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Bacillus megaterium strain NBAII 63: A potential biocontrol agent for the management of bacterial wilt of tomato caused by *Ralstonia solanacearum*

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Bacterial wilt caused by *Ralstonia solanacearum* (Smith*)* Yabuuchi *et al*. (1995) is primarily a soil borne disease widely distributed in the tropics, subtropics and warm temperate regions of the world (Buddenhagen 1962). *R. solanacearum* is a rod shaped, gram negative, β proteobacterium that causes bacterial wilt in more than 200 plant species including many economically important crops. In extreme cases the loss in yield due to the disease in tomato and egg plant is reported to be as high as 90 per cent (Sivakumar *et al*. 2011). Biological control can have an important role in the management of bacterial wilt as singly or more effectively by integration with other practices for effective disease management at the field level. Although there is a potential for managing the disease using biocontrol agents. *Bacillus* spp. such as *B. mesentericus*, *B. megaterium*, *B. subtilis*, and *B. mycoides* have been reported as active biocontrol agents (Doan and Nguyen 2006). *Bacillus* spp. and its related genera have been identified as potential biocontrol agent as they produce wide range of cyclic peptides active against various microorganisms. The present study was undertaken to identify the potential *Bacillus* species for the management of bacterial wilt of tomato under greenhouse and field conditions.

The pathogen causing bacterial wilt in tomato was isolated from infected tissue of tomato plants using Triphenyl Tetrazolium Chloride (TTC) medium. The pathogenicity of *Ralstonia solanacearum* was confirmed by root dipped inoculation method (Winstead and Kelman 1952) on the 20-day-old tomato seedlings. Koch's postulates were established through pathogenicity test. Rhizosphere colonizing *Bacillus* spp. were isolated from rhizosphere of various crops grown in different geographic areas and soil types of India as per the standard procedure. The bacterial colonies were characterized based on their morphology, gram staining and endospore staining for presence of spores and also growth under aerobic and anaerobic conditions (Norris *et al*. 1981, Sneath 1986).

One hundred isolates of *Bacillus* spp. were screened against *R. solanacearum* under *in vitro* by filter disc method (Dhingra and Sinclair 1995) and well method (Emami *et al*. 2006). Ten promising isolates of *Bacillus* spp. (NBAII 63, 7, 33, 56, 65, 79, 25, 71, 43 and 34) were further screened under pot culture condition against bacterial wilt to select the most promising one. The antagonists were applied to the seeds, seedling and soil. Tomato seeds (cv Selection 22) were initially surface sterilized with 1% sodium hypochlorite followed by five washings with sterile water. Seeds (15 seeds per isolate) were treated with each bacterial suspension (10^8 cfu/ml) prepared from 48 hr old *Bacillus* spp. culture on Nutrient Agar and were shade dried at 28±2°C for 1 h. Treated seeds were sown in the pots containing sterile potting mixture having river sand, soil and farmyard manure in the ratio of 1:1:1. The potting mixture was sterilized (121ºC and 15psi) for 1 hr on two consecutive days. After 20 days, the seedlings were uprooted carefully from the pots and roots were dipped in to the respective antagonistic bacterial suspension for 30 min. The treated seedlings were transplanted (3 seedlings per pot) in the pots containing sterile potting mixture drenched with the antagonistic bacterial suspension (20 ml/pot). Then the culture suspension of *R. solanacearum* (2×10^8 cfu/ml) were drenched evenly @ 20 ml/pot 5 days after transplanting. Three replications were maintained for each antagonistic bacterial treatment. The wilt incidence was calculated by counting the total number of plants and infected plants.

The most promising *Bacillus* isolate NBAII 63 was identified through 16S rDNA analysis using universal primers fD1 5´-GAGTTTGATCCTGGCTCA-3´ and rP2 5´-ACGGCTACCTTGTTACGACTT-3´.The nucleotide sequences of 16S rRNA were deposited in Genbank, NCBI and accession number assigned.

Talc formulation of *B. megaterium* was developed as per the standard procedure (Rangeshwaran *et al*. 2010) and bioefficacy of formulation was evaluated under green house and field condition for promotion of their growth and

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suppression of bacterial wilt in tomato. A pot culture experiment was conducted during 2010 with the following treatments by using completely randomized design (CRD) using cultivar sele.22. The treatments were: i. Seed treatment (ST), ii. Seedling root dip (SRD), iii. Soil application (SA), iv. Foliar spray (FS), v. Seed treatment + Seedling root dip, vi. Seed treatment+Soil application, vii. Seed treatment+Foliar spray, viii. Seedling root dip+Soil application, ix. Seedling root dip+Foliar spray, x. Soil application+Foliar spray, xi. ST+SRD+SA, xii. SRD+ SA+FS, xiii. ST+SRD+SA+FS, xiv. Streptomycin sulphate and xv. Control. One gram of formulation consisted of 10^8 cfu of bacteria when it was applied. For seed treatment, the seeds were initially surface sterilized with 1% sodium hypochlorite followed by five washings with sterile water. Seeds were treated with the talc formulation (4g/kg of seed) and dried under shade for 30 min and sown. After 20 days, the seedlings were uprooted carefully from the pots and roots were dipped in water containing talc formulation (10 g/L) for 1 h. The treated plants were transplanted at the rate of three plants per pot (30 cm diameter and /40 cm height) containing the sterile potting mixture having river sand, soil and farm yard manure in the ratio of 1:1:1. The potting mixture was sterilized (121°C and 15psi) for 1 hr for two consecutive days. For soil application, the talc formulation was applied 10 days after planting at the rate of 2.5 kg/ha. For foliar spray (FS), the talc formulation was thoroughly mixed in water (10 g/L) and allowed to settle for 1 h, filtered through muslin cloth and sprayed 10 days after transplanting. The culture suspension of *R. solanacearum* (10^8 cfu/ml) was drenched evenly @ 30 ml/pot at 15 days after transplanting. Root dipping and spraying with streptomycin sulphate (1 g/L) at 15 days after transplanting served as chemical check. Control was also maintained without any treatment. The efficacy of the bioformulations on various growth parameters, viz. root length, shoot length, wet weight and dry weight were recorded. The wilt incidence was calculated by counting the total number of plants and infected plants up to 90 days after transplanting. The bacterial population in the tomato rhizosphere was assessed at twenty days intervals by serial dilution technique.

A field experiment was conducted in completely Randomized Block Design of 4×5 m with three replications using the tomato cultivar sele. 22. Each plot contained 25 plants. The field experiment was conducted during 2011 in the research farm of National Bureau of Agriculturally Important Insects, Bengaluru. The tomato seedlings were raised on 10 June 2011 and planted and on 25 June. The soil type of the field experiment was red laterite. The treatments which were used for pot culture experiments were repeated again and the observations were recorded.

A total of 100 isolates *Bacillus* spp. were collected from rhizosphere soils of various crops. The bacterial colonies were characterized based on their morphology, gram staining and endospore staining for presence of spores. The bacterial cultures were maintained in glycerol stock. The typical wild type colonies *of R. solanacearum* which formed an irregularly-round, fluidal, white colonies with a pink centre were collected and stored in sterilized water. The inoculated tomato seedlings were showing drooping of the leaves followed by sudden wilting and complete collapse of the plants. The pathogen isolated from the inoculated plants resembled the original *R. solanacearum*. On re-inoculation the same symptoms were observed. Koch's postulates were established. Among the 100 *Bacillus* isolates, ten of them (NBAII 7, 25, 33, 34, 43, 56, 63, 65, 71 and 79) were found inhibitory against *R. solanacearum* under *in vitro*. The inhibition zone for all the ten isolates ranged from 4.1 to 15.5 mm by the filter disc method and 3.7 to 9.2mm by well method. *Bacillus* isolate NBAII-63 showed the highest inhibition zone of 15.5 mm in the filter method and 9.2 mm in the well method (Table 1). The inhibitory property of the isolates reflects the inherent potential of the organism to produce inhibitory metabolites against *R. solanacearum*. It is known that the extent of inhibition zone formation is related to the ability of the organism to produce inhibitory metabolites against the test organism (Sivaprasad 2002). The results of Table 1 further revealed that the isolate NBAII 63 performed better while subjecting under green house screening as compared to other isolates. Highest root growth (12 cm) and shoot growth (58.1 cm) and lowest wilt incidence (18.1%) were recorded when the tomato plants treated with the culture suspension of *Bacillus* isolate NBAII 63 as compared to control where the wilt incidence was recorded as 88.1% (Table 1). The *Bacillus* isolate NBAII 63 was selected as promising one based on the results of *in vitro* and *in vivo* experiments. The promising *Bacillus* isolate NBAII 63 was identified as *Bacillus megaterium* through 16S rDNA analysis and the Genbank accession number assigned is HQ 162492.

Table 1 Screening of *Bacillus* spp. against *Ralstonia solanacearum*

Isolates of *Bacillus* spp.	*In vitro* screening		*In vivo* screening (Green house)		
	Inhibition of *R. solanacearum* (mm)		Root length (cm)	Shoot length (cm)	Wilt incidence (%)
	Filter disc method	Well method			
NBAII 63	15.5	9.2	12.0	58.1	18.1
NBAII 7	9.4	7.2	8.0	52.4	23.6
NBAII 33	9.3	7.1	7.5	45.3	28.1
NBAII 56	7.4	6.7	7.0	44.2	27.2
NBAII 65	7.2	6.1	7.2	40.5	31.1
NBAII 79	5.4	5.5	6.7	42.2	35.3
NBAII 25	5.2	5.2	6.5	35.1	40.9
NBAII 71	4.7	5.1	6.0	33.3	45.3
NBAII 43	4.2	4.1	6.2	32.4	47.2
NBAII 34	4.1	3.7	5.5	31.2	54.1
Control	0.0	0.0	4.1	22.0	88.12
SEM	0.11	0.05	0.04	0.16	0.09
CD (P = 0.01)	0.45	0.20	1.6	0.51	0.39

Table 2 Effect of talc-based formulation of *Bacillus megaterium* strain NBAII 63 on the wilt incidence of tomato under green house condition

Treatment	Root length (cm)	Shoot length (cm)	Fresh weight (g)	Dry weight (g)	Wilt incidence (%)	Percentage reduction over control
Seed treatment (ST)	15.23	56.24	33.31	8.32	38.21	41.60
Seedling dip (SD)	15.34	55.13	31.23	7.80	41.32	36.84
Soil application (SA)	15.34	56.34	31.45	7.86	40.54	38.04
Foliar spray (FS)	16.21	54.23	30.14	7.53	43.25	33.89
Seed tmt+ seedling dip	16.45	61.14	37.23	9.30	35.31	46.03
Seed tmt+soil application	16.11	60.23	37.24	9.31	31.21	52.30
Seed tmt+foliar spray	15.31	61.45	36.56	9.14	37.42	42.80
Seed dip+soil application	16.92	60.25	36.14	9.03	32.23	50.74
Seedling dip+ foliar spray	15.21	61.74	36.21	9.05	38.53	41.11
Soil application +foliar spray	15.95	61.34	37.54	9.38	39.17	40.13
ST+SD+SA	18.23	63.56	37.25	9.31	28.23	56.85
SD+SA+FS	18.34	62.65	36.12	9.03	30.51	53.37
ST+SD+SA+ FS	21.12	63.21	38.29	9.57	26.21	59.94
Streptomycin sulphate	13.00	53.45	28.01	7.00	16.21	75.22
Control	8.00	27.12	15.21	3.80	65.43	0.00
SEM	0.08	0.18	0.05	0.07	0.93	
CD (P=0.05)	0.25	0.52	0.15	0.21	2.71	

Highest root length (21.12 cm), shoot length (63.21 cm), fresh weight (38.29 g) and dry weight (9.57 g) and highest reduction (59.94%) in wilt incidence were recorded under green house condition in the case of combined application as compared to single method of application such as seed treatment and soil application (Table 2). Among the single methods, seed treatment was found to be the best which resulted of wilt in 42% reduction of bacterial wilt incidence followed by soil application which resulted in 38% of wilt reduction as compared to other methods and control (Table 2). Nguyen *et al*. (2011) reported that *B. megaterium, P. guillermondii, E. cloacae* and *C. ethanolica* showed effectiveness in reducing the bacterial wilt when the antagonists were applied one week prior to transplanting of tomato seedlings. The present study also confirmed the efficacy of *B. megaterium* when the antagonist was applied as seed treatment and foliar spray. The results of the field experiment (Table 3) also revealed the same trend as that of green house experiment. Highest root length (26.2 cm), shoot length (74.1 cm), fresh weight (43.2 g) and dry weight (13.5 g) and lowest wilt incidence (22.3%) were recorded when all the four treatments were combined and applied which were significantly different from control where 50.7% of wilt incidence was observed. Among single methods, seed treatment was found to be the best and resulted in 33.3% of wilt incidence followed by soil application which resulted in 36.4% of wilt incidence as compared to other methods and control under filed condition (Table 3). The field experiment revealed that highest reduction (56.0%) of wilt incidence was recorded again where combined application method was followed as against single method of application such as seed treatment or soil application (Table 3).

Microorganisms that can grow in the rhizosphere are ideal for use as biocontrol agents, since the rhizosphere

Table 3 Effect of talc-based formulation of *Bacillus megaterium* strain NBAII 63 on the wilt incidence of tomato under field condition

Treatment	Seedling establishment (%)	Root length (cm)	Shoot length (cm)	Fresh weight (g)	Dry weight (g)	Wilt incidence (%)	Percentage reduction over control
Seed treatment (ST)	80.0	20.3	61.2	36.1	11.3	33.3	34.0
Seedling root Dip (SRD)	79.0	20.4	60.3	34.3	10.8	37.4	26.0
Soil application (SA)	79.0	20.4	61.3	34.5	10.8	36.4	28.0
Foliar spray (FS)	77.0	21.1	59.3	33.4	10.5	39.5	22.0
ST+SRD	82.0	21.5	66.1	40.3	12.3	31.1	38.0
ST+SA	83.0	21.1	65.2	40.4	12.3	26.1	48.0
ST+FS	82.0	20.1	66.4	39.6	12.1	37.4	26.0
SRD+SA	82.0	29.2	65.5	39.4	12.0	27.3	46.0
SRD+FS	80.0	20.1	66.4	39.2	12.0	33.3	34.0
SA +FS	79.0	20.5	66.3	40.4	12.3	34.2	32.0
ST+SRD+SA	85.0	23.3	68.5	40.2	12.3	23.3	54.0
SRD+SA+FS	83.0	23.4	67. 5	39.2	12.0	25.2	50.0
ST+SRD+SA+FS	88.0	26.2	74.1	43.2	13.5	22.3	56.0
Streptomycin sulphate	91.0	15.00	56. 5	30.1	10.0	15.4	69.0
Control	62.0	13.00	33.2	18.2	7.80	50.7	
SEM	0.54	0.05	0.39	0.05	0.05	0.86	
CD (P=0.05)	1.50	0.17	1.15	0.14	0.15	2.50	

provides the front-line defense against root attack by pathogens. According to Weller (1988), root-associated bacteria are an important group of beneficial microorganisms for controlling soil-borne pathogens and promoting plant growth promotion. Lwin and Ranamukhaarachchi (2006) showed that the application of bio-control agents also may affect the antagonist's action prior to pathogen attack. *B.megaterium*, *B. circulans*, *B. subtilis*, *B. polymyxa, B. sircalmous* could be referred as the most important strains of P solubilizers (Subbarao 1988). The application of biofertilizers containing the phosphate-solubilizing bacterium *B. megaterium* significantly increased the growth of *Zea mays* (Wu *et al*. 2005) and promoted growth of eggplant (Han and Lee 2005) pepper and cucumber (Han *et al*. 2006). In the present study good growth of tomato plants was achieved by the application of *B. megaterium*. Furthermore, like all *Bacillus* spp, *B. megaterium* has the ability to produce spores, which can be extremely resistant to high temperature, chemicals and UV radiation, and thus aid the survival of the bacterium in the natural environment (Roberts and Hitchins 1969). The present study confirmed that the wilt reduction was high when the *B. megaterium* was applied to the tomato plants through seed, soil and foliage. Liu and Sinclair (1993) reported that *B. megaterium* B153-2-2 colonized the rhizosphere of soybean under field conditions. In the present study also revealed that the introduced *B. megaterium* established well in the tomato rhizosphere and the population increased in all the treatments up to 40 days after transplanting and later decreased slowly. Significant increase (20 to 30 percent) in population at 40 days after transplanting in all the antagonist treated pots. Highest rhizosphere population (53.3 to 54.3×10^6 cfu/g of soil) was recorded at 40 days after transplanting when tomato plants raised separately from the antagonist treated seeds and antagonist treated soil as compared to the seedling root dip (49.4×10^6 cfu/g) and foliar spray (15.4×10^6 cfu/g). The population was further reached maximum to 67.3×10^6 and 71.2×10^6 cfu/g respectively under green house and field condition when the antagonist was applied as combination of all methods, i.e. seed treatment, soil application, seedling root dip and foliar spray (data was not shown). Multiplication and persistence of the organism in rhizosphere depend upon the competitive ability and adaptability of the organism (Brooks *et al*. 1994). The results of the present study clearly indicated that there is higher multiplication and persistence of the introduced *B. megaterium* in the tomato rhizosphere which is an essential criterion for the success of biological control. The highest disease suppression recorded in the present study may be due to the better root colonization, multiplication and persistence leading high build up of population of *B. megaterium.*

In the present study also the survival of the antagonist *B. megaterium* in the tomato rhizosphere was related in the management of the bacterial wilt disease. From this study it is concluded that application of *B. megaterium* (NBAII-63) in a combination approach such as seed treatment, seedling root dip, soil application and foliar spray significantly reduced the wilt incidence of tomato. This organism shows potential for use as a promising biological control agent in tomato.

SUMMARY

Among 100 isolates of *Bacillus* spp. screened under *in vitro* condition, ten of them were found inhibitory against *Ralstonia solanacearum* which causes bacterial wilt of tomato. *Bacillus* isolate NBAII-63 was selected as promising one based on *in vitro* and in *vivo* screening. The promising *Bacillus* isolate NBAII 63 was identified as *Bacillus megaterium* by 16S rDNA analysis. The bio-efficacy of talc formulation of *B. megaterium strain* NBAII-63 was evaluated under green house and field for plant growth promotion and suppression of bacterial wilt in tomato. A combined application of seed treatment, soil application, seedling root dip and foliar spray was found effective for the management of bacterial wilt of tomato. Combined application resulted in reduction of wilt incidence to 59.9% and 56.0% under green house and field conditions respectively. Streptomycin sulphate application recorded 75.0% reduction in wilt under green house and 69.0% under field conditions. Significant increase in growth parameters, viz. root length (26.2 cm) and shoot length (74.1 cm) of tomato plants were recorded under field condition due to application *B. megaterium* as compared to control where it was 13.0 cm and 33.2 cm respectively. The per cent increase in root and shoot length of tomato plants due to application of *B. megaterium* over control was 50.3 and 55.2 respectively. Highest rhizosphere population *B.megaterium* of 71.2×10^6 cfu/g was recorded in field condition at 40 days after transplanting when the antagonist was applied as a combination of seed treatment, seedling root dip, soil application and foliar spray. Application of *B. megaterium* strain NBAII-63 in a combination approach such as seed treatment, seedling root dip, soil application and foliar spray significantly reduced the wilt incidence of tomato. This organism shows potential for use as a promising biological control agent in tomato.

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