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Biocontrol Potential of Endophytic *Bacillus* Spp. Against *Meloidogyne Incognita* in Tomato

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ABSTRACT: *Plant samples were collected from different crops in Coimbatore district of Tamil Nadu in order to identify the most effective native isolates of endophytic Bacillus spp against root knot nematode, Meloidogyne incognita. Based on in vitro studies on inhibition of egg hatching and mortality of juveniles on exposure to the culture filtrate of Bacillus spp, five isolates viz. Bacillus weihenstephanensis (TSB4), B.subtilis (TSB5), B.cereus (TSB4D and CLB2D) and B. licheniformis (TSB3) were short listed as the most effective isolates and developed as talc formulation (2x10^{8}cfu/ml). The formulation is tested against M.incognita through laboratory assay and experiments under glasshouse conditions (30±2 °C).*

The experimental results indicated that B. weihenstephanensis isolate (TSB4) collected from the stem of tomato is proved to be most effective against M. incognita in tomato. The isolate showed the highest inhibitory effect in vitro exhibited the highest reduction in nematode population both in soil (52%) and root (67.76%) as well as nematode incidence in terms of gall index (76.25%) and increased fruit yield by 59.18 per cent in tomato under controlled conditions.

Key words: *Bacillus, culture filtrate, M. incognita, root knot nematode, talc formulation,*

INTRODUCTION

The most important vegetable crop tomato (*Solanum lycopersicum* Mill) widely cultivated in Tamil Nadu, India is affected by root knot nematode, *M. incognita* race3. Tomato fruit yield losses due to *Meloidogyne incognita* is assessed as 32 (Netscher and Sikora, 1990) and as high as 85 per cent (Sasser, 1979; Taylor and Sasser, 1978). The current research on nematode management particularly in vegetables like tomato is focused on the exploitation of nematode antagonists preferably on endophytic bacteria to contain the nematode disease since most of the members of the endophytic bacteria belonging to *Bacillus* spp are often considered as microbial factories for the production of a vast array of biologically active molecules potentially inhibitory for phytopathogens (Munif *et al.*, 2000; Vetrivelkalai *et al.*, 2010).

Therefore attempts were made to isolate and screen the biocontrol potential of indigenous endophytic *Bacillus* spp against *M.incognita* through laboratory assay and experiments under controlled conditions in the present study.

MATERIALS AND METHODS

The isolates of *B.weihenstephanensis*, *B.cereus*, *B.subtilis*, *B.licheninformis* collected from different plant parts *viz.* stem, leaves and roots of tomato, chilli, brinjal in Coimbatore district of Tamil Nadu (Quadt-Hallmann and Kloepper, 1996) were suitably designated after confirming the species identity of *Bacillus* through serial dilution method followed by biochemical (Krieg and Holt, 1984) and molecular characterization (Goto *et al.*, 2000).

Healthy tissues of leaves and stems of above plants were put in a beaker, soaked in distilled water and then drained. Tissues were rinsed with 70% ethanol for 30 sec. and then sterilized with 0.1% $HgCl_2$ for 3min. (Hallmann *et al.*, 1997). The tissues were then washed ten times with sterile water (Gagne *et al.*, 1987). The surface-disinfected tissues were aseptically macerated in a homogenizer. The macerated tissues were diluted ten times by adding nine volumes of sterile distilled water. Serial dilutions were made up to 10^{-6} by taking 1 ml of well-shaken suspension and 9 ml water in tubes. One ml. samples from the

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dilutions spread on plates with 15 ml. of melted Nutrient Agar (NA) medium in sterile Petri dishes were rotated gently in clockwise and anticlockwise direction and incubated at room temperature (28 ± 2°C) for 48h for the development of colonies of *Bacillus* spp. After incubation, the colonies of *Bacillus* spp., from the selective medium were subcultured to nutrient agar slants as well as nutrient agar Petri plates. The colonies formed were identified as *Bacillus* spp based on description given by Bergey's manual of Systematic Bacteriology (Krieg and Holt, 1984). Further through different bio-chemical as well as molecular characterisation tests, all the isolates collected in the present study were identified and confirmed as *Bacillus* spp (Table 1).

Table 1
Identification of *Bacillus* spp through Molecular Characterisation Based on Comparison of 3′ end 16S rDNA and 5′ end 16S - 23S ITS Nucleotide Sequences
(Goto *et al.*, 2000)

Bacillus spp	*Isolates*
Bacillus weihenstephanensis	TSB4
Bacillus cereus	CLB2D and TSB4D
Bacillus subtilis	TSB5
Bacillus licheniformis	TSB3

Nematode Bioassay *in vitro*

The per cent inhibition in egg hatching as well as juvenile mortality of *M.incognita* were studied by exposing to different concentrations of culture filtrate of *Bacillus* spp. The culture filtrate was prepared by inoculation of *Bacillus* culture in Nutrient Agar (NA) broth. The root knot nematode egg masses collected from pure culture maintained on tomato under glasshouse conditions were taken at the rate of one egg mass/ Petri plate (5cm) containing five ml. culture filtrate of the above *Bacillus* spp to study the influence of *Bacillus* spp on egg hatching of *M. incognita* at 24h interval for three days. Similarly 100 juveniles of *M. incognita* were transferred to Petri plate (5cm) containing different concentrations of culture filtrate of *Bacillus* spp. Observations on mortality of juveniles were taken at 24h interval for three days. In this study the broth and distilled water used as untreated control and all the treatments were replicated three times in Completely Randomized Design (Mendoza *et al.*, 2008).

Development of Talc Formulation of *Bacillus* spp

A loop full of *Bacillus* isolates were inoculated into nutrient broth separately present in 250ml. conical flask and incubated in a rotary shaker at 150 rpm for 48 h. at room temperature (28 ± 2 °C.). The broth containing $2x10^8$cfu/ml was used for the preparation of talc based formulation. For every 400 ml of bacterial suspension, one kg of the purified talc powder (sterilised at 105 °C for 12 h), 15 g of calcium carbonate (to adjust the pH to neutral/7) and 10 g of carboxy methyl cellulose (CMC) as an adhesive were mixed under aseptic condition. The mixed product was shade dried to reduce the moisture content to less than 20 per cent and then packed in polypropylene bags, sealed and stored at 4°C until used as described by Jayaraj *et al.*, (2006). At the time of application, the population of bacteria in talc formulation was assessed as 2.5-3 cfu/g (Omer, 2010 and Nakkeeran *et al.*, 2005).

Management of *M. incognita* in Tomato using *Bacillus* spp under Glasshouse Conditions

Experiments were conducted to assess the biocontrol potential of different species / isolates under glasshouse conditions to study the influence of different endophytic *Bacillus* spp in the management of *M. incognita* in tomato. Four weeks old healthy tomato seedlings (PKM-1) were transplanted @ 2 seedlings/pot filled with 10 kg stream sterilized pot mixture prepared with red soil, sand and farm yard manure in 2:1:1 proportion. Before transplanting the seedlings, talc formulation of *Bacillus* spp was applied in the pots at different doses of 1-5 g/pot followed by watering. Three weeks after planting the seedlings were thinned to one plant per pot and inoculated with freshly hatched out juveniles (J_2) of *M. incognita* (1 J_2/ g soil) obtained from the pure culture maintained on tomato by making three slanting holes to a depth of 2-3 cm around the seedlings. All the treatments were replicated four times in Completely Randomized Design (Mahgoob and El-Tayeb., 2010).

The experiments run twice were terminated at 180 days after transplanting (DAT) and the plants were removed with intact roots system and washed free of soil. Observations on nematode population/incidence and yield attributes were made.

RESULTS AND DISCUSSION

In vitro

Among the *Bacillus* spp, the culture filtrate of *B. weihenstephanensis* (TSB4) collected from tomato stem effectively inhibited egg hatching (100%) and caused the mortality of juveniles (90%) of root knot nematode *M. incognita in vitro*. The effectiveness of *Bacillus* spp in inhibiting egg hatching and causing mortality of juveniles of *M. incognita* was found to be directly proportional to the concentration of culture filtrate and time of exposure to all the species of *Bacillus*. Similarly the other species/ isolates *B. cereus* (CLB2D) isolated from chilli leaf inhibited egg hatching (99.11%) and it was followed by *B. subtilis* (TSB5) (98.78%) and *B. cereus* (TSB4D) (98.63%) and *B.licheniformis* (TSB3) (98.43%) in the present study. (Table 2)

Table 2
Influence of *Bacillus* spp on per cent egg hatching of *M. incognita in vitro*

Species/Isolates	*% inhibition in egg hatching*											
	25%				*50%*				*100%*			
	24 h	*72 h*	*120h*	*168h*	*24h*	*72h*	*120h*	*168h*	*24h*	*72h*	*120h*	*168h*
B. weihenstephanensis	100.00	98.72	98.71	99.61	99.47	99.71	98.97	98.97	100.00	100.00	99.74	99.74
(TSB 4)	(89.13)	(83.54)	(83.41)	(83.49)	(86.34)	(87.19)	(84.19)	(84.16)	(89.09)	(89.09)	(87.31)	(87.31)
B. cereus	99.11	99.42	99.22	99.22	100.00	99.71	99.61	99.61	100.00	100.00	99.61	99.61
(CLB2D)	(84.66)	(85.72)	(85.06)	(85.06)	(89.13)	(87.19)	(86.42)	(86.42)	(89.09)	(89.09)	(86.82)	(86.82)
B. subtilis	98.78	98.85	98.58	98.58	100.00	98.99	98.84	98.84	100.00	99.57	99.35	99.35
(TSB 5)	(84.66)	(83.88)	(83.18)	(83.18)	(89.13)	(84.28)	(83.81)	(83.81)	(89.09)	(86.24)	(85.44)	(85.44)
B. cereus	98.63	98.47	98.07	98.07	100.00	97.99	98.58	98.71	100.00	99.57	98.97	98.71
(TSB4D)	(82.88)	(83.24)	(982.01)	(82.01)	(89.13)	(81.86)	(83.18)	(83.81)	(89.09)	(86.24)	(83.88)	(83.49)
B.licheniformis	98.43	99.19	98.60	98.58	100.00	100.00	99.61	99.48	100.00	99.85	99.61	99.61
(TSB3)	(83.31)	(88.14)	(83.18)	(83.18)	(89.13)	(89.09)	(86.42)	(87.60)	(89.09)	(89.09)	(86.82)	(86.12)
Broth	56.13	60.71	93.33	93.33	56.13	60.71	93.33	93.33	56.13	60.71	93.33	93.33
	(48.54)	(50.67)	(76.74)	(76.74)	(48.54)	(52.48)	(76.74)	(76.74)	(48.54)	(76.74)	(75.07)	(75.07)
Control	190.00	233.33	260.00	265.00	190.00	233.33	260.00	265.00	190.00	233.33	260.00	265.00
	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
SEd	2.11	0.96	0.79	0.62	2.01	0.69	0.62	0.62	0.17	0.20	0.23	9.00
CD (P=0.05)	4.39	2.02	1.66	1.29	4.17	1.45	1.29	1.29	0.36	0.42	0.48	18.66

Figures in parenthesis are arc sine transformed values.

With regard to influence of *Bacillus* spp on juveniles of *M .incognita* it is observed that the most effective isolates belonging to four species of *Bacillus* had effect to cause mortality of juveniles at all the concentrations of culture filtrate and time of exposure used in the present study and it was ranged from 25 to100 per cent. Among them, *B. weihenstephanenesis* (TSB4) recorded the highest per cent (90%) mortality of J_2 of *M. incognita* and it was followed by *Bacillus cereus* isolate CLB2D (88.97%). Similar effect was noticed with other species *viz*. *B. subtilis* isolate TSB5 (88.53%), *B. cereus* isolate TSB4D (87.46%) and *B. lichenifomis* isolate TSB3 (80.66%). (Table 3)

Influence of talc formulated *Bacillus* spp against *M. incognita* on tomato under glasshouse conditions

All the four species of *Bacillus viz B. weihenstephanenesis, B.cereus, B. subtilis and B. licheniformis* had effect in checking root knot nematode, *M. incognita* population in tomato compared to untreated control. In general the degree of nematode control was found to be increased with increase in the doses of talc formulated *Bacillus* spp used in the present study. Among them *B. weihenstephanensis* (TSB4) applied @ 5g/ plant was found to be most effective in suppressing *M.incognita* population and lowering the gall index. The treatment registered the lowest number of root knot nematode females (86.23%), egg masses/ g root (88.81%), gall index (80%) and eggs per egg mass (67.46%) as well as nematode population in soil (59.48%) The results of the present findings fall in line with the report of suppression of phytonematodes including *M.incognita* in different crops due to the application of *Bacillus* spp as made by earlier workers (Kloepper *et al*.,1991; Kloepper *et al*.,1999 ; Siddiqui and Shaukat. 2003; Siddiqui. 2004 and Siddiqui. 2006) (Table 4).

Several reports demonstrated that endophytic bacteria are plant-associated bacteria that colonize and persist in various healthy plants, such as fruits, vegetables, stems and roots (Mclnroy and Kloepper, 1995; Sturtz *et al*., 1997). Some of these bacteria are known to increase nutrient availability, produce growth hormones, convey stress tolerance, induced systemic resistance, or deter plant pathogens (Hallmann *et al*., 1997; Buchenauer, 1998). Kavita *et al*; (2012) also proved that the biocontrol activity of *Bacillus* strains against plant pathogens is associated

Table 3
Influence of *Bacillus* spp on per cent mortality of J2 of *M. incognita in vitro*

Species/Isolates	*% mortality of juveniles*											
	25%				*50%*				*100%*			
	24 h	*72 h*	*120h*	*168h*	*24h*	*72h*	*120h*	*168h*	*24h*	*72h*	*120h*	*168h*
B. weihenstephanensis	57.66	64.66	71.66	75.00	58.33	65.00	72.66	78.33	68.33	73.33	81.00	90.00
(TSB 4)	(50.44)	(49.60)	(57.85)	(60.08)	(49.83)	(53.06)	(58.50)	(63.48)	(56.13)	(58.94)	(64.17)	(69.56)
B. cereus	53.33	63.33	68.33	75.00	62.66	66.00	71.33	79.33	22.33	73.33	80.00	88.97
(CLB2D)	(47.91)	(52.75)	(55.79)	(60.07)	(52.59)	(54.71)	(58.13)	(64.51)	(60.41)	(59.48)	(68.73)	(71.95)
B.subtilis	40.00	60.00	70.00	83.33	70.00	70.00	78.00	85.00	72.33	73.33	80.00	88.53
(TSB 5)	(39.20)	(50.77)	(56.80)	(66.03)	(56.80)	(56.89)	(62.10)	(67.36)	(58.30)	(58.95)	(63.56)	(70.20)
B. cereus	31.00	33.33	37.66	40.00	31.66	41.00	47.33	50.00	44.32	47.33	51.33	87.46
(TSB 4d)	(33.81)	(35.23)	(37.78)	(39.21)	(34.21)	(39.80)	(43.46)	(44.99)	(41.74)	(43.46)	(45.76)	(45.76)
B. licheniformis	45.00	59.33	69.00	80.00	68.33	71.66	76.60	83.33	73.33	75.00	81.00	80.66
(TSB3)	(42.11)	(50.39)	(50.19)	(63.46)	(56.19)	(57.86)	(61.24)	(67.02)	(59.96)	(58.95)	(64.22)	(69.90)
Broth	6.00	7.33	8.66	8.67	6.00	7.33	8.66	8.67	6.00	7.33	8.66	8.67
	(13.86)	(15.41)	(16.87)	(16.99)	(13.86)	(15.41)	(16.87)	(16.99)	(13.86)	(15.41)	(16.87)	(16.99)
Control	0.00	0.00	1.00	3.00	0.00	0.00	1.00	3.00	0.00	0.00	1.00	3.00
	(0.28)	(0.28)	(4.71)	(9.54)	(0.28)	(0.28)	(4.71)	(9.54)	(0.28)	(4.71)	(4.71)	(9.54)
SEd	4.08	2.24	5.09	2.20	2.38	2.39	2.19	3.14	2.61	1.95	1.30	1.69
CD (P=0.05)	8.47	4.64	10.56	4.58	4.94	4.97	4.54	6.51	5.42	4.06	2.69	3.51

Figures in parenthesis are arc sine transformed values.

with the ability to produce lipopeptide antibiotics that exhibit a wide spectrum of antinemic activity. Therefore Ankit Kumar *et al.,*(2011) opined that *Bacillus* spp competitively colonize the roots of plant and can act as biofertilizer and /or antagonists (biopesticides) or simultaneously both. Hence biological control using *Bacillus* spp to suppress plant disease offers a promising alternative to the use of synthetic chemicals.

Plant Growth Parameters

It is evident that the suppression of root knot nematode population / incidence followed by the application of *Bacillus* spp as biocontrol agent resulted in increase in plant biomass in terms of length and weight of shoot and root of tomato. In the present study the most effective isolate of *B. weihenstephanensis* against *M.incognita* improved the plant growth characters *viz.* height (36.96%) and weight (47.29%) of shoot and length (55.44%) and weight (66.31%) of root of tomato (Fig.1). Similar observations made by Siddiqui *et al*., (2009) on their experimentation with *Bacillus* spp for the management of nematodes and subsequent plant growth confirmed the present results of the study. Fang *et al*., (2009) also already proved that *B.weihenstephanenesis* is effective against *Bursaphelenchus xylophilus* . Further in support of the present study it is documented that other bacterial endophytes like *Pseudomonas* spp, *Rhizobium* spp, *Microbacterium esteraomaticu, Kocuria variance* having inhibitory effect on root knot nematode in different crops enhanced plant growth as observed in the present study (Munif *et al.,* 2000).

Fruit Yield

There was remarkable significant increase in fruit yield followed by the soil application of different dosage of *Bacillus* spp in talc formulation used for the management of *M. incognita* in tomato. Positive correlation exists between increase in fruit yield of tomato and dosage of *Bacillus* spp used in the present study. The more effective endophyte *B. weihenstephanensis* @ 5g/ plant compared to *B.subtilis, B. cereus* and *B.licheniformis* recorded the highest fruit yield of 305.65 g with 59.18 per cent increase over untreated control (257g/ plant).

It seems that this is the first report on the effectiveness of *B.weihenstephanensis* in the management of root knot nematode in tomato. Therefore it is suggested that in-depth study will be useful to develop the TSB4 isolate of *B.weihenstephanensis* as biopesticide for the management of nematodes.

Hence it is concluded that the indigenous isolates of endophytic *B.weihenstephanenesis, B.cereus, B.subtilis, B.licheniformis* collected from Coimbatore district of Tamil Nadu had inhibitory effect over *M. incognita* . Among the four species of *Bacillus* isolated and experimented on tomato, it was observed that the indigenous isolate of talc formulated TSB4 of

Table 4
Influence of *Bacillus* spp on *M. incognita* under glasshouse conditions

Bacillus spp/ isolates with doses	*No. of females /g root*	*No. of egg masses/ g root*	*No. of eggs / egg mass*	*Gall index*	*Nematode population (250cc)*	*Fruit yield / plant (g)*
B.weihenstephanenesis (TSB4)						
1g	33.51 (35.32)	22.21 (28.08)	28.45 (32.17)	43.00 (40.96)	36.12 (36.94)	40.65 (39.50)
2g	35.94 (36.83)	29.62 (32.94)	41.37 (40.02)	55.00 (47.87)	41.79 (40.21)	48.94 (44.38)
3g	44.62 (41.90)	37.03 (37.47)	45.91 (42.65)	63.00 (51.94)	43.87 (41.47)	53.96 (47.27)
4g	55.36 (48.08)	53.69 (47.12	49.70 (44.82)	69.10 (56.27)	46.18 (42.80)	57.67 (49.32)
5g	67.76 (55.41)	69.13 (56.30)	53.25 (46.86)	76.25 (60.84)	52.00 (46.14)	59.18 (50.30)
SEd	2.34	2.73	1.74	2.39	1.19	2.70
CD (P=0.05)	5.00	5.81	3.72	5.10	2.54	5.75
B. cereus (CLB2D)						
1g	16.52 (23.94)	24.91 (29.93)	30.98 (33.70)	30.50 (33.51)	20.98 (31.28)	24.91 (29.93)
2g	27.26 (31.47)	36.25 (37.01)	35.40 (35.70)	32.45 (34.67)	37.44 (37.71)	36.25 (37.01)
3g	33.05 (35.08)	42.57 (40.72)	37.80 (37.93)	34.62 (36.04)	46.69 (43.09)	42.57 (40.72)
4g	47.92 (43.80)	46.71 (42.66)	47.00 (43.27)	40.25 (39.37)	52.61 (46.50)	46.71 (42.66)
5g	57.11(49.03)	50.79 (45.45)	55.50 (48.16)	48.56 (44.17)	56.74 (48.87)	50.79 (45.45)
SEd	2.15	0.96	6.90	1.19	2.33	0.96
CD (P=0.05)	4.59	2.05	14.71	2.54	4.97	2.05
B. subtilis (TSB5)						
1g	13.23 (21.27)	17.89 (24.96)	28.12 (32.00)	37.30 (37.63)	28.53 (34.62)	19.34 (24.02)
2g	23.13 (28.72)	21.57 (27.65)	32.80 (34.93)	41.40 (40.04)	28.91(34.77)	27.65 (30.81)
3g	29.33 (25.53)	30.86 (33.63)	38.61 (38.41)	44.90 (42.06)	32.35 (35.58)	35.56 (35.93)
4g	34.94 (36.22)	46.28 (42.86)	43.97 (41.53)	47.65 (43.65)	34.34 (36.79)	41.59 (40.12)
5g	49.58 (44.75)	55.55 (48.18)	48.26 (43.87)	61.30 (51.53)	43.78 (40.06)	46.23 (42.81)
SEd	1.56	1.70	1.12	1.41	0.58	15.92
CD (P=0.05)	3.32	3.62	2.39	3.02	1.25	33.93
B.cereus (TSB4D)						
1g	14.81(22.54)	14.81 (22.54)	29.93 (29.46)	22.47 (26.98)	25.60 (30.39)	14.15 (21.60)
2g	16.04(23.58)	16.04 (23.58)	37.01 (32.08)	24.75 (29.82)	28.19 (32.06)	23.02 (28.52)
3g	28.08(31.98)	28.08 (31.98)	40.72 (36.40)	29.10 (38.24)	29.91(33.13)	31.41 (34.02)
4g	41.98(40.37)	41.98 (40.37)	42.66 (38.07)	42.90 (40.91)	32.47 (34.73)	35.26 (36.39)
5g	48.75(44.28)	48.75 (44.28)	45.45 (41.11)	49.25 (44.56)	39.07 (38.68)	39. 58 (38.98)
SEd	1.80	1.85	1.80	3.50	0.53	3.75
CD (P=0.05)	3.94	3.94	4.00	7.46	1.13	8.01
B. licheniformis (TSB3)						
1g	9.91 (18.27)	10.95 (16.76)	20.97 (27.00)	18.75 (25.64)	24.29 (29.53)	11.30 (19.29)
2g	20.65 (26.97)	12.34 (20.54)	27.79 (31.80)	22.25 (28.12)	26.49 (30.97)	20.13 (26.55)
3g	23.96 (29.27)	21.61 (27.67)	34.24 (34.58)	30.00 (33.30)	27.24 (30.71)	32.04 (34.44)
4g	31.79 (34.31	33.94 (35.62)	35.27 (36.43)	41.80 (40.27)	30.92 (33.78)	37.68 (37.86)
5g	39.66 (39.02)	44.43 (41.79)	41.15 (39.89)	50.15 (45.08)	36.45 (37.15)	40.81 (39.70)
SEd	1.81	1.65	2.20	0.85	0.51	2.43
CD (P=0.05)	3.86	3.52	4.70	1.81	1.10	5.19
Untreated control	60.50	40.50	345.00	5.00	400.00	257.00

Figures in parenthesis are arc sine transformed values.

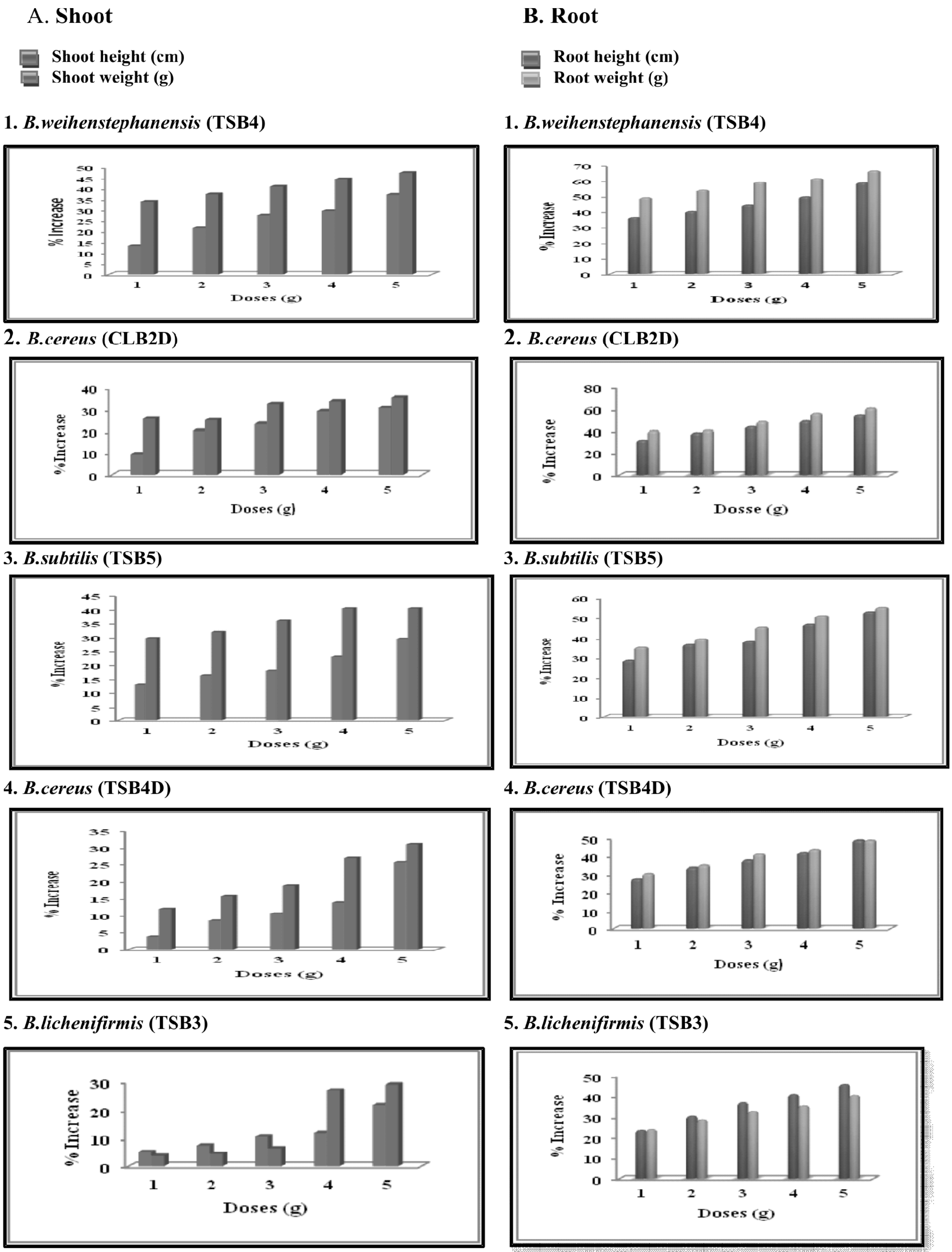

Figure 1: Influence of *Bacillus* spp on plant growth of tomato

B.weihenstephanensis was most effective for the management of *M.incognita* and enhancing the fruit yield of tomato.

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