# Enhancing the performance of chilli (*Capsicum annuum*) through twin role of plant growth promotion and disease suppression via *Bacillus subtilis*-based bioformulation

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#### **ABSTRACT**

Considering the majority of studies confined *in vitro* evaluation of microbial bioagents against diseases, a study was carried out during 2018–21 at Assam Agricultural University, Jorhat, Assam, (*in vivo* and field studies) to assess bio-efficacy of *B. subtilis* (strain LB22)-based liquid bioformulation against bacterial wilt (*Ralstonia solanacearum*) and anthracnose (*Colletotrichum gloeosporioides*) diseases of chilli (*Capsicum annuum* L.). *In vivo* efficacy of *B. subtilis* formulation (inclusive treatment involving seed treatment, seedling dip, soil treatment and foliar spray) showed significant suppressiveness against both the diseases coupled with enhanced growth and yield attributes. Subsequently, the field evaluation of *Bacillus subtilis* formulation comparing standard chemicals also edged over the latter by 15.45% in terms of yield gains in 2-years of study. A significant reduction was found in disease severity during field evaluation of *Bacillus subtilis* formulation on account of induced resistance via upregulated synthesis of plant defense-enzymes (Polyphenol oxidase, peroxidase, phenylalanine ammonia-lyase, and  $\beta$ -1,3-glucanase) by 2.3–11.0 folds with their peaks mostly expressed within 24–72 h. These results put forward a conceptual framework for delivery mechanism of a microbial bioagent in a formulation mode, having the potential to be effective against a number of other diseases as well.

Keywords: B. subtilis, Bioformulation, Chilli, Induced defense enzymes, Plant growth promotion

Chilli (Capsicum annuum L.), belonging to family Solanaceae and genus Capsicum, grown in an area of 21.2 thousand ha with a production and productivity of 20.1 thousand tonnes and 0.948 tonnes/ha, respectively. It is considered a widely popular spice worldwide for its hot and pungent flavour due to abundance of phenolic compounds like capsaicin (8-methyl-N-vanillyl-6-nonenamide), depending upon genotypes (Yang et al. 2021, Bal et al. 2022). The crop is attacked by a number of diseases; of them, bacterial wilt (Ralstonia solanacearum) and chilli anthracnose (Colletotrichum sp.) are known to incur maximum yield losses up to 50% (Saxena et al. 2016, Rahman et al. 2023). To date, systemic chemical pesticides are used as a quick remedy to control these diseases, but their long-term use is frequently associated with unstable

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crop responses coupled with development of resistance in the pathogens (Bora and Bora 2020, Choudhary *et al.* 2022). Microbial bioagents as an alternative approach have attracted the attention of many researchers in recent years due to their environmentally friendly solutions minimizing the undue reliance on chemical-based interventions (Srivastava *et al.* 2022, Suma *et al.* 2023).

Bacillus subtilis, is a ubiquitous bacterium known for its ability to produce an array of bioactive metabolites, growth hormone IAA, and siderophores, in addition to nutrient solubilization (Bora and Bora 2021) and resistance to antibiotic, rifampicin (Prihatiningsih et al. 2019, Miljakovic et al. 2020). Antagonistic effects of B. subtilis against various diseases of crops like wheat, sugarbeet, sweet potato, etc. are widely reported (Xie et al. 2021, Wang et al. 2021). In chillies, B. subtilis was observed as an effective biocontrol agent against Aspergillus flavus coupled with prolonged shelf life (Yuan et al. 2023) and Colletotrichum gleoesporioides OGC1 and Colletotrichum capsici causing anthracnose rot (Ashwini and Srividya 2014, Kumar et al. 2021). However, amidst these successes, the commercial success of a biocontrol agent lies in developing

a formulation capable of displaying consistent field responses (Saikia *et al.* 2021). In this background, we employed a carefully designed approach involving a bioformulation carrying *Bacillus subtilis* LB22 in a nutrient-rich medium followed by pot/field response evaluation of chilli plants using different inoculation techniques against two major diseases (bacterial wilt and anthracnose rot) in relation to growth and yield performance of chilli, so known for maximum diversity in northeast India.

### MATERIALS AND METHODS

Pot experiment setup: Liquid bioformulation of B. subtilis LB22 (NCBI Accession no. ON386193 and NBAIM Accession no. as NAIMCC-TB-3886) was collected from authors' Biocontrol Laboratory, Department of Plant Pathology, Assam Agricultural University, Jorhat, Assam. The PGPR strain LB22 showing in vitro biocontrol potential (Saikia et al. 2023) was screened for its growth promotion ability and bio-efficacy through in silico experiments on chilli plants.

A systematic pot experiment was set-up under greenhouse conditions (23-32°C, relative humidity of 65–75% and photoperiod of 6.9–7.5 h) at Assam Agricultural University, Jorhat, Assam for two consecutive seasons (October 2018–February 2019 and March 2019–July 2019) on chilli cultivar Pusa Jwala to evaluate in vivo PGP-efficacy and bioefficacy of B. subtilis-based liquid bioformulation (AAU-Bioguard) against diseases of chilli. The soil (sandy loam) was sterilized for 1 h in an autoclave for 3 days (121°C, 15 PSI pressure). While, the pots (30 cm  $\times$  25 cm × 25 cm) after washing were sterilized with ethyl alcohol (70% for 2 min) followed by UV- treatment in a laminar airflow for 30 min for 3 days. The experiment consisted of 7 treatments, viz. T<sub>1</sub>, Control (only pathogen inoculation); T<sub>2</sub>, Seed treatment (ST) at 1%; T<sub>3</sub>, ST + seedling dip treatment (SD) at 1%; T<sub>4</sub>, ST + SD + soil application (SA) at 30-DAP at 1%; and  $T_5$ , ST + SD + SA at 30-DAP + Foliar application (FA) at 60-DAP with 5 replications in completely randomized design.

Seed treatment for 2 h was done in surface sterilized chilli seeds with uniform size and shape. A 10 ml of bioformulation was mixed with 2% carboxymethylcellulose (100 ml) solution, later the slurry was used for treatment. After treatment, the seeds were air dried for 1 h at room temperature (25°C) to enable bacterial colonization. Treated seeds were sown in pre-sterilized pots. Subsequently, seedling root dip treatment was given to 30-days-old seedlings rinsed with deionized water for 10- min followed by dipping the roots for 1 h in the formulation before transplanting; while 1% solution was used for both soil application as well as a foliar application. Bacterial wilt pathogen R. solanacearum (GenBank Accession No.: OQ743450) and Anthracnose pathogen C. gleosporiodes (GenBank Accession No: OM202512) were collected from the Culture Bank of the Department of Plant Pathology, Assam Agricultural University, Jorhat, Assam. R solanacearum was inoculated by root inoculation technique (Winstead and Kelman 1952)

with a suspension of bacterial inocula adjusted to  $1 \times 10^8$  cfu/mL and, suspension of *C. gleosporioides* conidia was inoculated ( $1 \times 10^5$  conidia/ml) into wounded leaves of chilli plants by pin-prick method (Jena *et al.* 2021).

Assessment of growth promotion and disease incidence: Yield and growth attributing characters, viz. number of leaves, fresh shoot weight (g), fresh root weight (g), shoot dry weight (g), root dry weight (g), plant height (cm) and yield (t/ha) were recorded. Bacterial wilt in chilli was recorded as per cent Wilt Incidence (PWI). Fruit rot was assayed as Per cent Disease Index (PDI) (Mckinney 1923), where fruit rot disease rating scale proposed by Chester (1959) was followed.

Field experiment setup: The treatments which were found to be best in pot experiments were selected for onward field evaluation. The three treatments selected for the experiment comprising T<sub>1</sub> (Absolute control); T<sub>2</sub> (LB22); and T<sub>3</sub> (Chemical treatment) were evaluated in anthracnose-wilt sick plot for two consecutive seasons (October, 2019-March, 2020 and October 2020-March, 2021) using single-factor randomized block design. The LB22-based formulation (T<sub>2</sub>) was applied as ST + SD + SA at 30-DAP and foliar spray at 60-DAP. Under T<sub>3</sub>, two chemicals applied consisted of Mancozeb 75% WP and Streptomycine (Streptomycin Sulphate 90% + Tetracycline Hydrochloride 10%). The first spraying of Mancozeb (@2.5 g/L of water) was done 30-DAP followed by two sprays at 20 days intervals. Streptomycine was applied as soil drenching @100 ppm at 15 days after transplanting. Each treatment contained five replications and each replication contained 16 number of plants. The performance of the treatments was recorded in terms of disease incidence and plant growth promotion.

Studies on bioagent-induced host defense: One gram leaf samples from each replicate was collected after the aapplication of bioformulation and chemical treatment (30 DAP), homogenized with 2 ml of 0.1 M sodium citrate buffer (pH 5.0) at 48°C and centrifuged for 20 min at 10,000 rpm. The supernatant crude enzyme extract was used for assaying  $\beta$ -1,3-glucanase (Pan *et al.* 1991) activity. Enzyme extracted in 0.1 M sodium phosphate buffer (pH 7.0) was used for the estimation of peroxidase (PO) (Smith and Hammerschmidt 1988), polyphenol oxidase (PPO) (Mayer *et al.* 1965) and phenylalanine ammonia-lyase (PAL) (Ross and Sederoff 1992).

## RESULTS AND DISCUSSION

Crop growth response and diseases suppression: In the pot experiment, all the plant growth-promoting parameters (plant height, shoot dry weight, root dry weight and number of leaves/plant) were observed significantly higher in LB22- treated plants compared to control (Table 1). These observations supported the growth promotion ability of tested microbial antagonist. An improvement in overall growth response of host plant was associated with subsequently significant reduction in incidence of wilt and anthracnose diseases. Treatment  $T_5$  as a complete package

of inoculation method comprising ST+RD+SA+FA of LB22-based formulation showed the lowest bacterial wilt incidence (16.15%) and PDI (15.21%) of fruit rot followed by  $T_4$  and  $T_3$ , respectively, in decreasing order (Table 1). Further, the best treatment of LB22 formulation when applied under field conditions exhibited a consequential effect of disease suppression and plant growth promotion (Table 2), and collectively led to an increase in chilli yield by 57.36% over untreated control (T<sub>1</sub>) with lowest wilt and fruit rot incidence (Table 2). According to Jinal et al. (2020), plants treated with B. subtilis (SSR2I) strain reduced the bacterial wilt incidences by 29% and subsequently raised the root length by 48.37% and shoot length by 34.42% compared to control plants treated with only R. solanacearum. The previous findings of Ramzan et al. (2016) reported significant in vivo response of B. subtilis in improving the plant growth in mungbean plants compared to untreated control. Bacillus spp. possess multiple PGP traits expressed through P-solubilization, IAA-production and siderophore production in addition to exhibiting in vitro significant growth inhibition against the phytopathogenic fungi, Colletotrichum capsicias (Natarajan et al. 2012). The bioconcontrol potential of Bacillus spp. have been reported to be direct antibiosis through synthesis of different antimicrobial metabolites, enzymes, volatiles NRPs besides its aggressive colonization mediated through quorum sensing (Feng et al. 2020).

Response on triggering defense enzymes: A bioagent communicates with plant functioning via regulation of plant defense pathways and activating defense enzymes. The changes in dynamics of defense-related enzymes revealed significantly higher enzymatic activities (P=0.05) associated

Table 1 Effect of application of *Bacillus subtilis* (LB22) on plant growth and disease incidence parameters in chilli grown in pot experiment (Pooled data of two seasons)

Bie	ometric r	Disease incidence				
Treatment	Plant height (cm)	Shoot dry weight (g)	Root dry weight (g)	No of leaves	PWI	PDI of fruit rot
$T_1$	104.00	13.16	5.40	194.61	89.50 (68.55)	57.00
$T_2$	116.20	18.66	7.48	223.33	36.20 (32.65)	51.68
$T_3$	121.33	21.43	8.00	240.36	28.98 (24.62)	42.33
$T_4$	126.62	25.36	8.50	240.66	9.45 (18.02)	23.66
$T_5$	129.66	28.00	8.80	288.31	8.10 (16.15)	15.21
C D (P=0.05)	12.41	4.10	0.80	29.82	5.82	8.77

Treatment details are given under Materials and Methods. Data in parenthesis indicate transformed arc sine values. PWI, per cent wilt incidence; PDI, per cent disease incidence.

with response of B. subtilis LB22 and chemical-treated plants compared to untreated control. The PAL activity of leaves was recorded significantly higher up to 48 h following bioagent treatment (2.9 folds higher as compared to control) with subsequent onward downturn (Fig. 1a). While, enzymes like PO and PPO-levels inflated steadily and reached their highest activities within 72 h following inoculation treatment. Maximal significant PO- activity was recorded with T2, 5.1-folds higher than untreated naturally infected plants as control (Fig. 1b). The highest levels of PPO was achieved at 72 h of application in T<sub>2</sub>, which later declined at 96 h, indicating 2.3-folds higher over control and T3 as well (Fig. 1c). B. subtilis treated plants showed 11-folds higher levels of  $\beta$ -1,3-glucanase in leaves at 72 h after application over control (Fig. 1d). These observations support the distinctive involvement of host plant defense while interacting with B. subtilis through increased production of defense-related proteins, thereby, challenging the bacterial wilt and anthracnose pathogens, in addition to their known direct antagonism. According to previous studies (Jinal et al. 2020) Bacillus sp. strain M10 and SSR21 along with their putative catalase protein (KatA) act as an effective biocontrol agent to shield chilli plants from C. capsici via different defense enzymes in form of PAL, PO and PPO. These *Bacillus*-induced defense enzymes (Kashyap et al. 2021) are reported for their additional biopriming effect on chilli seeds as an innate immunity against Ralstonia solanacearum, the bacterial wilt causing pathogen. Earlier, Jayapala et al. (2019) observed an increase in concentration of defense-related enzymes that correlated significantly as a consequential response of reduction in incidence of anthracnose disease due to upregulation of defense-related enzymes coupled with accumulation of phenolic compounds. Bioagent induced defense widely characterized by deposition of callose-induced thickening of cell wall as important biological barrier (Hyder et al. 2020, Srivastava et al. 2022), accumulation of phenolic compounds in addition to improved secretion of antioxidant enzymes as

Table 2 Field efficacy of *Bacillus subtilis* LB22 on plant growth promotion and suppression in diseases of chilli (Pooled data of two seasons)

Treatment	Dry	Dry	Yield	PWI	PDI
	root wt	shoot wt (g)	(q/ha)	(BW)	(Anthracnose)
$T_1$	1.32	15.00	35.01	59.19 (51.55)	35.34
$T_2$	2.95	27.43	79.12	16.21 (22.95)	14.87
$T_3$	2.92	26.54	72.91	17.03 (23.98)	20.10
CD (P=0.05)	0.56	2.27	10.01	8.41	6.87

Treatment details are given under Materials and Methods. Data in parenthesis indicate transformed arc sine values. PWI, per cent wilt incidence; PDI, per cent disease incidence.

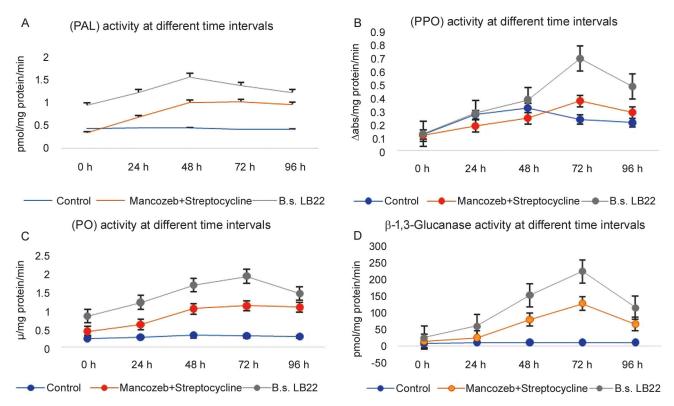


Fig. 1 Changes in defense enzymes activity in chilli plants in response to different treatments.

superoxide dismutases, increased phosphate solubilization, increased secretion of gibberellic acids (Ferrusquia-Jimenez *et al.* 2022, Bora *et al.* 2022) and pathogenesis related proteins known as chitinase, PR-1 /PR-2 coupled with hike in plant defense enzymes (Gowtham *et al.* 2018) as a part of host defense response.

Hence, our study, demonstrated that Bacillus subtilis strain LB22-based liquid bioformulation serves a dual role as growth promoter and bioprotectant against bacterial wilt and anthracnose of chilli, by inducing a strong immune response against infection of both the pathogens. However, such diverse mode of action through direct antagonism, immuno-triggering and growth enhancement need inclusive study on the signaling pathways. It will be interesting to decode the dichotomy of signaling molecules towards plant growth promotion and disease resistance towards plant response to microbial antagonist spatially as well as temporally. Such studies put forth a possibility of developing microbial bioagents-mediated chilli production system in a complete organic framework, with more technical knowhow on mass multiplication protocol, shelf life and standard operating procedures for various methods of inoculation of bioagents. Such a production system would be way forward towards developing a climate neutral production system keeping the ecological balance intact.

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