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Research Article

Screening and identification of potential *Bacillus* spp. for the management of bacterial wilt of brinjal

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ABSTRACT: Among 100 isolates of *Bacillus* spp. screened *in vitro* against *Ralstonia solanacearum* that causes bacterial wilt of brinjal, ten were found inhibitory to *R. solanacearum*. *Bacillus megaterium* isolate NBAII 63 was highly inhibitory with 29.20 mm of inhibition zone against *R. solanacearum* as compared to the other nine strains of *Bacillus*. Six of them were identified by 16S rDNA analysis. The bioefficacy of talc formulation of *B. megaterium* was evaluated under greenhouse for plant growth promotion and suppression of bacterial wilt in brinjal. The bacterial wilt was effectively managed by *B. megaterium* through different methods of applications. A combination of four methods (seed treatment + soil application + seedling root dip + foliar spray) was the most effective. Maximum root length (23.42 cm), shoot length (65.21 cm), fresh weight (40.39 g), dry weight (10.33 g) and highest wilt reduction (50.54%) was recorded in the combination method. Among single application methods, seed treatment was effective exhibiting 41% reduction of bacterial wilt followed by soil application which gave 36% wilt reduction. The bacterial wilt reduction in chemical control (streptomycin sulphate) was 71%. Good growth of the brinjal plants was recorded due to application of *B. megaterium*. Highest rhizosphere population of 67.0 x 10^6 cfu/g was recorded in brinjal at 40 days after transplanting when the antagonist was applied by combining the different application methods.

KEY WORDS: *Bacillus* spp., *Ralstonia solanacearum,* bioefficacy, talc formulation, brinjal

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INTRODUCTION

Bacterial wilt disease caused by *Ralstonia solanacearum* is one of the most devastating diseases of solanaceous vegetable crops in India and affects more than 450 plant species across the world. There is a typical browning of vascular tissues in roots, stems and tubers. The bacterium exists in different races and biovars. In extreme cases, the loss in yield due to the disease in egg plant and tomato has been reported to be as high as 80 and 90 per cent respectively. As the disease is widely distributed, it has a wide host range and is mainly soil borne; it is difficult to control with chemicals and cultural practices (Grimault *et al*., 1993). Biological control can play an important role in the management of bacterial wilt either singly or by integration with other practices at the field level. Although there is a potential of managing the disease through the use of biocontrol agents, no comprehensive studies were made to exploit bacterial antagonists especially *Bacillus* spp. against the bacterial wilt disease. *Bacillus* spp. such as *B. mesentericus*, *B. megaterium*, *B. subtilis*, and *B. mycoides* have been reported as effective biocontrol agents (Shekhawat *et al*., 1993; Doan and Nguyen, 2006) and more specifically for the management of control several root and foliar disease (Kloepper, 1997; McSpadden–Gardener and Fravel, 2002; Sally and Peter, 2004). The present study was undertaken to identify the potential *Bacillus* species for the management of bacterial wilt of brinjal under greenhouse condition.

MATERIALS AND METHODS

Isolation of pathogen

Bacterial wilt pathogen of brinjal, *R. solanacearum* was isolated from infected tissue of brinjal (egg plant) plants using triphenyl tetrazolium chloride (TTC) medium (Kelman, 1954). The collar portion of infected plants were cut into two or three pieces and put into sterile water. The bacterial ooze coming out of the cut end was streaked on to TTC medium. The growth of the pathogen was observed after 48 hours. The pathogenicity of the bacterial isolates was confirmed by root dipped inoculation method (Winstead and Kelman, 1952) on the 20-day-old brinjal seedlings. The roots of the seedlings were dipped in 10^7 cfu /ml concentration of

R. solanacerum suspension. Inoculated seedlings were transplanted in 10 cm pots filled with sterilized soil and placed at 30ºC in green house. Symptom developments were observed at 14 days after inoculation.

Isolation of rhizobacteria

Rhizosphere colonizing *Bacillus* spp. were isolated from rhizosphere of various crops grown in different geographic areas and soil types of India. Fresh roots (1 g) were suspended in 10 mL of sterile water and vortexed for five minutes. The suspension was held at 75°C for 20 min to kill all vegetative microbial cells. The suspension was then plated on to Nutrient Agar plates and incubated at 37°C for 24 h. The growths of bacterial colonies were recorded. 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).

In vitro* screening of *Bacillus* spp. against *R. solanacearum

One hundred isolates of *Bacillus* spp. were screened against *R. solanacearum in vitro* by filter disc method (Dhingra and Sinclair, 1995). *R. solanacearum* suspension was first inoculated on to nutrient agar plates by spread plate. Antagonists were supplied as filter paper disks of 5 mm in diameter which were soaked with 20 µl of the antagonist suspension. Three replications of each isolate including control with sterile water were maintained. The discs were placed in the petriplates immediately. The effect of antagonism was measured 2 days after incubation.

Identification of promising *Bacillus* isolates

Among the ten promising *Bacillus* isolates, six of them were identified through 16S rDNA analysis. Bacterial DNA was isolated using the HiPurA genomic DNA isolation kit from HiMedia. 16S rRNA gene was amplified from the genomic DNA of bacteria using universal primers fD1 5´-GAGTTTGATC CTGGCTCA-3´ and rP2 5´-ACGGCTACCTTGTTA CGACTT-3´. Purified PCR products were sequenced; homology search of the 16S rRNA sequences was done using the BLAST function of NCBI Genbank. The 16S rRNA sequences were aligned with other *Bacillus* sp. 16S rRNA sequences obtained from Genbank, NCBI, USA using Clustalx software (Higgins *et al*., 1992). The nucleotide sequences of 16S rRNA were deposited in Genbank, NCBI, USA and obtained the accession numbers of the six *Bacillus* isolates.

Bioefficacy studies with talc based formulation of *Bacillus megaterium* isolate NBAII 63 against bacterial wilt of brinjal under green house condition

The most promising *Bacillus* isolate *B. megaterium* NBAII 63 was used for formulation development and pot culture studies. 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 condition for promotion of their growth and suppression of bacterial wilt in brinjal. A pot culture experiment was conducted with the following treatments by using completely randomized design (CRD) with three replications. 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.

The treatments were imposed as seed treatment, seedling root dip, soil and foliar application. 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 on two consecutive days. For soil application, the talc formulation was applied 10 days after planting at the rate of 5 g/kg of soil. 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^{-1}) was drenched evenly @ 30 ml pot^{-1} at 15 days after transplanting. Root dipping and spraying with streptomycin sulphate (1g/L) at 15 days after transplanting served as chemical check. Control was also maintained with out any treatment. The efficacy of the bio formulations 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. The bacterial population in the brinjal rhizosphere was assessed at monthly intervals by serial dilution technique.

RESULTS AND DISCUSSION

Collection of rhizosphere bacteria

Totally 100 isolates of bacteria 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 cultures were stored in sterile water.

Collection of pathogen and pathogenicity test

The typical wild type colonies *of R. solanacearum* which formed an irregularly-round, fluidal, white colonies with a pink centre (Kelman, 1954) were collected and stored in sterilized water. The inoculated brinjal plants were showing drooping of the leaves followed by sudden wilting and complete collapse of the plants.

In vitro screening of antagonistic bacteria against *R. solanacearum*

Among the 100 *Bacillus* isolates, ten of them (NBAII 63,7,33,56,65,79,25,71,43 and 34) were found inhibitory against *R. solanacearum*. *Bacillus* isolate NBAII-63 showed the highest inhibition zone of 29.20 mm followed by NBAII-7 with 17.40 mm, NBAII-33 with 15.30mm with 15.30mm and NBAII-56 with 13.40mm, NBAII-65 with 6.20mm and NBAII-34 with 4.1mm (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 inhibitory activity of ten isolates of *Bacillus* of this present study could be due to high production of antimetabolites against *R. solanacearum.*

Identification of promising *Bacillus* spp.

Six promising *Bacillus* isolates were identified through 16S rDNA analysis and the accession numbers assigned (Table 1and Fig. 1). The identified species were NBAII-63 (*Bacillus megaterium*), NBAII-25 (*B. subtilis*), NBAII-7 (*B. cereus*), NBAII-71 (*B. cereus*), NBAII-33 (*B. cereus*) and NBAII- 65 (*B. megaterium*).

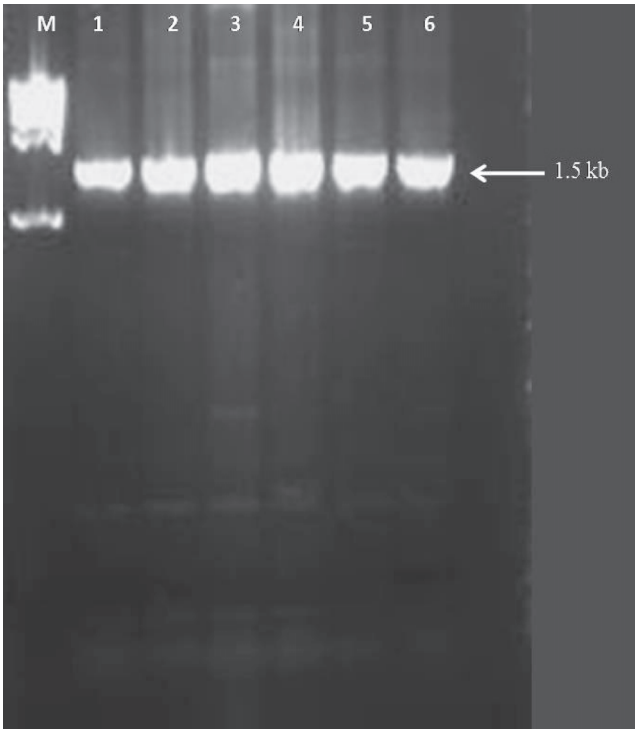

Fig. 1. 16S rRNA amplification of 1.5kb (M = 1 kb DNA ladder; Lane 1 = NBAII-63; Lane 2 = NBAII-7; Lane 3 = NBAII-25; Lane 4 = NBAII-71; Lane 5 = NBAII-33; Lane 6 = NBAII- 65)

Table 1. Identification of *Bacillus* spp. through 16S rDNA analysis

Sl. No.	*Bacillus* isolates	Identified species	Gen bank accession number
1	NBAII-33	*Bacillus cereus*	HQ162491
2	NBAII-63	*Bacillus megaterium*	HQ162492
3	NBAII-25	*Bacillus subtilis*	HQ162493
4	NBAII-7	*Bacillus cereus*	HQ162494
5	NBAII-71	*Bacillus cereus*	HQ162495
6	NBAII-65	*Bacillus megaterium*	HQ162496

Bioefficacy studies with talc based formulation of *Bacillus megaterium* isolate NBAII 63 against bacterial wilt of brinjal under green house condition

The bacterial wilt of brinjal disease was effectively managed by *B. megaterium* through different methods of applications. All four application methods were evaluated individually as well as in combination. Among single methods, seed treatment was found to be the best and resulted in 41% reduction of bacterial wilt followed by soil application with 36% reduction as compared to other methods and control (Table 2). A combination of all four methods was the most effective approach for the bacterial wilt disease management in brinjal. Maximum root length (23.42 cm), shoot length (65.21 cm), fresh weight (40.39 g) and dry weight (10.33 g) and highest reduction (50.54%) in wilt incidence were recorded in the case of combination approach as compared to single method of application such as seed treatment and soil application (Table 2). The bacterial wilt reduction in chemical control (streptomycin sulphate) was 71%. 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 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.

Microorganisms that can grow in the rhizosphere are ideal for use as biocontrol agents, since the rhizosphere provides the front-line defense against root attack by pathogens. Pathogens encounter antagonism from rhizophere microorganisms before and during primary and secondary root infection. 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. Bacteria are more effective in phosphorus solubilization than fungi (Alam *et al.*, 2002). Phosphorus solubilizing bacteria mainly *Bacillus, Pseudomonas* and *Enterobacter* are very effective for increasing the plant available P in soil as well as the growth and yield of crops. *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

Table 2. Effect of talc-based formulation of *Bacillus megaterium* isolate NBAII 63 against bacterial wilt of brinjal under greenhouse condition

Treatments	Root length (Cm)	Shoot length (Cm)	Fresh weight (g)	Dry weight (g)	Wilt incidence (%)	Percentage reduction Over control
Seed treatment (ST) – 4g/kg	17.31	58.24	35.34	9.52	39.42	40.55
Seedling root dip (SRD) 10g/L	17.37	57.13	33.33	8.90	40.15	36.05
Soil application (SA)5 g/kg of soil	16.69	58.34	33.55	8.96	40.23	35.92
Foliar spray (FS) 10g/L	17.18	56.23	32.44	8.73	42.27	32.68
ST + SRD	17.67	63.14	39.43	10.50	33.14	47.22
ST + SA	17.18	62.23	39.34	10.61	32.63	48.03
ST + FS	15.57	62.45	38.56	10.74	33.15	47.20
SRD + SA	16.77	62.25	38.44	10.83	33.45	46.72
SRD+ FS	16.88	63.74	38.31	10.95	35.25	43.87
SA + FS	16.37	63.34	39.34	10.18	34.24	45.46
ST + SRD + SA	19.63	65.56	39.35	10.66	32.71	47.90
SRD + SA + FS	19.75	64.65	38.22	10.32	33.45	46.72
ST + SRD + SA + FS	23.42	65.21	40.39	10.33	31.05	50.54
Streptomycin sulphate (1g/L)	14.45	55.45	31.34	7.98	18.21	70.99
Control	9.23	29.42	18.34	5.68	62.79	0.00
CD (P = 0.05)	2.15	3.01	1.23	2.67	3.12	

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 brinjal plants was achieved by the application of *B. megaterium*. It has previously been tested and has shown the potential to inhibit or suppress a range of plant diseases, occurring on both the roots and aerial parts of the plant (Liu and Sinclair, 1992; Jung and Kim, 2005). 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 brinjal plants through seed, soil and foliage. The earlier reports (Wydra and Semrau, 2005; Nguyen and Ranamukhaarachchi, 2010) also highlighted that comparable *R. solanacearum* wilt disease reduction and growth and yield increase associated with biocontrol agents *Bacillus* spp.

The introduced *B. megaterium* established well in the brinjal rhizosphere and the population increased in all the treatments up to 40 days after transplanting and later decreased slowly. The population of the antagonist was not observed in the chemical treated and control pots. Significant increase (20 to 30 percent) in population at 40 days after transplanting in all the antagonist treated pots. Highest rhizosphere population (48.31 to 51.12 x 10^6 cfu/g of soil) was recorded at 40 days after transplanting when brinjal plants raised separately from the antagonist treated seeds and antagonist treated soil as compared to the seedling root dip (46.46 x 10^6 cfu/g) and foliar spray (14.53 x 10^6 cfu/g). The population was further reached maximum to 64.75 x 10^6 cfu/g when the antagonist was applied as combination of all methods i.e. seed treatment, soil application, seedling dip and foliar spray. It was noticed that there was more than 60 percent decrease in population of *Bacillus* spp. at 60 days after transplanting in all the treatments (Table 3). Fewer incidences in the combined approach were mainly due the survival and multiplication of the antagonist in rhizosphere. *B. megaterium* is often found in rhizospheres and phylloplanes and is able to thrive under different nutrient conditions. Competent antagonistic bacteria should be able to establish themselves in the plant rhizosphere at population densities sufficient to produce beneficial effect after planting. Efficient antagonistic bacteria survive in the rhizosphere by making use of the exudates secreted by the plant root, proliferate, colonize the entire root system and compete with the indigenous microorganisms (Bloemberg and Lugtenberg, 2001). Multiplication and persistence of the organism in

Table 3. Population of *Bacillus megaterium* in brinjal rhizosphere under greenhouse condition

Treatments	Population (10^6 cfu/g of soil) days after transplanting		
	20	40	60
Seed treatment (ST)	39.91	48.31	13.22
Seedling root dip (SRD)	37.13	46.46	14.32
Soil application (SA)	41.34	51.12	18.02
Foliar spray (FS)	12.57	14.53	5.50
ST + SRD	42.25	53.24	19.14
ST + SA	37.33	47.34	17.33
ST + FS	41.44	51.45	15.82
SRD + SA	42.51	53.56	17.21
SRD + FS	36.22	51.29	16.14
SA + FS	41.35	51.38	16.35
ST + SRD + SA	42.51	52.32	17.25
SRD + SA + FS	42.22	53.24	14.26
ST + SRD + SA + FS	46.17	64.75	19.13
CD (P = 0.05)	0.71	0.61	0.52

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 brinjal 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 multiplication and persistence leading high build up of population of *B. megaterium.* Presence and survival of *B. megaterium* in the soil, roots and rhizosphere of tomato plants have been observed and their role in the control of bacterial wilt have been well documented (Nishijima *et al*., 2005). In the present study also the survival of the antagonist *B. megaterium* in the brinjal rhizosphere was related in the management of the bacterial wilt disease of brinjal. From this study it is concluded that application of *B. megaterium* (NBAII-63) in a combination approach such as seed treatment, root dip, soil application and foliar spray significantly reduced the wilt incidence of brinjal. This organism shows potential for use as a promising biological control agent in brinjal.

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