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Evaluation of *Bacillus subtilis* for the management of dry root rot and vascular wilt of chickpea

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Abstract
The present study was conducted to evaluate the efficacy of a novel rhizospheric *Bacillus* strain with multifaceted biocontrol efficacy for the effective management of wilt and root rot of chickpea. Among the 30 *Bacillus* isolates screened, the strain CaB5 was selected based on *in vitro* inhibition, growth promotion and presence of antibiotic biosynthetic genes. *Bacillus subtilis* CaB5 was formulated in talc in which the population of *Bacillus* was found to be stable at 10^8 till 105 days. Glass house studies revealed that CaB5 (seed treatment @ 10 g/kg seed + basal application @ 2.5 kg/ha followed by soil application of CaB5 at 45 days after sowing) reduced wilt and root rot in chickpea by 72.0 and 61.1 per cent respectively. The pooled analysis of two field experiments revealed that the same treatment reduced the wilt and root rot in field by 60.5 and 62. 8 per cent respectively.

Keywords: Chickpea, root rot, wilt, biocontrol, *Bacillus subtilis*

1. Introduction
Chickpea (*Cicer arietinum* L.) is the third most important food legume of the world with developing countries accounting for 96% of the total cultivated area. India is the largest producer as well as consumer of chickpea in the world. The cultivated area of chickpea in India is 8.3 Mha with a production of 7.7 MT and productivity of 928 kg/ha (Anonymous, 2015) [1]. Chickpea production is limited by several biotic and abiotic factors. Among the biotic stresses, soil-borne diseases viz., root rot and vascular wilt caused by *Rhizoctonia bataticola* (Taub). Butler and *Fusarium oxysporum* f. sp. *ciceri* respectively are the most important in the Indian subcontinent. Pulse growers generally adopt synthetic fungicides as the primary means of reducing soil borne diseases. The irrational selection and use of synthetic chemicals led to contamination of environment with undesirable side effects on non-target organisms. Hence scientists have started focussing on biological methods to protect crop plants from invasion and infection by pathogens (Liu *et al*., 2007) [2]. The use of antagonistic microbes or their secondary metabolites is considered to be a practicable technology for the management of plant diseases (Han *et al*., 2005) [3]. Bacillus species are outstanding biocontrol agents with efficient root colonization, multiple modes of action and promising ability to sporulate (Kloepper *et al*., 2004) [4]. Turner and Backman (1991) [5] found that *Bacillus* sp. colonized the root surface, increased plant growth and caused lysis of fungal mycelia. Their endospore-forming ability also makes these bacteria one of the best candidates for developing efficient biopesticide. The present study was aimed at assessing the biocontrol potential of *Bacillus subtilis* strain CaB5 isolated from chickpea rhizosphere in reducing the root rot and wilt incidence at field level.

Materials and Methods
Bacterial cultures used in the study
The *Bacillus* culture CaB5 was isolated from the chickpea rhizosphere soil of Tamil Nadu by serial dilution plate technique (Johnson and Curl, 1972) [6]. The culture was characterised by biochemical and molecular techniques and identified as *B. subtilis* strain CaB5 (GenBank Accession number KP412481.1). *Bacillus subtilis* strain CcB7 was isolated from pigeonpea rhizosphere of Tamil Nadu (Accession number KR265028.1). The *Bacillus* isolate EPCO16 was obtained from Culture Collection Section, Department of Plant Pathology, Tamil Nadu Agricultural University (TNAU), Coimbatore, India.

Development of talc-based formulation and shelf life study
Talc-based powder formulation of the *Bacillus* subtilis strain CaB5 (selected based on their antagonistic potential against root rot and wilt pathogens under *in vitro* conditions, growth

promotion and antibiotics production) were developed as described by Vidhyasekaran and Muthamilan (1995) [7]. Four hundred ml of 72-h-old bacterial culture in their respective medium with a population of 9×10^8 cfu ml^{-1} was mixed with 1 kg of talc containing 15 g of calcium carbonate and 10 g of CMC. Moisture content of the product was reduced to 20 per cent by shade drying and it was packed in white polythene bags. The population of bacteria in the product was assessed for 120 days at 15 days interval.

Population dynamics of *Bacillus* spp in chickpea rhizosphere

A glass house experiment was designed to study the population dynamics of *Bacillus* in the chickpea rhizosphere. The virulent isolates of *F. oxysporum* f. sp. *ciceri* and *R. bataticola* were mass multiplied in sand-maize medium and mixed separately with potting mixture. The experiment was conducted in completely randomized block design replicated three times and four pots were maintained for each replication. Surface sterilized seeds of chickpea (Co4) were sown in pots containing pathogen inoculated potting mixture at the rate of three seeds per pot. Four pots were maintained for each replication. Two *B. subtilis* isolates *viz*., CcB7 and EPCO16 and a standard chemical Carbendazim were also used as checks. One gram of rhizosphere soil was collected from treated plants at monthly interval till three months after sowing. The population dynamics of *Bacillus* was assessed through serial dilution technique at 10^{-6} dilution in Nutrient Agar Medium. The treatments are as given below

T_1	Seed treatment @ 10 g kg^{-1} + basal application of *B. subtilis* CcB7 @ 2.5 kg ha^{-1}
T_2	Seed treatment @ 10 g kg^{-1} + basal application of *B. subtilis* CaB5 @ 2.5 kg ha^{-1}
T_3	Seed treatment @ 10 g kg^{-1} + basal application of *B. subtilis* EPCO16 @ 2.5 kg ha^{-1}
T_4	T_1 + soil application of *B. subtilis* CcB7 @ 2.5 kg ha^{-1} at 45 DAS
T_5	T_2 + soil application of *B. subtilis* CaB5 @ 2.5 kg ha^{-1} at 45 DAS
T_6	T_3 + soil application of *B. subtilis* EPCO16 @ 2.5 kg ha^{-1} at 45 DAS
T_7	Seed treatment + soil drenching with carbendazim 0.1% at 45 DAS
T_8	Control

The population of *Bacillus* spp in all the treatments were enumerated by serial dilution technique at monthly interval up to 90 days.

Field trial

To assess the efficacy of rhizospheric *Bacillus* strain CaB5 against chickpea root rot and wilt pathogens, field trials were conducted at two locations in Tirupur district of Tamil Nadu, India during 2014-15. The experiment comprised of eight treatments and three replications in RBD with a spacing of 45 cm between rows and 10 cm between plants and a plot size of 5x5 m. Cultivation practices were followed as per the crop production guide recommendation of Tamil Nadu Agricultural University, Coimbatore, India (http://agritech.tnau.ac.in/). The biometric observations were made on plant growth characters like number of plant height, pods per plant and 100 seed weight. The treatment details were the same as used for glasshouse trial.

Statistical analysis

All the experiments were analysed independently. The treatment means were compared by Duncan's Multiple Range Test (DMRT). The package used for analysis was IRRISTAT version 92-1 developed by the International Rice Research Institute Biometrics unit, The Philippines.

Results and Discussion

Bacillus subtilis strain CaB5 isolated from chickpea rhizosphere was tested positive for the presence of five lipopeptide antibiotic genes viz., surfactin, iturin A, iturin D, fengycin and Bacillomycin D. It was also proven to be the best among 30 isolates showing maximum *in vitro* inhibition of root rot and wilt pathogens as well as maximum vigour index (Smitha *et al*., 2015) [8]. Hence it was used for field trial studies.

The selected *B. subtilis* strain CaB5 was formulated in the inert carrier talc and the initial population in the formulation was assessed to be 72.66 x 10^8 cfu/ml. The population of *Bacillus* in the formulation was assessed at 15 days intervals for a period of four months. The cfu was maintained at 10^8 till 105 days of storage and at 120 days it was 5 x 10^7 cfu/ml (Fig.1).

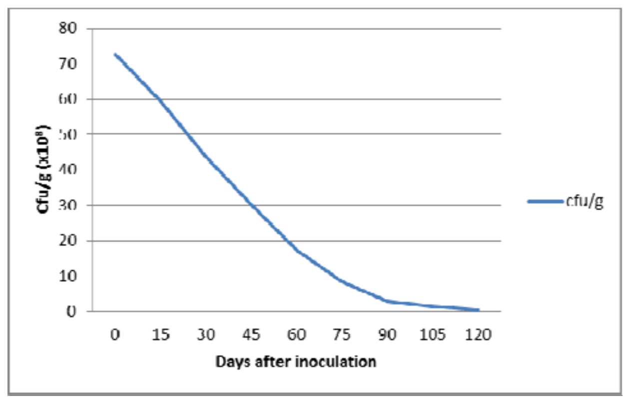

Fig 1: Survival of *B. subtilis* strain CaB5 in talc formulation

Population dynamics of *Bacillus* spp in the chickpea rhizosphere

The population of *Bacillus* spp. was enumerated at monthly intervals up to
90 days under glasshouse conditions. The observations on the population revealed that there was a gradual increase in the *Bacillus* spp population in all the treatments wherein *Bacillus* was amended (i.e. except carbendazim and untreated check). The population of *Bacillus* was significantly higher in T_5, seed treatment + basal application of *B. subtilis* CaB5 followed by application of CaB5 at 45 DAS. The *Bacillus* population was 51x10^8 cfu/g at 90 DAS in this treatment (Fig. 2). The results clearly indicate that there is enhanced *Bacillus* population in all treatments except standard chemical check and untreated control. It also indicates that the chickpea rhizosphere favours the isolate CaB5 compared to other *Bacillus* isolates collected from different rhizospheres.

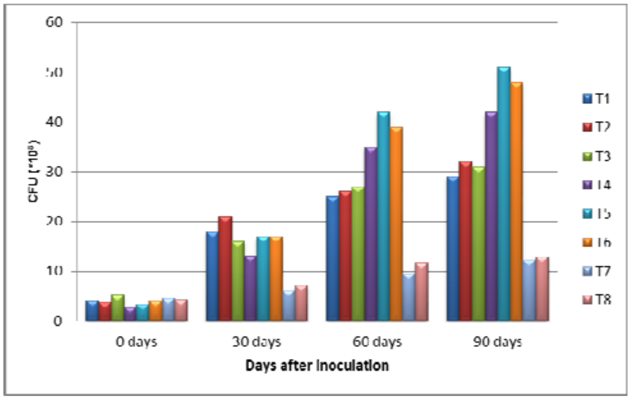

Fig 2: Effect of the formulation on the population dynamics of *Bacillus* spp. in chickpea rhizosphere

Glass House and Field Trials

The results of the glass house experiment revealed that seed treatment with talc based formulation of *B. subtilis* CaB5 @ 10 g/kg seed + basal application of CaB5 @ 2.5 kg/ha followed by soil application of CaB5 at 45 DAS reduced the wilt and root rot incidence in chickpea by 72.0 and 61.1 per cent respectively compared to Carbendazim which reduced wilt and root rot by 80.03 and 68.97 per cent respectively. The pod yield per plant was found to be significantly higher in biocontrol treated plants compared to chemical treatment as well as untreated control (Table 1). The pooled analysis of two field experiments revealed that seed treatment of CaB5 @10 g/kg seed + basal application of CaB5 @ 2.5 kg/ha followed by soil application of CaB5 at 45 DAS reduced the wilt and root rot incidence by 60.5 and 62. 8 per cent respectively (Table 2).

Table 1: Effect of *Bacillus subtilis* on root rot and wilt of chickpea – Pot culture

Treatment	Wilt**		Yield/ plant (g)*	Root rot**		Yield/ plant (g)*
	Per cent incidence*	Per cent reduction over control		Per cent incidence*	Per cent reduction over control	
T$_1$	36.25 (37.02)^{e}	47.80	4.50^{d}	46.98 (43.27)^{e}	41.68	4.90^{de}
T$_2$	35.11 (36.33)^{de}	49.44	4.16^{e}	42.44 (40.65)^{d}	47.32	4.81^{e}
T$_3$	33.33 (35.26)^{d}	52.00	4.36^{e}	43.21 (41.90)^{de}	46.36	5.11^{cde}
T$_4$	22.37 (28.22)^{c}	67.79	6.97^{a}	36.11 (36.93)^{c}	55.18	5.30^{a-d}
T$_5$	19.44 (26.16)^{b}	72.00	6.20^{bc}	31.33 (34.03)^{b}	61.11	5.05^{cde}
T$_6$	21.50 (27.62)^{bc}	69.04	6.50^{b}	33.98 (35.65)^{bc}	57.82	4.98^{cde}
T$_7$	13.87 (21.86)^{a}	80.03	5.90^{c}	25.50 (29.99)^{a}	68.97	5.25^{b-e}
T$_8$	69.44 (56.46)^{f}		3.41^{f}	80.56 (63.91)^{f}		3.12^{f}

*Mean of three replications
Means in a column followed by same superscript are not significantly different according to DMRT.
Values in parentheses are arcsine transformed values
**Wilt – Pot soil amended with wilt pathogen – *Fusarium oxysporum f. sp. ciceri*
**Root rot - Pot soil amended with wilt pathogen – *Rhizoctonia bataticola*

Table 2: Effect of *Bacillus subtilis* on chickpea wilt and root rot under field condition

Treatment	Chickpea**				
	Wilt		Root rot		Yield kg/ha
	Per cent incidence *	Reduction Per cent	Per cent incidence*	Reduction Per cent	
T$_1$	18.7(25.7)^{e}	46.4	11.1(19.5)^{d}	50.2	684.0^{e}
T$_2$	17.1(24.3)^{d}	51.0	10.4(18.8)^{cd}	53.4	697.0^{e}
T$_3$	18.2(25.2)^{de}	47.8	11.3(19.6)^{d}	49.3	693.0^{e}
T$_4$	15.3(23.0)^{c}	56.2	9.5(17.9)^{c}	57.4	727.0^{d}
T$_5$	13.8(21.7)^{b}	60.5	8.3(16.7)^{b}	62.8	793.0^{b}
T$_6$	15.4(23.2)^{c}	55.9	9.8(18.2)^{c}	56.0	758.0^{c}
T$_7$	7.7(16.2)^{a}	77.9	4.8(12.6)^{a}	78.5	820.0^{a}
T$_8$	34.9(36.2)^{f}	-	22.3(28.1)^{e}	-	563.0^{f}

*Mean of three replications **Chickpea : Pooled data of two field trials
Means in a column followed by same superscript are not significantly different according to DMRT
Values in parentheses are arcsine transformed values

T$_1$ -Seed treatment @10g/kg+ basal application of *B. subtilis* CcB7@ 2.5kg/ha
T$_2$- Seed treatment @10g/kg + basal application of *B. subtilis* CaB5@ 2.5kg/ha
T$_3$ - Seed treatment @10g/kg + basal application of *B. subtilis* EPCO16@ 2.5kg/ha
T$_4$ - T$_1$+ soil application of *B. subtilis* CcB7at 45 DAS @ 2.5kg/ha
T$_5$ – T$_2$+ soil application of *B. subtilis* CaB5 at 45 DAS @ 2.5kg/ha
T$_6$ – T$_3$+ soil application of *B. subtilis* EPCO16 at 45 DAS @ 2.5kg/ha
T$_7$ - Seed treatment @2g/kg + soil application @0.1% twice basal and 45 DAS
T$_8$ – Control

The use of antagonistic bacteria is reported as a powerful strategy to suppress soil-borne pathogens due to their ability to colonize the rhizosphere and ability to antagonise the pathogen by multiple modes of action. *Bacillus* spp. are recognized as safe biocontrol agents specifically as seed protectants and antifungal agents (Asaka and Shoda, 1996; Stein, 2005) [9, 10]. The results of the present study shows that *Bacillus subtilis* isolated from the chickpea rhizosphere were promising in their *in vitro* efficacy against wilt and root rot pathogens of chickpea. The production of secondary metabolites by *Bacillus* spp. including antibiotics of low molecular weight and natural cyclic peptides have been related to the inhibition of many fungal species (Romero *et al*., 2007) [11]. The strain CaB5 of *B. subtilis* has already been reported to produce five different lipopeptide antibiotics viz., surfactin, iturin A, iturin D, bacillomycin D and fengycin D (Smitha *et al*., 2015).

The commercial exploitation of a biological control depends on proper formulation of the efficient biocontrol agent in a suitable carrier material. A successful biocontrol agent must survive formulation and storage, and must be a competitive and aggressive colonizer after inoculation (Beatty and Jensen, 2002; Selim *et al*., 2005) [12, 13]. In the present study, the efficient strain of *B. subtilis* CaB5 was formulated in talc based carrier for glasshouse and field studies. The assessment of the bacterial population at regular intervals revealed that the population remained stable in the range of 10^8 till 105 days after storage at room temperature and then it gradually decreased. The studies of Narasimhan and Shivakumar (2015) [14] revealed that the population level of the antagonist was stable in talc at 1.6×10^8 at 30°C till the 180th day.

Talc based formulation of the most efficient *Bacillus* isolate from chickpea rhizosphere *viz*., CaB5 was tested for its efficacy in reducing root rot and wilt diseases under glasshouse conditions. Seed treatment along with soil application as basal and 45 DAS was found to be very efficient in reducing wilt and root rot. The reduction of *Fusarium* infection in field following the application of *Bacillus* spp. have been reported by many workers (Khan *et al*., 2001; Schisler *et al*., 2002; Crane *et al*., 2013) [15-17]. Chickpea seedlings were significantly protected against Fusarium wilt by *Bacillus* spp. Rb29 and reduced the disease severity and incidence caused by *F. oxysporum* f. sp. *ciceri* (Zaim *et al*., 2013) [18].

Results of the study showed that native *Bacillus* strains were more efficient in reducing disease incidence compared to strains isolated from other rhizospheres. An important trait of plant rhizobacteria is their ability to effectively colonize the rhizosphere and maintain a stable relationship with the surface of plant roots which again depends on their abilities to take advantage of a specific environment or on their abilities to adapt to changing conditions. The microorganisms isolated from the root or rhizosphere of a specific crop may be better adapted to that crop and may provide better control of diseases than organisms isolated from other plant species. Such plant associated microorganisms make better biocontrol agents because they are already closely associated with and adapted to the plant and the particular environmental conditions in which they must function.

From this study, the *Bacillus subtilis* strain CaB5 having multifaceted biocontrol efficacy *viz.,* novel lipopeptide production and efficient rhizosphere competence were identified. These *B. subtilis* strain CaB5 was found to be promising in reducing the wilt and root rot of chickpea glasshouse and field conditions.

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