INDUCTION OF SYSTEMIC RESISTANCE BY *BACILLUS SUBTILIS* ISOLATE Bs_t AGAINST *ROTYLENCHULUS RENIFORMIS* IN TOMATO*

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Summary. A rhizobacterium, *Bacillus subtilis* (isolate Bs_t) was tested for its ability to induce systemic resistance against *Rotylenchulus reniformis* in tomato. A split root system was used to keep the inducer (rhizobacterium) and the challenger (nematode) spatially separate and observations on nematode penetration, its multiplication and growth characters were recorded. The results show that the nematode penetration was reduced by 44.5% in the split root in which one half received a bacterial cell suspension (10^{10} cells/ml) as a soil drench and a week after the other half was inoculated with the nematode. All growth characters measured were increased to some extent by the bacterium, but only fresh shoot length and fresh shoot weight significantly differed from the control. The nematode multiplication rate was reduced significantly when the bacterial soil drench was applied a week before nematode inoculation but not in those treatments that received simultaneous inoculations of the bacterium and the nematode. The results indicate that Bs_t could induce systemic resistance in tomato against *R. reniformis*.

Although several specific mechanisms by which rhizobacteria inhibit fungal or bacterial pathogens have been demonstrated (Weller, 1988), few have been reported against nematodes. Production of toxic metabolites that apparently affect hatching, recognition, penetration and multiplication of nematodes (Oostendorp and Sikora, 1986; Keel *et al.*, 1991; Westcot and Kluepfel, 1993; Neipp and Becker, 1999), modifying microflora of rhizosphere of antagonistic plants (Kloepper *et al.*, 1999) and induced systemic resistance (Hasky-Gunther *et.al.*, 1998) are thought to be the possible mechanisms implemented by rhizobacteria against nematodes. Systemic induced resistance has been reported in several host-pathogen systems (Kuc, 1990) and is defined as the process of active resistance dependent on physical or chemical barriers of the host plant, activated by biotic or abiotic inducing agents (Kloepper *et al.*, 1992). Induced resistance to diseases in tomato has been reported by Hammerschmidth and Kuc (1995). It was also established that plants from tomato seeds treated with a biogenic elicitor (e.g. lipoglycoprotein) had induced resistance to *Meloidogyne incognita* in Russia (Zinovieva *et al.*, 1989).

Kiyohara (1986) reported that pre-immunisation with an avirulent strain of the pine wilt nematode, *Bursaphelenchus xylophilus*, induced systemic resistance against the same nematode. Ogallo and McClure (1995) indicated that infection of tomato or pyrethrum plants with incompatible or mildly virulent *Meloidogyne* species induced resistance in the plants such that the reproduction of challenge inoculum of normally compatible *Meloidogyne hapla* was highly suppressed. Hasky-Gunther *et al.* (1998) were the first to demonstrate induced systemic resistance mechanism of action by *Agrobacterium radiobacter* (G12) and *Bacillus sphaericus* (B43) against *Globodera pallida*. The present study investigated whether *Bacillus subtilis* (Bs_t) can induce systemic resistance in tomato against the reniform nematode, *Rotylenchulus reniformis*.

MATERIALS AND METHODS

Nematode culture. Rotylenchulus reniformis Linford *et* Oliveira inoculum obtained from naturally infected roots of castor (*Ricinus communis* L.) plants were multiplied by inoculating single egg mass into the rhizosphere of cowpea [*Vigna unguiculata* Walp.(L.)] plants (cv. Pusa Komal).

Bacterial culture. Roots of tomato (*Lycopersicon esculentum* Mill.) collected from the Indian Agricultural Research Institute fields were first shaken gently to remove loose soil particles and then cut into small segments. One g of the root segments was agitated in 10 ml of 0.1 M $MgSO_4$ for 15 min to separate bacteria from the roots. The suspension obtained was aseptically streaked on Tryptic Soy Agar contained in Petri plates. These were incubated at 28 °C for 48 h. All morphologically distinct colonies from each Petri plate were selected and aseptically streaked onto a new medium. This process was repeated at least three times to obtain a pure culture. Pure cultures were subsequently identified as *Bacillus subtilis* (Ehrenberg, 1835) Cohn, 1872 and designated as Bs_t isolate, stored at 4-5 °C and subcultured at monthly intervals. The colony forming units (cfu) per ml were determined using a dilution plating method.

Split root experiment. Tomato seedlings were initially

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grown in a steam-sterilized soil-sand mixture (3:1) in 15 cm diam earthen pots for 21 days after sowing. They were then removed gently from the pots and the main root tips were carefully cut with a pair of sterilized scissors to stop growth and promote production of lateral roots. Then the root system was equally divided into two parts and each part planted in two adjacent plastic bags. Every care was taken to establish these split seedlings by enclosing the unsplit portion of each plant (crown) with an ice cream cup open at both ends.

Bacterial inoculation. Twenty-five ml of bacterial suspension (10^{10} cells/ml) were poured around each plant at transplanting time (after splitting the roots) as a soil drench and then covered with the removed soil. A bacterial suspension was prepared by scraping the bacterial colonies from the media and mixing thoroughly in sterile distilled water. The number of bacterial cells/ml was ascertained before inoculations were made. Plants receiving 25 ml of sterile distilled water served as the control.

Nematode inoculation. The hand-picked egg masses of the nematode, collected from the culture pots, were kept in 5 cm diam Petri plates containing sterile distilled water and incubated at 25 ± 2 °C with the water changed every 24 hrs. The nematode suspension collected after a week, was poured into a graduated cylinder and the number of immature females and males/ml was ascertained. The top layer of the soil around the plant base was carefully removed to expose the fine roots and the nematode inoculation was performed by pouring two immature females and males of *R. reniformis* per g soil with a pipette. After inoculation, the removed soil was replaced.

The glasshouse experiment consisted of 10 treatments indicated in (Table I) and was terminated 83 days after sowing. Length, fresh and dry weights of shoot and root were recorded. The soil and root balls were depotted into a pan containing water. Egg masses were separated from nematode infected plants by a strong jet of tap water and then collected on a 100 mesh sieve. The number of egg masses per root system was counted under a stereomicroscope. The average number of eggs per egg mass was estimated after dissolving the gelatinous matrix in 0.5% NaOCl for 10-15 min. The final nematode population was extracted from 250 cm^3 soil using Cobb's sieving and decanting technique followed by modified Baermann funnel (Flegg, 1967). Also, a multiplication factor (Pf/Pi) was calculated by dividing the sum of egg masses per root system, eggs per egg mass and the nematode population in soil (Pf), by the initial inoculum density (Pi). The data were subjected to analysis of variance (ANOVA) using MSTAT-C software (Michigan State University Version 2.10) and differences among treatment means were determined with Duncan's multiple range test at a probability level of 5%.

RESULTS

Fewer nematodes were counted in tomato roots exposed to nematode inoculum a week after bacterial inoculum applied as a soil drench, in both unsplit and split roots compared to seedlings of both unsplit and split root inoculated with nematode alone (Table I). Nematode invasion of seedlings of both unsplit and split root systems exposed simultaneously to bacteria and nematodes was at par with other treatments. The number of nematodes that

Table I. Effect of *Bacillus subtilis* on penetration of *Rotylenchulus reniformis* into split/unsplit roots of tomato seedlings.

Treatment	Penetration	Per cent reduction over control
Bacterium (soil drench) + nematode one week after bacterization - split root	24.0 a** (4.87)*	44.5
Bacterium (soil drench) + nematode one week after bacterization - unsplit root	23.0 a (4.70)	51.0
Simultaneous inoculation of bacterium and nematode - split root	33.6 ab (5.77)	22.4
Simultaneous inoculation of bacterium and nematode - unsplit root	33.0 ab (5.71)	29.0
Nematode alone – split root (Control)	43.3 b (6.55)	-
Nematode alone - unsplit root (Control)	46.3 b (6.77)	-
LSD (0.05)	(1.39)	

Figures in parentheses represent square-root transformed values; ** within a column, data followed by the same letter are not significantly different ($P \geq 0.05$)

Table II. Effect of *B. subtilis* on growth characters of tomato split and unsplit root plants infested with *R. reniformis*.

Treatment	Shoot			Root		
	Length (cm)	Fresh weight (g)	Dry weight (g)	Length (cm)	Fresh weight (g)	Dry weight (g)
Nematode alone – split root	51.2 e*	27.2 c	2.95	18.1	3.3 bc	0.75
Nematode alone – unsplit root	53.7 de	27.5 c	3.00	17.2	3.0 c	0.80
Bacterium alone – split root	80.2 ab	40.2 ab	3.92	17.8	5.7 a	1.00
Bacterium alone – unsplit root	88.7 a	43.0 a	4.47	19.3	5.8 a	1.00
Bacterium + nematode (one week after bacterial inoculation) – split root	71.5 abcd	36.7 abc	3.75	16.7	5.4 ab	1.02
Bacterium + nematode (one week after bacterial inoculation) – unsplit root	74.2 abc	39.7 ab	4.15	17.3	5.4 ab	0.97
Bacterium + nematode (simultaneous) – split root	64.0 bcde	32.2 abc	3.52	18.5	5.2 ab	0.92
Bacterium + nematode (simultaneous) – unsplit root	66.5 bcde	32.2 abc	3.47	19.2	5.2 ab	0.92
No nematode + no bacterium – split root (control)	58.7 cde	30.5 bc	3.25	18.0	4.0 abc	0.90
No nematode + no bacterium – unsplit root (control)	60.5 cde	30.7 bc	3.30	18.3	4.1 abc	0.95
LSD (0.05)	16.76	10.21	N.S.	N.S.	1.94	N.S.

Within a column, data followed by the same letter are not significantly different ($P \geq 0.05$).

Table III. Influence of soil drench application of *B. subtilis* cell suspension (10^{10} cells/ml) on the multiplication of *R. reniformis* on split/unsplit roots of tomato plants.

Treatment	Nematode multiplication				
	No. of egg masses/root	No. of eggs/ egg mass	Soil population/ kg soil	Total	Pf/Pi (Multiplication rate)
Bacterium + nematode (one week after bacterization) – split root	52.0 a*	81.7	4176.0	8049.5 a	3.8 a
Bacterium + nematode (one week after bacterization) – unsplit root	49.7 a	83.2	4016.7	7360.5 a	3.6 a
Bacterium + nematode (simultaneous) – split root	70.0 ab	90.0	5280.5	10579.0 bc	5.2 bc
Bacterium + nematode (simultaneous) – unsplit root	68.7 ab	86.5	4952.0	12031.0 cd	6.0 c
Nematode alone – split root	73.5 b	101.0	5079.2	10802.0 bc	5.4 bc
Nematode alone – unsplit root	78.2 b	92.2	4075.7	13574.2 d	6.7 c
LSD (0.05)	19.4	N.S.	N.S.	2489.0	1.5

* Within a column, data followed by the same letter are not significantly different ($P \geq 0.05$).

invaded unsplit roots receiving the nematode one week after the bacterium inoculation, and its corresponding treatment with split root, unsplit root seedlings receiving simultaneous inoculations of bacteria and nematodes, and split root seedlings receiving simultaneous inoculations of bacteria and nematodes decreased by 51, 44.5, 29 and 22.4%, respectively, over the control (Table I).

Of all the plant growth parameters recorded, significant increase was observed only in shoot length, fresh shoot weight and fresh root weight (Table II). The highest increase in shoot length (88.7 cm), fresh shoot weight (43 g), and fresh root weight (5.8 g) was found in unsplit root plants receiving the bacterium alone. The minimum increase in shoot length (51.2 cm) and fresh shoot weight (27.2 g) was found in split root plants receiving nematode alone (control), while the fresh root weight (3 g) was found in unsplit root plants receiving nematode alone.

The data on nematode multiplication (Table III) revealed lesser production of egg masses/root, eggs/egg mass, nematode population in soil, total nematode population and multiplication rate in all the treatments that received bacterium compared to unsplit or split roots of tomato seedlings infected with nematodes alone. Significantly, fewer egg masses were formed on both unsplit (49.7) and split (52.8) root of tomato seedlings inoculated with the nematode, one week after bacterial inoculation, as compared to unsplit or split root of seedlings inoculated with the nematode alone. The highest reduction in egg mass production recorded was 46.2% in the treatment that received nematode one week after bacterial inoculation as soil drench, around the unsplit root system followed by 29.6% in the split root of its corresponding treatment. In the case of simultaneous inoculation of the bacterium and nematode, the reduction in egg mass production was 10.4 and 3.7%, respectively in both unsplit and split roots. The data recorded on number of eggs in egg masses and the nematode population in soil were, however, found non-significant, although small reductions were observed in treatments that received bacterial inoculations.

Regarding total nematode population and its multiplication rate (Pf/Pi), the reduction trend was similar to that of egg mass production except for the difference that the treatment that had received nematodes, after one week of the bacterium inoculation in both unsplit and split root; simultaneous inoculations of the bacterium and nematode in split root, and unsplit root inoculated with nematode alone were significantly different from each other. Further, the nematode multiplication rate was suppressed (46.2%) in the treatment that received seed bacterization followed by a soil drench with the bacterium and the nematode, one week after the bacterium inoculation on unsplit root, compared to the control. This was followed by 29.6, 10.4, and 3.7% respectively, with treatments that received the nematode

one week after bacterial inoculation on split root, simultaneous inoculation of the bacterium and nematode on unsplit root, and its corresponding split root.

DISCUSSION

The results of the present study show that the rhizobacterium (Bs_t)-mediated induced-systemic-resistance significantly reduced nematode penetration by 44.5%. This phenomenon was recorded in split roots where one half received the nematode, while its corresponding half received bacterial cell suspension as a soil drench, one week before nematode inoculation. In its corresponding unsplit roots nematode penetration was reduced by 51%. However, simultaneous inoculations of the bacterium and the nematode showed no significant reduction in nematode penetration, although 22.4 and 29% reduction was observed in split and unsplit roots, respectively. Furthermore, splitting of roots did not adversely effect nematode penetration.

All measured growth characters were improved to some extent, though some growth characters showed no significant increase compared to the control. Root length was an exception that showed no uniform trend.

Nematode multiplication rate was significantly lower when soil drenching with the bacterium was done one week before nematode application. Simultaneous treatments (split and unsplit), however, did not show this effect. Possibly it may be due to early colonization of the roots by the bacterium when applied one week prior to nematode inoculation, leading to induced systemic resistance in tomato plants by eliciting some chemicals that modify root exudates affecting penetration of the nematode. Our results corroborate the findings of Kerry (2000), who also found that induced resistance reduced invasion, but once inside roots the nematodes develop normally.

Separation of the nematode and the bacterium spatially on split roots of individual plant fail to compete for specific sites or nutrients as well as lectin-carbohydrate bindings. The recorded results can probably be explained by induced systemic resistance. Similar phenomena were reported by Hasky-Gunter *et al.* (1998).

The present study suggests that *B. subtilis* (Bs_t) is able to reduce the nematode penetration and multiplication through induced systemic resistance but it requires detailed studies on the biochemical and physiological basis of this phenomenon.

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