis*		100	100
Plant (control)		100	100
L S D at :	0.05%	10.9	12.7
	0.01%	15.5	17.8

In conclusion, *B. subtilis* treatment proved to be effective for controlling of *R. solani*, and it should be considered as a possible means to retard disease development. The use of *B. subtilis* antagonists also showed promise, especially as seed treatment.

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بسم الله الرحمن الرحيم

المقاومة البيولوجية لفطر *رايزوكتونيا* فى الفاصوليا باستخدام *باسيلس ساتلس*

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تم أستعمال أربعة أوساط غذائية بغرض دراسة الأمكانية التضادية للعامل الحيوى لبكتريا (*B. subtilis*) تجاه الفطر الممرض (*R. solani*) وكان أكثر هذه الأوساط الغذائيه فعالية وسط كنج.

كل من المعلق الخلوى و راشح الخلايا كانا لهما أمكانية تضادية تجاه الفطر الممرض (*R. solani*) على الرغم أن راشح الخلايا كان أكثر تضادية. أستعمال العامل الحيوى فى شكله المعد كان فعال جدا تجاه الفطر الممرض (*R. solani*) و كان عند معالجة البذور به أكثر فعاليه مقارنة بمعالجة التربه به.

من الجدير بالذكر انه تم ملاحظة عدم وجود فرق معنوى (أحصائى) بين فعالية العامل الحيوى والمبيد الكميائى فى الأمكانية التضادية تجاه الفطر الممرض خاصة عند أستعمالهما بمعالجة البذور مما يعطى أمكانية لاستخدام ذلك التطبيق فى الأنتاج الحيوى للفاصوليا الخضراء.

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