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RESEARCH ARTICLE

# Deciphering the Role of *Bacillus subtilis* var. *amyloliquefaciens* in the Management of Late Blight Pathogen of Potato, *Phytophthora infestans*

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**Abstract** An oomycete fungus *Phytophthora infestans* (Mont.) de Bary, is an incitant of destructive late blight disease on potato, causes severe yield loss worldwide. A study was undertaken for the management of the diseases by using the antagonistic bacterium. Twenty-five isolates of antagonistic bacteria were collected from various parts of Tamil Nadu from rhizospheric soils of Solanaceous crops potato, tomato and *Solanum nigrum.* These isolates were tested in vitro for its efficacy against *P. infestans* along with the FZB 24 strain of *Bacillus subtilis* var. *amyloliquefaciens* of M/S. Novozymes South Asia Ltd. *B. subtilis* var. *amyloliquefaciens* (FZB 24) showed a maximum inhibition of 35.56% against *P. infestans*. Based on this initial screening, the FZB 24 was used for further investigation under glass house and field conditions for the late blight disease management in potato. The glasshouse studies revealed that the application of corn flour formulation of *B. subtilis* var. *amyloliquefaciens* (FZB 24) as foliar spray @ 0.2% at 7 days interval for six times had shown significant reduction of 49.7% late blight severity over control. In addition, the potato plants from the above treatment showed higher activity of defence related enzymes such as peroxidase, polyphenol oxidase and phenylalanine ammonia lyase, which lead to higher accumulation of total phenols in comparison to untreated control plants. Under field conditions, *B. subtilis* var. *amyloliquefaciens* (FZB 24) treated potato plots registered significantly less severity of late blight disease which recorded higher tuber yield of 15.5 t/ha as against 9.4 t/ha in control.

**Keywords** *Bacillus* · Induced systemic resistance · *Phytophthora infestans* · Late blight of potato · Biochemical study · Biological control

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## Introduction

Late blight disease of potato caused by Oomycete mould, *Phytophthora infestans* (Mont.) de Bary is a destructive pathogen worldwide [1]. It has a historic evidence of Irish famine during the year 1845–1850, as a result of which several millions died and several millions of people migrated to other country [2, 3]. In India, late blight disease was first reported from Nilgiris hills of Tamil Nadu during the year 1870s and later in North India. If the weather conditions are favorable, an uncontrolled epidemic will

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lead to 100% loss. It is reported that, worldwide around 4 billons loss is incurred annually due to this disease outbreak [4–6].

The management of late blight disease heavily depends on the multiple applications of fungicides at different stages of the crop. However, the use of chemicals for disease control has brought a situation where there is an imbalance in the microbial population and also development of the resistant races of the pathogen against the fungicides [7]. Thus, an alternative method with an eco-friendly base is highly in need nowadays for the crop disease management. One such way of crop disease management is the use of plant growth promoting rhizobacteria (PGPR) as biocontrol agents [8].

*Bacillus* and *Pseudomonas* are commonly used PGPR for the late blight disease management. These PGPR are found effective both in vitro and in vivo against the pathogen [9]. It is also evident that strains of *Bacillus* are potential elicitors of induced systemic resistance (ISR) and reduce the disease severity [10]. When the PGPRs are applied to the plant systems the plants exhibit the resistance through the rhizobacteria mediated ISR [11]. Thus, *Bacillus* is known to be the best biocontrol agents through antibiosis or ISR and also it forms endospore in soil for long time.

With this background, an attempt was carried out for the late blight disease management in potato with an objective to manage the disease under glasshouse conditions and field conditions using antagonistic *Bacillus.*

## Material and Methods

### Isolation of *Phytophthora infestans*

Potato leaves exhibiting characteristic symptoms of late blight were collected during 2012–2013 from the Nanjanad farm of Horticultural Research Station, Ooty, Tamil Nadu (India) placed in ice-coolers by keeping it inside the ziploc bag and taken to the laboratory for isolation. The infected leaf tissues showing late blight symptoms were cleaned and kept in a plastic bag for 2–4 days at 20 °C to promote sporangia formation. *P. infestans* was isolated from the infected leaves on V8 agar medium (V8 Juice—200 ml, $CaCO_3$—3 g, agar—15 g and distilled water—800 ml) under controlled laboratory conditions. The leaflets with single lesion were cut into small pieces of 1–1.5 cm and surface sterilized with 0.1% $HgCl_2$ for 1 min, then washed with sterile distilled water thrice and transferred to 9 cm diameter Petri dishes containing V8 agar medium. The plates were incubated at 18 °C for 5 days in the dark [12, 13]. Using single hyphal tip method, fungal cultures were purified and pure cultures were used for further studies. The fungus was identified based on the morphological characteristics of mycelium and sporangia [14].

### Isolation of Antagonistic Bacteria from Rhizosphere Soil

Rhizospheric soil was collected from different places of Tamil Nadu for the isolation of antagonistic bacteria. The plants were collected with intact roots and the excess soil adhering on roots was removed gently. Ten gram of soil was transferred to 250 ml Erlenmeyer flask containing 100 ml sterile distilled water. The suspension was mixed thoroughly, the antagonist in the suspension was isolated by serial dilution plate method. One ml of aliquot was pipetted out from the each final dilutions of $10^{-6}$, $10^{-7}$ and $10^{-8}$ is poured into sterilized Petri dish containing nutrient agar medium and it was incubated for 24 h at a temperature 28 ± 2 °C. The colonies were viewed under UV light at 366 nm. Colonies with characteristics of *Bacillus* were isolated individually and purified by streak plate method [15] on nutrient agar medium. The pure cultures were maintained on nutrient agar slants at 4 °C.

### Isolation of Endophytic Antagonistic Bacteria

Source plants were manually collected and brought to the laboratory. Sections (2–3 cm long) of root, stem and leaf were taken just below the soil line (where the vegetation begins) for younger plants and 5–10 cm below the soil line for older plants. Samples were surface sterilized and washed in four changes of 0.02 M phosphate buffer solution. A sterile check was kept by adding measured quantity of 0.1 ml aliquot from the final buffer wash to 9.9 ml of Tryptic Soya Broth. Selected samples were titrated in 9.9 ml of buffer in a sterile pestle and mortar. Then, it was serially diluted and plated on tryptic soya agar (TSA). Representatives of colony morphology were transferred to tryptic soya agar plated as pure cultures [16]. The FZB 24 strain of *Bacillus subtilis* var. *amyloliquefaciens* was supplied by M/S. Novozymes South Asia (P) Ltd., Bangalore.

### Molecular Characterization of Antagonistic Bacteria

The genomic DNA from all the isolates of *Bacillus* species was isolated using the standard protocol of cetyl trimethyl ammonium bromide (CTAB) method [17] with slight modifications [18]. The DNA was stored at −20 °C for further use. To confirm the isolates as *Bacillus* species, 16S rDNA intervening sequence specific BCF1 (5′CGGGAGG CAGCAGTAGGGAAT3′); BCR2 (5′CTCCCCAGGCGG AGTGCTTAAT3′) primers (Operon, Inc., Alameda, CA) were used to get an amplicon of 546 bp size [19]. PCR was conducted with a total reaction volume of 25 µl in

Eppendorf Master Cycler, German with the settings of a hold of 2 min at 95 °C, 40 cycles of 1 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C and a final extension of 5 min at 72 °C. Then the PCR products were resolved on 2% agarose at 50 V, stained with ethidium bromide (0.5 µg/ml), which was further photographed and analyzed using gel documentation system (Alpha Innotech Corporation, San Leandro, California).

### In vitro Screening of Antagonistic Bacterial Strains Against *P. infestans*

Antagonistic bacterial strains (rhizobacteria and endophytes) along with the *B. subtilis* var. *amyloliquefaciens* (FZB 24) were tested for their antagonistic activity against mycelial growth of *P. infestans* by following the dual culture technique [20]. Mycelial disc (8 mm diameter) of 96 h old culture of *P. infestans* was placed at one side of the Petri plate containing PDA medium at 10 mm away from the periphery. Bacterial cultures were streaked onto the medium exactly opposite to the mycelial disc 10 mm away from the periphery. Those plates were incubated at room temperature (28 ± 2 °C) for 10 days. The efficiency of the antagonistic bacterial strains were assessed based on the inhibition zone formed against the late blight pathogen.

### Testing the Efficacy of Antagonistic *Bacillus subtilis* var. *amyloliquefaciens* (FZB 24) Against Late Blight Disease Under Glass House Conditions

Pot culture experiment was conducted to determine the efficiency of corn flour based formulation of *B. subtilis* var. *amyloliquefaciens* (FZB 24) against late blight pathogen under glass house condition. The experiment was conducted with three replications in a completely randomized design (CRD). Potato seed tubers cv. Kufri Jyothi were sown in earthen pots at the rate of three tubers per pot. The potted plants were grown in greenhouse maintained at 28 °C with 85% RH. The sporangial suspension harvested from the late blight pathogen culture plate was incubated at 6 °C for 2 h to promote zoospore release. Corn flour based formulation of *B. subtilis* var. *amyloliquefaciens* (FZB24) with minimum population of $1 \times 10^{10}$ cfu/g (FZB 24—13% and Corn starch—87% w/w)] obtained from M/S. Novozymes South Asia Pvt. Ltd. was delivered as foliar spray in three different concentrations at two different set of intervals. Plants treated with metalaxyl MZ 72% WP @ 0.25% and *Chaetomium globosum* (Stanes) at 1% served as standard chemical and biocontrol check respectively. Inoculated and unionoculated controls were also maintained separately. The effect of the treatments on the intensity of late blight disease was observed 10 days after inoculation using 1–9 scale and then percent disease index was calculated [21]. Leaf tissues were collected from the treated plants at different time of intervals for studying the induced systemic resistance.

### Testing the Effect of Antagonistic *B. subtilis* var. *amyloliquefaciens* (FZB 24) Against Late Blight Disease of Potato Under Field Conditions

Two field trials were conducted with three replications during 2012–2013 at Nanjanad farm and Woodhouse farm of Horticulture Research Station, Ooty to determine the efficacy of antagonistic *B. subtilis* var. *amyloliquefaciens* (FZB 24) in two different seasons. The seed tubers (cv. Kufri Jyothi) were sown at a spacing of 60 × 20 cm in a randomized block design with a plot size of 3 × 4 m. Natural incidence of late blight diseases was recorded at different stages of crop growth and biometric observations were made on the plant height and number of branches. The yield and individual tuber weight were also recorded at the time of harvest.

### Induction of Defence Enzymes

#### *Extraction of Enzymes*

The leaf tissues were immediately homogenized with liquid nitrogen which was collected from bacterized and control potato plants. Extraction was done with appropriate buffer at 4 °C from 1 g of homogenized sample. The homogenate was centrifuged for 20 min at 10,000 rpm. Protein extracts prepared from potato plant tissues were used for estimation of defense enzymes.

#### *Assay of Peroxidase (PO)*

PO activity was determined using the crude enzyme preparations which were diluted to give changes in absorbance at 470 nm of 0.1–0.2 U/min absorbance [22]. Activity was expressed as the increase in absorbance at 420 nm/min/g of fresh tissue.

#### *Assay of Polyphenoloxidase (PPO)*

Polyphenoloxidase activity was determined and the activity was expressed as change in absorbance at 490 nm/min/g of fresh tissue [23].

#### *Assay of Phenylalanine Ammonia-Lyase (PAL)*

The PAL activity was determined as the rate of conversion of L-phenylalanine to *trans*-cinnamic acid at 290 nm [24] and was expressed on fresh weight basis as nmol *trans*-cinnamic acid $min^{-1}$/g of fresh tissue.

### Estimation of Total Phenols

The estimation of total phenol accumulation was estimated by calculating the content of the total soluble phenols and expressed as catechol equivalents $g^{-1}$ tissue weight [25].

### Activity Gel Electrophoresis

*Peroxidase (PO) and Polyphenol Oxidase (PPO)*

The expression pattern of different isoforms of peroxidases in different treatments was studied using the activity gel electrophoresis [26]. The isoform patterns of PPO was also studied in different treatments and visualized as dark brown discrete bands [27].

### Statistical Analysis

IRRISTAT version 92 designed by the International Rice Research Institute Biometrics unit, the Philippines was used for analyzing the data with 5% level of significance [28].

## Results and Discussion

### Isolation and Pathogenicity

*Phytophthora infestans* was isolated on the V8 agar medium from the late blight affected portion of potato leaf showing symptoms of small circular to irregular brown lesions with white fuzzy pathogen sporulation on the underside. *P. infestans* was isolated from potato leaves showing single lesions in V8 agar medium [29].

Spore suspension ($10^4$ sporangia $ml^{-1}$) was sprayed on the potato plants using hand atomizer. Typical small circular to irregular brown lesions with white fuzzy pathogen sporulation on the underside of the leaves appeared on the susceptible cv. Kufri Jyothi upon artificial inoculation after 7 days of inoculation under glasshouse conditions. The sporangial suspensions were applied in drops on the leaflets of potato at 18 °C and typical symptoms of the pathogen infection was observed [30].

### Isolation and Molecular Characterization of Antagonistic Bacteria

Six rhizospheric and 18 endophytic strains of bacteria were isolated and selected from solanaceous plants viz., potato, tomato and *Solanum nigrum*. Molecular characterization using PCR based on 16S rDNA region showed amplification of 546 bp with all the 25 isolates including FZB 24 (M/S. Novozymes South Asia Pvt. Limited, Bengaluru) (Fig. 1) and were sequenced. Sequence results showed that 13 isolates of *B. subtilis*, 3 isolates of *B. amyloliquefaciens*, 3 isolates of *B. licheniformis* and each one isolate of *B. endophytics*, *B. pumilus*, *B. aryabhattai*, *B. cereus*, *B. megaterium*, *B. parabrevis* were isolated; and they were deposited in NCBI database (Supplementary Table 1). Similarly, 125 bacteria were isolated from rhizospheric soil and among them, six isolates were identified as *Bacillus* species and one was *Paenibacillus polymyxa* based on 16S rDNA sequencing [31].

### In Vitro Screening of Antagonistic Bacterial Strains Against *P. infestans*

In vitro efficacy of all the twenty-five antagonistic bacterial isolates was assessed against *P. infestans* through dual culture technique (Table 1). Among the isolates, FZB 24 showed a maximum percent inhibition of 35.6% and was selected for the further studies in the management of late blight disease. The isolates of *B. subtilis* and *B. amyloliquefaciens* viz., AB05, AB10, AB11, AB12, and AB17 were able to reduce the mycelial growth of *P. infestans* by more than 70% under in vitro [31]. Similarly, among 83 isolates, 14 isolates comprising mostly *Pseudomonas* and *Bacillus* sp. were able to inhibit the growth of *P. infestans* under in vitro [32].

### Testing the Efficacy of Antagonistic *B. subtilis* var. *amyloliquefaciens* (FZB 24) Against Late Blight Disease Under Glass House Conditions

The corn flour based formulation of *B. subtilis* var. *amyloliquefaciens* (FZB 24) was applied to potato as foliar spray with different concentrations at two different intervals to determine the efficacy against late blight pathogen (*P. infestans*) under glasshouse conditions along with a fungicide check of metalaxyl MZ-72% WP at 0.25% and biocontrol check of *C. globosum* (Symbion C®). Among the treatments, foliar application of *Bacillus* (FZB 24) @ 0.2% at 7 days interval with 6 applications recorded a lower late blight intensity of 34.1%, which was found to be 49.68% reduction in disease intensity over control (Table 2). Application of *Bacillus* (FZB 24) also enhanced the plant growth over untreated control. The above treatment recorded a higher plant height of 84.2 cm at 60 days after sowing as against 76.3 cm in untreated control at 60 days after sowing. Artificial inoculation of *P. infestans* to tomato plants treated with bacterial isolates showed significant reduction in the severity of late blight disease [31].

### Testing the Effect of Antagonistic *Bacillus amyloliquefaciens* (FZB 24) Against Late Blight of Potato Under Field Conditions

Field experiments revealed that the intensity of late blight was significantly less in FZB 24 treated plot as foliar spray

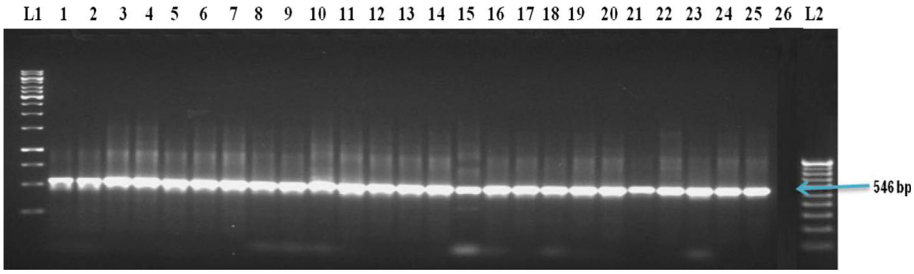


**Fig. 1** Molecular characterization of antagonistic bacteria based on 16S rDNA region

L1. 1kb marker
1. SB 1
2. SB 2
3. SB 3
4. SB 4
5. SB 5
6. SB 6
7. SB 7
8. SB 8
9. SB 9
10. SB 10
11. SB 11
12. SB 12
13. SB 13
14. SB 14
15. SB 15
16. SB 16
17. SB 17
18. SB 18
19. SB 19
20. SB 20
21. SB 21
22. SB 22
23. SB 23
24. SB 24
25. FZB 24
26. Pf1
L2.100bp ladder

**Table 1** In vitro efficacy of bacterial antagonistic isolates against *P. infestans*

| Isolates | Radial growth of *Phytophthora infestans* (mm)* | Percent inhibition over control |
|---|---|---|
| *Bacillus* sp.—SB 1 | $63.00^{ijk}$ | 30.00 |
| *Bacillus* sp.—SB 2 | $68.00^{def}$ | 24.44 |
| *Bacillus* sp.—SB 3 | $61.70^{j–m}$ | 31.44 |
| *Bacillus* sp.—SB 4 | $66.00^{fgh}$ | 26.67 |
| *Bacillus* sp.—SB 5 | $65.00^{ghi}$ | 27.78 |
| *Bacillus* sp.—SB 6 | $63.00^{ijk}$ | 30.00 |
| *Bacillus* sp.—SB 7 | $64.00^{hij}$ | 28.89 |
| *Bacillus* sp.—SB 8 | $61.50^{j–n}$ | 31.67 |
| *Bacillus* sp.—SB 9 | $74.00^{b}$ | 17.78 |
| *Bacillus* sp.—SB 10 | $67.00^{efg}$ | 25.56 |
| *Bacillus* sp.—SB 11 | $63.50^{h–k}$ | 29.44 |
| *Bacillus* sp.—SB 12 | $64.00^{hij}$ | 28.89 |
| *Bacillus* sp.—SB 13 | $65.50^{ghi}$ | 27.22 |
| *Bacillus* sp.—SB 14 | $62.40^{ijkl}$ | 30.67 |
| *Bacillus* sp.—SB 15 | $70.00^{cd}$ | 22.22 |
| *Bacillus* sp.—SB 16 | $69.00^{de}$ | 23.33 |
| *Bacillus* sp.—SB 17 | $59.50^{mno}$ | 33.89 |
| *Bacillus* sp.—SB 18 | $59.00^{no}$ | 34.44 |
| *Bacillus* sp.—SB 19 | $68.00^{def}$ | 24.44 |
| *Bacillus* sp.—SB 20 | $65.50^{ghi}$ | 27.22 |
| *Bacillus* sp.—SB 21 | $72.00^{bc}$ | 20.00 |
| *Bacillus* sp.—SB 22 | $61.00^{k–n}$ | 32.22 |
| *Bacillus* sp.—SB 23 | $60.00^{l–o}$ | 33.33 |
| *Bacillus* sp.—SB 24 | $59.00^{no}$ | 34.44 |
| *B. amyloliquefaciens*—FZB 24 | $58.00^{o}$ | 35.56 |
| Control | $90.00^{a}$ | – |

Means in a column followed by same superscript letters are not significantly different according to DMRT

* Mean of three replications

@ 0.2% at 7 days interval with 6 applications. The average late blight intensity was 32.65% in the above treatment was found to be 45.9% reduction in disease severity over control (Table 3). The yield attributing parameters viz., seed tuber germination percentage, plant height and number of branches per plant were also recorded high in the same treatment by registering a maximum tuber yield of 15.5 t/ha. Field trials performed with *Bacillus* strain FZB 42 under standard conditions resulted in 8.3% increase in yield of potato tubers [33]. Similarly, spraying *Bacillus* strains (BB11 and FH17) mixture at 15 l/ha ($10^{10}$ cfu/ml) under field conditions was found to be 83.2% efficient for bell pepper blight caused by *Phytophthora* and also increased 31.2% of yield of the tuber [34].

FZB 24 treated plants had shown 46–50% reduction in disease severity over control plots both under glass house and field conditions though FZB 24 recorded only 35% reduction on the mycelial growth over the control. The results led the authors to study the mechanism of action of FZB 24 through which enhanced reduction in disease was obtained by plants. Hence, biochemical analysis of defence enzymes which are involved in the induced resistance was studied.

## Induction of Defence Enzymes

### *Assay of PO, PPO and PAL*

It has been demonstrated that PAL, a key enzyme involved in the phenylpropanoid pathway leading to the synthesis of *trans*-cinnamic acid, is the precursor of defense products [35]. Similarly, the PO and PPO are known to involve in the synthesis of phenolics which could restrict the entry of plant pathogens. Hence, the PO, PPO and PAL activities were measured in FZB 24 bioformulation pretreated potato leaves challenge

**Table 2** Effect of *B. subtilis* var. *amyloliquefaciens* (FZB 24) on the severity of late blight of potato under glasshouse conditions

| Treatment details | Plant height at 60 DAS (cm)* | Percent disease index (PDI)* | Percent reduction over control |
|---|---|---|---|
| Foliar spray with *B. subtilis* var. *amyloliquefaciens* (FZB 24) @ 0.05% at 7 days interval (6 applications) | 80.80$^{bcd}$ | 49.12 (44.49)$^{b}$ | 27.52 |
| Foliar spray with *B. subtilis* var. *amyloliquefaciens* (FZB 24) @ 0.1% at 7 days interval (6 applications) | 82.60$^{abc}$ | 46.32 (42.89)$^{c}$ | 31.65 |
| Foliar spray with *B. subtilis* var. *amyloliquefaciens* (FZB 24) @ 0.2% at 7 days interval (6 applications) | 84.20$^{a}$ | 34.10( 35.72)$^{e}$ | 49.68 |
| Foliar spray with *B. subtilis* var. *amyloliquefaciens* (FZB 24) @ 0.05% at 14 days interval (3 applications) | 81.50$^{abcd}$ | 40.12 (39.31)$^{d}$ | 40.80 |
| Foliar spray with *B. subtilis* var. *amyloliquefaciens* (FZB 24) @ 0.1% at 14 days interval (3 applications) | 80.40$^{cd}$ | 46.24 (42.84)$^{c}$ | 31.76 |
| Foliar spray with *B. subtilis* var. *amyloliquefaciens* (FZB 24) @ 0.2% at 14 days interval (3 applications) | 82.50$^{abc}$ | 48.74 (44.28)$^{b}$ | 28.08 |
| Foliar spray with Metalaxyl MZ-72 @ 0.25% at 7 days interval (6 applications) | 78.70$^{de}$ | 16.94 (24.30)$^{g}$ | 75.00 |
| Foliar spray with *Chaetomium globosum* (Stanes) @ 1% at 7 days interval (6 applications) | 83.60$^{ab}$ | 30.56 (33.55)$^{f}$ | 54.90 |
| Untreated control | 76.300$^{e}$ | 67.77 (55.41)$^{a}$ | – |

Means in a column followed by same superscript letters are not significantly different according to DMRT

Values in parentheses are arcsine transformed value

* Mean of three replications

**Table 3** Effect of *B. subtilis* var. *amyloliquefaciens* (FZB 24) treatments on the plant growth parameters, tuber yield and late blight intensity of potato under field conditions

| Treatment | Growth parameters | | Disease intensity | | Tuber yield (t/ha)* |
|---|---|---|---|---|---|
| | Plant height (60 DAS) (cm)* | No. of branches/ plant (60 DAS)* | PDI | % reduction over control | |
| Foliar spray with *B. subtilis* var. *amyloliquefaciens* (FZB 24) @ 0.05% at 7 days interval (6 applications) | 65.347$^{e}$ | 65.347$^{e}$ | 45.410 (42.36)$^{b}$ | 24.77 | 10.900$^{b}$ |
| Foliar spray with *B. subtilis* var. *amyloliquefaciens* (FZB 24) @ 0.1% at 7 days interval (6 applications) | 66.960$^{c}$ | 66.960$^{c}$ | 39.277 (38.78)$^{b}$ | 34.93 | 12.170$^{ab}$ |
| Foliar spray with *B. subtilis* var. *amyloliquefaciens* (FZB 24) @ 0.2% at 7 days interval (6 applications) | 70.570$^{a}$ | 70.570$^{a}$ | 32.657 (34.73)$^{c}$ | 45.90 | 15.497$^{a}$ |
| Foliar spray with *B. subtilis* var. *amyloliquefaciens* (FZB 24) @ 0.05% at 14 days interval (3 applications) | 65.207$^{e}$ | 65.207$^{e}$ | 40.697 (39.52)$^{b}$ | 32.58 | 14.567$^{a}$ |
| Foliar spray with *B. subtilis* var. *amyloliquefaciens* (FZB 24) @ 0.1% at 14 days interval (3 applications) | 65.977$^{de}$ | 65.977$^{de}$ | 39.510 (38.81)$^{b}$ | 34.54 | 11.107$^{b}$ |
| Foliar spray with *B. subtilis* var. *amyloliquefaciens* (FZB 24) @ 0.2% at 14 days interval (3 applications) | 66.810$^{cd}$ | 66.810$^{cd}$ | 40.170( 39.31)$^{b}$ | 33.45 | 12.337$^{ab}$ |
| Foliar spray with Metalaxyl MZ-72 @ 0.25% at 7 days interval (6 applications) | 63.860$^{f}$ | 63.860$^{f}$ | 18.720 (25.57)$^{e}$ | 68.99 | 15.060$^{a}$ |
| Foliar spray with *Chaetomium globosum* (Stanes) @ 1% at 7 days interval (6 applications) | 69.260$^{b}$ | 69.260$^{b}$ | 26.330 (30.82)$^{d}$ | 56.38 | 15.307$^{a}$ |
| Untreated control | 63.090$^{f}$ | 63.090$^{f}$ | 60.360 (51.05)$^{a}$ | 0.00 | 9.397$^{b}$ |

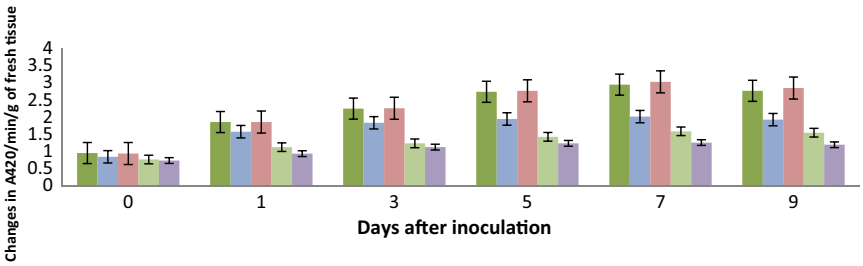


**Fig. 2** Induction of peroxidase activity in potato plants upon treatment with *B. subtilis* var. *amyloliquefaciens* (FZB 24) challenged with *P. infestans* under glasshouse conditions

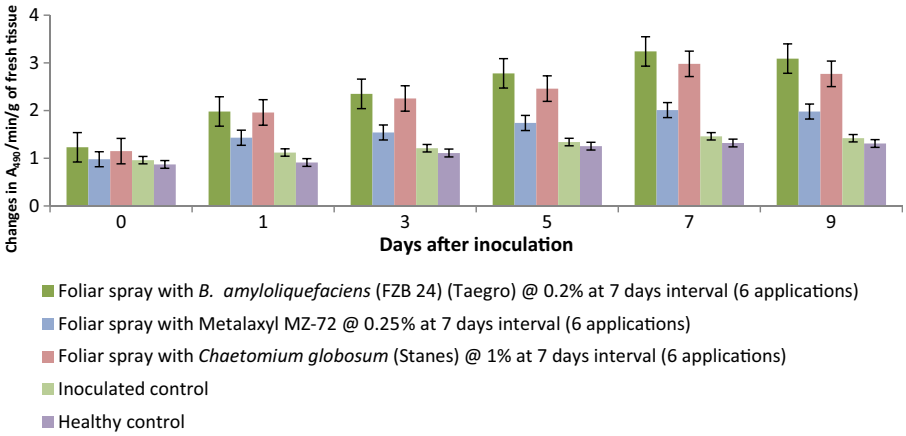


**Fig. 3** Induction of polyphenol oxidase activity in potato plants upon treatment with *B. subtilis* var. *amyloliquefaciens* (FZB 24) challenged with *P. infestans* under glasshouse conditions

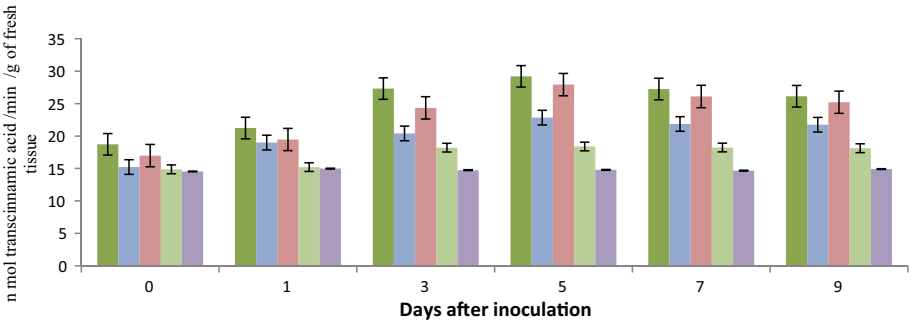


**Fig. 4** Induction of phenylalanine ammonia lyase activity in potato plants upon treatment with *B. Subtilis* var. *amyloliquefaciens* (FZB 24) challenged with *P. infestans* under glasshouse conditions

inoculated with late blight pathogen *P. infestans*. Foliar application of FZB 24 @ 0.2% at 7 days interval with 6 applications increased the activity of PO, PPO and PAL in potato against *P. infestans* (Figs. 2, 3, 4). The activity of PO and PPO was found to be gradually increased up to 7th day of inoculation but the activity of PAL was only up to 5th day of inoculation and declined thereafter. Similarly it was reported that endophytic *B. subtilis* (FZB 24) is increasing the activity of PO, PPO, PAL by foliar application in rice against *R. solani* pathogen [36]. It is also evident that ISR is elicited by *B. subtilis* through the production of phenylalanine ammonia lyase (PAL) and peroxidase [37]. The plants that are treated with *Bacillus megaterium* L8 had higher levels of PAL than the control when these plants were inoculated with the pathogen *Pythium aphanidermatum* and the levels were higher as compared to that of control [38].

## Activity Gel Electrophoresis of PO and PPO

The native PAGE analysis of PO for the samples collected on 7th day after inoculation from antagonistic bacterial strain FZB 24 from the above treatment challenged with *P. infestans* found to have 5 isoforms of PO (PO2 to PO6), whereas only 2 isoforms in the non-bacterized plants (PO4 and PO5) (Fig. 5). Similarly in the case of PPO 6 isoforms viz., PPO1, PPO3, PPO4, PPO5, PPO6 and PPO7 were observed from the same treatment whereas lesser isoforms were induced in inoculated and uninoculated control plants (Fig. 6).

## Estimation of Total Phenols

The level of total phenol accumulation was found to be more in antagonistic bacterial strain FZB 24 treated potato plants @ 0.2% at 7 days interval with 6 applications. The

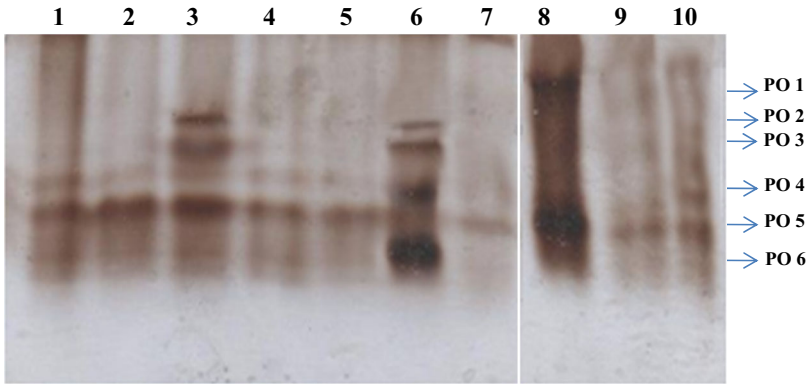


**Fig. 5** Expression of PO isoforms in potato plants upon treatment with *B. subtilis* var. *amyloliquefaciens* (FZB 24) challenged with *P. infestans* under glasshouse conditions

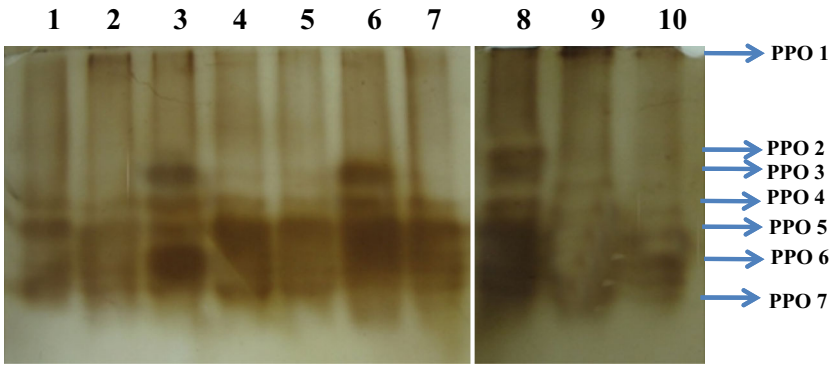


**Fig. 6** Expression of PPO isoforms in potato plants upon treatment with *B. subtilis* var. *amyloliquefaciens* (FZB 24) challenged with *P. infestans* under glasshouse conditions

| | |
|---|---|
| Lane1 | Foliar spray with *B. amyloliquefaciens* (FZB 24) (Taegro) @ 0.05% at 7 days interval (6 applications) |
| Lane2 | Foliar spray with *B. amyloliquefaciens* (FZB 24) (Taegro) @ 0.1% at 7 days interval (6 applications) |
| Lane3 | Foliar spray with *B. amyloliquefaciens* (FZB 24) (Taegro) @ 0.2%/ha at 7 days interval (6 applications) |
| Lane4 | Foliar spray with *B. amyloliquefaciens* (FZB 24) (Taegro) @ 0.05% at 14days interval (3 applications) |
| Lane5 | Foliar spray with *B. amyloliquefaciens* (FZB 24) (Taegro) @ 0.1% at 14days interval (3 applications) |
| Lane6 | Foliar spray with *B. amyloliquefaciens* (FZB 24) (Taegro) @ 0.2% at 14days interval (3 applications) |
| Lane7 | Foliar spray with metalaxyl MZ-72 @ 0.25% at 7 days interval (6 applications) |
| Lane8 | Foliar spray with *Chaetomium globosum* (Stanes) @ 1% at 7 days interval (6 applications) |
| Lane9 | Inoculated control |
| Lane10 | Healthy control |

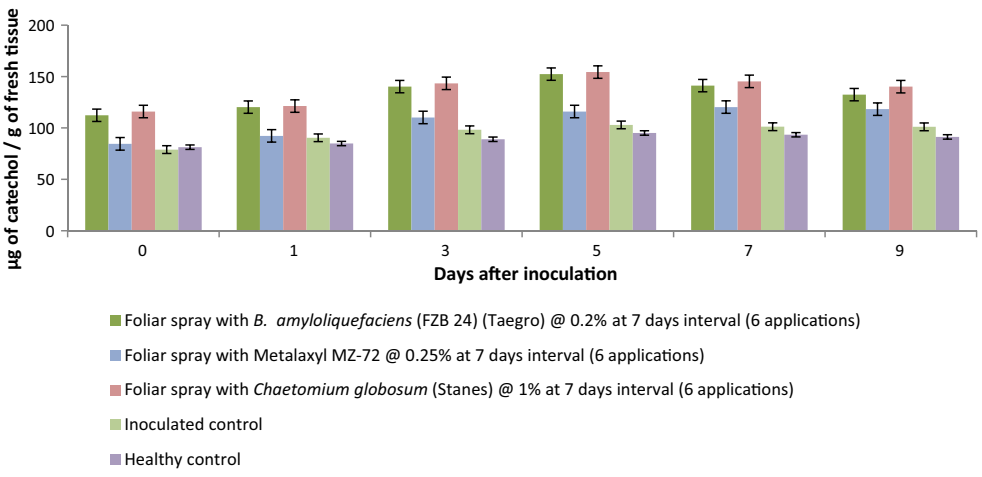


**Fig. 7** Accumulation of total phenols in potato plants upon treatment with *B. Subtilis* var. *amyloliquefaciens* (FZB 24) challenged with *P. infestans* under glasshouse conditions

accumulation of total phenols on 5th day after inoculation with *P. infestans* was found to be maximum (Fig. 7). The phenol accumulation was found to be less in inoculated control and uninoculated healthy control, as compared to that of bacterized plants. Increased accumulation of total phenols in *B. subtilis* treated chilli plants at third day after challenge inoculation with pathogen was observed as compared to *C. capsici* treated and untreated control [39].

## Conclusion

Overall, applications of *B. subtilis* var. *amyloliquefaciens* (FZB24) at regular intervals on potato crop protect the plants against late blight disease throughout the crop growth as the regular application at weekly interval supplies required load of antagonistic bacterium. Higher level expression of defense related PO, PPO, PAL enzymes and timely accumulation of these enzymes at the infection site certainly prevent the colonization of pathogen in potato plants.

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