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# Management of Bacterial Leaf Blight Disease in Rice with Endophytic Bacteria

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**Abstract:** Forty bacterial endophytes were isolated from different plant sources and tested for their efficacy against *Xanthomonas oryza* pv. *oryzae* inciting bacterial leaf blight disease in rice. Out of these, isolates *viz., Bacillus subtilis* var. *amyloliquefaciens* (FZB 24), EPB 9, EPB10, EPCO 29 and EPCO 78 recorded a significantly higher inhibition of *X. oryzae* pv. *oryzae* over control *in vitro*. Among these efficient endophytes EPB 18, EPB 11, EPCO 74, FZB24 and EPB 10 were promote the plant growth of rice seedlings significantly over the other isolates and control. In the present study, rice plants (cv. ADT39) applied with FZB 24 through seed treatment @ 4g/kg + seedling dip @ 4g/l + soil application @ 500g/ha + foliar application @ 500g/ha recorded the lowest severity of bacterial leaf blight (31.36 %) with a per cent reduction of 40 over control under glasshouse conditions. In addition, the *B. subtilis* (FZB 24) treated rice plants registred higher induction of defence related enzymes *viz.,* peroxidase, polyhenol oxidase and phenylalanine ammonia lyase and resulted in higher accumulation of total phenols compared to untreated control plants. The endophytes treated rice plots registered a significantly lower intensity of bacterial leaf blight (2.80%) compared to untreated control plots (19.82%), which also recorded a higher grain and straw yield.

**Abbreviation:** Xoo - *Xanthomonas oryzae* pv. *oryzae; Xam - Xanthomonas axonopodis* pv. *mavacearum;* PSA – Potato sucrose agar; ACC - 1-aminocyclopropane-1-carboxylic acid; DAPG – 2,4- di-acetylphloroglucinol

**Key words:** Endophytes · Bacterial leaf blight of rice · *Bacillus* · Induced systemic resistance · *Xanthomonas oryza* pv. *oryzae*

## INTRODUCTION

Rice is the most important staple food crop of more than half of the world population. Bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* Ish. is found worldwide and particularly destructive in Asia. The disease was endemic in Bihar (Srivastava and Rao [1]) and Tamil Nadu (Rajagopalan *et al*. [2]). Reduction in rice yield may be as high as 50 per cent was also recorded, when the crop was severely infected (Mew *et al*. [3]). It became a destructive disease of rice in Punjab and appeared in epiphytotic form in Ludhiana, Jalandhar, Patiala and Sangur districts and caused about 30 per cent yield loss (Chahal, [4]). Scientists have focused on biological methods to protect crops from invasion and infection by the pathogen (Liu *et al*. [5]). The management of pathogens by the use of antagonistic microorganisms or their secondary metabolites is now considered to be a feasible disease control technology (Han *et al*. [6]).

Plants are constantly involved in interactions with a wide range of bacteria. These plant-associated bacteria colonize the rhizosphere (rhizobacteria), the phyllosphere (epiphytes) and inside the plant tissues (endophytes). Endophytes are sheltered from environmental stresses and microbial competition by the host plant and they seem to be ubiquitous in plant tissues, having been isolated from flowers, fruits, leaves, stems, roots and seeds of various plant species (Kobayashi and Palumbo [7]). Several bacterial endophytes have been shown to support plant growth and increase nutrient uptake by providing phytohormones (Kang *et al*. [8]) and biologically fixing nitrogen (Jha and Kumar [9]).

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Endophytic bacteria can not only promote plant growth and act as biocontrol agents, but also produce nature products to control plant diseases (Guan *et al*. [10]) and reducing disease severity (Senthilkumar *et al*. [11]).

*Bacillus* species are among the most common bacteria found to colonize plants endophytically (Lilley *et al*. [12], Mahaffee and Kloepper, [13]) and it is likely that their endophytic ability could play a role in the biocontrol of vascular plant pathogens. Endophytic bacteria from potato tissue was found to be antagonistic towards *Clavibacter michiganensis* ssp. *sepidonicus*, the casual agent of bacterial ring rot of potato (Van Buren *et al*. [14]) and against *Erwinia carotovora* var. *atroseptica* causing potato soft rot (Sturz and Matheson, [15]). Some endophytic bacteria such as *P. fluorescens* 89B-61 induces systemic resistance against *P. syringae* pv. *lachrymans* (Liu *et al*. [16]). Reiter *et al*. [17] screened the endophytic bacteria against *E. carotovora* ssp. *atroseptica* revealed that 38 per cent of the endophytes protected tissue culture potato plants from black leg disease. With this background, the present study was carried out to isolate, screen and field evaluation of the effective endophytic bacterial strains for the management of bacterial leaf blight disease of rice.

## MATERIALS AND METHODS

**Bacterial Pathogen:** Rice leaves showing typical bacterial leaf blight symptoms were collected for isolation of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) from wetlands of Tamil Nadu Agricultural University, Coimbatore. The collected diseased leaves were ground with autoclaved pestle and mortar, suspended in 200 ml of sterile saline for 2 h. The suspensions were serially diluted 4 x 1:10 in test tubes. Aliquots of 0.05 ml were spread on to mXOS (Modified XOS agar; Di *et al*. [18]; Gnanamanickam *et al*. [19]) medium and incubated at 28 ± 2°C for 3-5 days. The pathogenicity of the isolated *Xoo* was confirmed by clip inoculation. Thirty-day-old rice plants grown in greenhouse with fully expanded leaves were clip inoculated with the sterile scissors dipped in bacterial suspension ($10^8$ cfu $ml^{-1}$) (Kauffman *et al*. [20]). For inoculum preparation, the 36 h old bacterial cultures were multiplied on PSA for 48 h, centrifuged at 5000 x g for 10 min and the bacterial pellets were washed with sterile saline. Cell suspensions were adjusted to $10^8$ cfu $ml^{-1}$ (Thompson [21]). *Xoo* was maintained on rice cultivar ADT 39 in a greenhouse and on PSA (Sakthivel *et al*. [22]) and nutrient agar under laboratory conditions.

**Isolation of Endophytes:** Source plants were manually uprooted and brought to the laboratory. Root, stem and leaf sections (2-3cm long) were made using a sterile scalpel. The root samples were taken just below the soil line for younger plants and 5-10cm below the soil line for older plants. Stem samples were first weighed and surface sterilized with hydrogen peroxide (20%) for 10 min. and rinsed four times with 0.02 M potassium phosphate buffer (pH 7.0). Root samples were surface disinfected with sodium hypochlorite (1.05%) and washed in four changes of 0.02 M phosphate buffer solution. Measured quantity of 0.1 ml aliquot from the final buffer wash was removed and transferred in 9.9 ml tryptic soya broth to serve as sterile check. Samples were discarded, if growth was detected in the sterile check within 48 h. Selected samples were triturated in 9.9 ml of buffer in sterile pestle and mortar. The triturate was serially diluted in potassium phosphate buffer solution and plated on Tryptic Soya Agar (TSA). Representatives of colony morphology were transferred to fresh TSA plated as pure cultures (McInroy and Kloepper [23]).

**Antagonism of Endophytic Bacterial Strains Against Xoo:** Cell suspension of *Xoo* was prepared in the sterile distilled water to a concentration of $10^7$ cfu / ml. One ml of the bacterial cell suspension (*Xoo*) was mixed with 19 ml of nutrient agar (NA) medium and poured onto the sterile Petri dishes. After solidification, sterile paper discs (6mm diameter) were placed on the surface of the medium at 1 cm away from the side of the Petri dish and 5µl of the endophytic bacterial culture in NA broth of 4h old was applied to each disc. The plates were incubated at 37±2°C and the inhibition of bacterial growth was measured 48h after the treatment (Salah *et al*. [24]).

**Plant Growth Promotion by Endophytes**

**Preparation of Bacterial Inoculum:** Endophytic bacteria were grown on KB broth with constant shaking at 100 Xg for 48 h at room temperature (28±2°C). Bacterial cells were harvested by centrifugation at 12,000 Xg for 15 min and bacterial cells were resuspended in Phosphate Buffer (0.01 M, pH 7.0). The concentration was adjusted to approximately $10^8$ cfu $ml^{-1}$ (OD595=0.3) with a spectrophotometer and used as bacterial inoculum (Thompson [21]).

**Seed Bacterization:** Rice seeds (cv. ADT 39) were surface sterilized with 2% sodium hypochlorite for 30 sec, rinsed in sterile distilled water and dried overnight under a sterile

air stream. Endophytic bacterial strains, inoculated into their respective broths and bacterial suspension was prepared as mentioned above. The required quantity of seeds was soaked in bacterial suspension containing $3X10^8$ bacteria $ml^{-1}$ for 2 h and dried under shade.

**Plant-Growth Promotion:** The plant-growth promoting activity of the bacterial endophytic strains was assessed on the basis of seedling vigour index as determined by the standard roll towel method (ISTA [25]). Twenty five seeds were kept on presoaked germination paper. The seeds were held in position with another presoaked germination paper strip on top of them and gently pressed. The polythene sheet along with the seeds was then rolled and incubated in a growth chamber for 14 days. Three replications were carried out for each treatment. The root and shoot length of individual seedlings was measured and seed germination percentage calculated.

The vigour index was calculated using the formula of Baki and Anderson [26]:

Vigour index = % germination X seedling length (shoot length + root length)

**Glasshouse Study**

**Application of Bioformulations:** Bioformulation containing *B. subtilis* var. *amyloliquefaciens* was used to treat the rice plants. Rice seeds (ADT 39) were soaked in double the volume of sterile distilled water containing the formulation. After 24 h, the suspension was drained off and the seeds were dried under shade for 30 min and sown in plastic pots along with untreated control seeds. For seedling dip, seedlings were removed 15 days after sowing, tied in bundles and their roots were dipped in water containing bioformulation of *B. subtilis* (FZB 24) for 30 min. The seedlings were transplanted in plastic pots containing 0.014 $m^3$ soil at the rate of two seedlings per hill and four hills per pot (Nandakumar *et al*. [27]) and were watered regularly to maintain a 1-cm water level. Soil application of bioformulation was applied at the time of planting under glasshouse conditions. The foliar spray was given with bioformulation dissolved in water and the suspension was sprayed. Foliar spray with Streptomycin sulphate + Tetracycline @ 600ppm + Copper oxy chloride @ 0.25% served as a chemical check.

**Bacterial Leaf Blight Disease Assessment:** Three replications were maintained for each treatment in completely a randomised design under glasshouse conditions. The effectiveness of the treatments on the intensity of bacterial leaf blight disease was observed fifteen days after pathogen inoculation, with a 0-9 scale of the Standard Evaluation System for rice (IRRI [28]) and the per cent disease indices were calculated using the formulae of McKinney [29]:

$$PDI = \frac{\text{Sum of all numerical ratings}}{\text{Total number of leaves graded}} \times \frac{100}{\text{Maximum grade}}$$

In addition, growth parameters like plant height and number of tillers were also recorded at different time intervals. Plant samples were also collected at different time intervals from different treatments to study the induced systemic resistance from the following treatments.

$T_1$ - ST - Seed treat with *B. s.* var. *amyloliquefaciens* (FZB24) @4g/kg
$T_2$ - SD - Seedling dip with *B. s.* var. *amyloliquefaciens* (FZB24) @4g/l
$T_3$ - SA + FA - Soil + Foliar application with *B. s.* var. *amyloliquefaciens* (FZB24) @500g each /ha
$T_4$ - ST + SD + SA + FA - Seed treat with FZB24@4g/kg + Seedling dip with FZB24@4g/l + Soil application @500g/ha + Foliar spray @ 500g each /ha with FZB24@30 Days After Transplanting
$T_5$ - Chemical - Foliar spray with Streptomycin sulphate + Tetracycline @ 600ppm + COC @ 0.25% as chemical check
$T_6$ - IC - Inoculated control
$T_7$ - HC - Healthy control

**Induced Systemic Resistance:** Plants were carefully uprooted without causing any damage to root and leaf tissues at different time intervals viz., BI (Before Inoculation), 0, 12, 24, 48, 72, 96 and 120 h after challenge inoculation. Plant samples were homogenized with liquid nitrogen in a pre-chilled mortar and pestle. The homogenized plant tissues were used immediately or were stored in deep freezer (-70°C) until used for biochemical analysis. Variation in activity of defense related enzymes, phenylalanine ammonia lyase (PAL), peroxidase (PO) and polyphenol oxidase (PPO) was determined by enzyme assays according to Ross and Sederoff [30], Hammerschmidt *et al*. [31] and Mayer *et al* [32] respectively. The enzyme activity was expressed as nmoles of cinnamic acid $min^{-1}g$ $protein^{-1}$ and change in absorbance $min^{-1}$ $mg^{-1}$ of protein respectively.

Accumulation of total phenols was also estimated according to Zieslin and Ben Zaken [33] and it is expressed in mg of catechol/g of fresh tissue. Each of the enzyme assays were atleast repeated three times to obtain consistent results.

**Native Polyacrylamide Gel Electrophoresis Analysis:** The isoform profiles of PO and PPO were studied by discontinuous native polyacrylamide gel electrophoresis (PAGE) (Laemmli [34]). The protein extract was prepared by homogenising 1 g of leaf sample in 2 ml of 0.1 M sodium phosphate buffer pH 7.0 and centrifuged at 16,000g for 20 min at 4°C. The samples were loaded into 8% polyacrylamide gels (Sigma, USA). After electrophoresis, PO isoforms were visualised by soaking the gels in staining solution containing 0.05% benzidine (Sigma Aldrich, Mumbai, India) and 0.03% $H_2O_2$ in acetate buffer (20 mM, pH 4.2) (Nadolny and Sequeira [35]). For assessing the PPO isoform profiles, the gels were equilibrated for 30 min in 0.1% p-phenylene diamine, followed by the addition of 10 mM catechol in the same buffer (Jayaraman *et al*. [36]).

**Testing the Efficacy of Endophytes Against Bacterial Leaf Blight of Rice under Field Conditions:** Two different field trials with rice cultivar ADT 39 were laid out in two different seasons under randomized block design with three replications with a plot size of 4X3 m. The treatments details are as follows.

| T. No. | Treatment Details |
|---|---|
| $T_1$ | Seed treat with FZB24@2g/kg |
| $T_2$ | Seed treat with FZB24@4g/kg |
| $T_3$ | Seedling dip with FZB24@2g/l |
| $T_4$ | Seedling dip with FZB24@4g/l |
| $T_5$ | Soil + Foliar app with FZB24@250g each /ha |
| $T_6$ | Soil + Foliar app with FZB24@500g each /ha |
| $T_7$ | T3 + Foliar app with FZB24@250g/ha |
| $T_8$ | T4 + Foliar app with FZB24@500g/ha |
| $T_9$ | T1 + T3 + Soil application @250g/ha + Foliar spray @ 250g each /ha with FZB24@30 Days After Transplanting |
| $T_{10}$ | T2 + T4 + Soil application @500g/ha + Foliar spray @ 500g each /ha with FZB24@30 Days After Transplanting |
| $T_{11}$ | Streptomycin sulphate + Tetracycline @ 600ppm + COC @ 0.25% |
| $T_{12}$ | Control |

The observations on per cent incidence of bacterial leaf blight were recorded on 90 days after transplanting. The growth parameters such as plant height and number of tillers were recorded at different intervals and the grain and straw yield was also recorded at the end of the crop.

**Statistical Analysis:** The statistical analysis of the data were made using package IRRISTAT version 92 of International Rice Research Institute Biometrics unit, Philippines.

## RESULTS

**Isolation and Pathogenicity:** Xanthomonas oryzae pv. *oryzae* (*Xoo*) were isolated on the mXOS agar medium from the infected portion of leaf showing characteristic symptoms of water soaked yellowish lesions with wavy margins on leaf blades. *Xoo* was artificially inoculated on the leaves of susceptible rice variety ADT 39 by leaf clip method under glass house conditions. Symptoms of water soaked lesions with wavy margin were observed at 10 days after inoculation of the pathogen.

**Isolation of Endophytes:** Twenty six endophytic bacterial strains were isolated from different field crop plants *viz.,* rice, greengram, cotton, redgram and ragi, medicinal plants such as neem and noni, weed plants *viz., Trianthema, Acalypha, Tribulus*, *Cactus*, *Opuntia, Aloe* and *Agave*. FZB24 strain of *Bacillus subtilis* var. *amyloliquefaciens* was obtained from Novozymes South Asia Pvt. Ltd. Another thirteen strains of endophytes were obtained from the culture collection centre of Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore.

***In vitro* Screening:** Forty endophytic bacterial isolates were screened against *Xoo* to test their efficacy of inhibition. Among the isolates, FZB24, EPB 9, EPB 10, EPCO 29 and EPCO 78 showed the maximum inhibition halo of 20mm diameter followed by EPB 6, EPB 7, EPB 14 and EPCO 16 with an inhibition halo of 19 mm diameter (Table 1).

**Endophytic Bacterial Strains on Plant Growth Promotion of Rice:** Rice seeds were treated with 40 endophytic bacterial strains separately and their effect on plant growth was studied by roll towel method *in vitro*. Endophytic bacterial isolates *viz*., EPB 18, EPB 11, EPCO 74, FZB24 and EPB 10 were found to increase the vigour index of the rice seedling significantly compared to untreated control. A maximum vigour index of 3343 was recorded by the EPB 18 isolate which was followed by the isolates *viz.,* EPB 11, EPCO 74, FZB24 and EPB 10 with vigour index of 3225, 3127, 3035 and 3023 respectively. The untreated control has a very less vigour index of 1168 (Table 1).

Table 1: Effect of bacterial endophytic isolates against *Xanthomonas oryzae* pv. *oryzae* and its growth promoting activity on rice *in vitro*

| | | | Growth promotion activity | | | |
|---|---|---|---|---|---|---|
| S.No | Isolates | Inhibition zone of Antagonistic activity (mm) | Root length (cm) | Shoot length (cm) | Germination (%) | Vigour index* |
| 1 | EPB 1 | 15.0 efg | 20.81 | 7.95 | 96 | 2761 mn |
| 2 | EPB 2 | 15.0 efg | 19.58 | 7.93 | 94 | 2586 o |
| 3 | EPB 3 | 14.0 fg | 20.90 | 7.20 | 100 | 2810 jkl |
| 4 | EPB 4 | 12.0 hi | 20.20 | 7.70 | 100 | 2790 lm |
| 5 | EPB 5 | 16.2 cde | 21.70 | 7.50 | 100 | 2920 h |
| 6 | EPB 6 | 19.0 ab | 21.30 | 9.33 | 98 | 3002 ef |
| 7 | EPB 7 | 19.0 ab | 21.70 | 8.00 | 100 | 2970 fg |
| 8 | EPB 8 | 15.6 def | 18.54 | 8.21 | 98 | 2621 o |
| 9 | EPB 9 | 20.0 a | 19.51 | 8.52 | 100 | 2803 klm |
| 10 | EPB 10 | 20.0 a | 22.06 | 8.17 | 100 | 3023 de |
| 11 | EPB 11 | 15.0 efg | 23.54 | 8.71 | 100 | 3225 b |
| 12 | EPB 12 | 10.6 ijk | 19.09 | 8.49 | 84 | 2317 s |
| 13 | EPB 13 | 17.0 cd | 20.20 | 8.39 | 100 | 2859 i |
| 14 | EPB 14 | 19.0 ab | 19.11 | 8.12 | 100 | 2723 n |
| 15 | FZB 24 | 20.0 a | 21.90 | 8.45 | 100 | 3035 de |
| 16 | EPB 15 | 0.0 p | 20.95 | 8.06 | 82 | 2379 r |
| 17 | EPB 16 | 15.0 efg | 15.99 | 8.31 | 100 | 2430 q |
| 18 | EPB 17 | 17.0 cd | 20.44 | 8.00 | 100 | 2844 ijk |
| 19 | EPB 18 | 14.0 fg | 23.88 | 9.55 | 100 | 3343 a |
| 20 | EPB 19 | 14.2 fg | 19.50 | 8.60 | 100 | 2810 jkl |
| 21 | EPC 5 | 14.0 fg | 19.35 | 8.66 | 100 | 2801 klm |
| 22 | EPC 8 | 17.8 bc | 19.39 | 7.90 | 100 | 2729 n |
| 23 | EPCO 16 | 19.0 ab | 20.15 | 8.39 | 100 | 2854 ij |
| 24 | EPCO 26 | 11.0 ij | 18.87 | 8.24 | 96 | 2603 o |
| 25 | EPCO 29 | 20.0 a | 15.60 | 6.41 | 100 | 2201 t |
| 26 | EPCO 30 | 4.0 n | 12.91 | 6.87 | 86 | 1701 v |
| 27 | EPCO 74 | 11.0 ij | 22.63 | 8.64 | 100 | 3127 c |
| 28 | EPCO 78 | 20.0 a | 16.63 | 8.64 | 94 | 2375 r |
| 29 | EPCO 81 | 2.0 o | 20.99 | 8.48 | 100 | 2947 gh |
| 30 | EPCO 95 | 14.6 efg | 19.51 | 9.74 | 100 | 2925 gh |
| 31 | EPCO 96 | 7.8 m | 12.74 | 8.13 | 98 | 2045 u |
| 32 | EPCO 43 | 8.0 m | 11.40 | 6.81 | 84 | 1529 w |
| 33 | EPCO 60 | 3.5 gh | 17.60 | 8.30 | 86 | 2227 t |
| 34 | EPB 20 | 2.2 hi | 19.91 | 6.83 | 90 | 2407 qr |
| 35 | EPB 21 | 8.3 lm | 19.90 | 8.55 | 92 | 2617 o |
| 36 | EPB 22 | 12.3 hi | 21.60 | 8.10 | 100 | 2970 fg |
| 37 | EPB 23 | 10.0 jkl | 21.00 | 7.70 | 100 | 2870 i |
| 38 | EPB 24 | 9.2 klm | 20.44 | 8.00 | 88 | 2503 p |
| 39 | EPB 25 | 9.0 klm | 13.00 | 6.01 | 88 | 1673 v |
| 40 | EPB 26 | 8.0 m | 20.7 | 8.06 | 90 | 2588 o |
| 41 | Control | 0.0 p | 8.70 | 5.90 | 80 | 1168 x |

* Mean of three replications.

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

**Efficacy of FZB24 under Glasshouse Conditions:** Bioformulation with endophytic *Bacillus* (FZB24) was applied to rice with different methods of application as described earlier to determine its efficiency against bacterial leaf blight pathogen, *Xoo* under glasshouse conditions along with chemical check of Streptomycin sulphate @ 600ppm + Copper oxy chloride @ 0.25%. Among the treatments, seed treatment @ 4g/kg + seedling dip @ 4g/l + soil application @ 500g/ha + foliar spray @ 500g/ha with *B. subtilis* (FZB24) recorded a lower intensity of 31.36 per cent BLB, which was 39.81 per cent reduction in intensity over control (Table 2).

**Effect of Endophytic Bioformulations on Plant Growth Parameters of Rice under Glass House Conditions:** Application of bioformulations enhanced the plant growth in rice under glasshouse conditions compared to untreated control and chemical treatment. The growth

Table 2: Effect of *B. subtilis* (FZB24) treatments on the plant growth parameters and bacterial leaf blight intensity of rice (ADT39) under glasshouse conditions on 75 DAT

| T. No. | Treatment | Plant height* (cm) | No. of Tillers* | Bacterial leaf blight* (PDI) | % reduction over control |
|---|---|---|---|---|---|
| $T_1$ | Seed treat with FZB24@2g/kg | 56.99$^{abc}$ | 7.07$^{bcd}$ | 46.33$^{c}$ (42.89) | 11.09 |
| $T_2$ | Seed treat with FZB24@4g/kg | 57.37$^{abc}$ | 7.84$^{a}$ | 36.49$^{f}$ (37.15) | 29.98 |
| $T_3$ | Seedling dip with FZB24@2g/l | 56.80$^{abc}$ | 6.89$^{cd}$ | 50.33$^{b}$ (45.18) | 3.42 |
| $T_4$ | Seedling dip with FZB24@4g/l | 59.10$^{ab}$ | 6.87$^{cd}$ | 44.29$^{d}$ (41.71) | 15.01 |
| $T_5$ | Soil + Foliar app with FZB24@250g each /ha | 59.25$^{ab}$ | 7.14$^{bc}$ | 41.39$^{e}$ (40.03) | 20.57 |
| $T_6$ | Soil + Foliar app with FZB24@500g each /ha | 57.75$^{abc}$ | 6.71$^{de}$ | 33.96$^{I}$ (35.64) | 34.83 |
| $T_7$ | T3 + Foliar app with FZB24@250g/ha | 55.45$^{cd}$ | 7.15$^{bc}$ | 36.11$^{g}$ (36.93) | 30.70 |
| $T_8$ | T4 + Foliar app with FZB24@500g/ha | 57.86$^{abc}$ | 7.42$^{b}$ | 33.16$^{j}$ (35.15) | 36.37 |
| $T_9$ | T1 + T3 + Soil application @250g/ha + Foliar spray @ 250g each /ha with FZB24@30 DAT | 56.15$^{bc}$ | 7.19$^{bc}$ | 34.93$^{h}$ (36.22) | 32.97 |
| $T_{10}$ | T2 + T4 + Soil application @500g/ha + Foliar spray @ 500g each /ha with FZB24@30 DAT | 59.84$^{a}$ | 8.16$^{a}$ | 31.36$^{k}$ (34.05) | 39.82 |
| $T_{11}$ | Streptomycin sulphate @600ppm + COC 0.25% | 57.23$^{abc}$ | 6.93$^{cd}$ | 12.71$^{l}$ (20.88) | 75.61 |
| $T_{12}$ | Control | 53.10$^{d}$ | 6.36$^{e}$ | 52.11$^{a}$ (46.21) | 0.00 |

* Mean of three replications.

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

Values in parentheses are arcsine-transformed values

paramaters *viz*., number of active tillers per hill and plant height, were significantly higher in the treatment combination of seed treatment @ 4g/kg + seedling dip @ 4g/l + soil application @ 500g/ha + foliar spray @ 500g/ha with the endophytic *Bacillus* FZB24 than other treatment combinations (Table 2).

The treatment combination with seed treatment @ 4g/kg + seedling dip @ 4g/l + soil application @ 500g/ha + foliar spray @ 500g/ha with *B. subtilis* (FZB24) recorded higher plant height of 59.84cm against 53.10cm on 75 days after transplanting in untreated control. Similarly in case of number of productive tillers, the combination treatment of seed treatment @ 4g/kg + seedling dip @ 4g/l + soil application @ 500g/ha + foliar spray @ 500g/ha with *B. subtilis* (FZB24) recorded higher number of tillers than other treatments. Least number of tillers was observed in the untreated control plants (Table 2).

**Enhanced Activity of Defence Enzymes:** The study of disease resistance in rice plants treated with *B. subtilis* (FZB24) bioformulation revealed the higher activity and expression of defence-related proteins against Bacterial leaf blight pathogen. The activity of PO and PPO were measured in leaves from *Xoo* inoculated and *B. subtilis* (FZB24) bioformulations pretreated rice plants. *B. subtilis* (FZB24) bacteria in different method of application differed in their ability to stimulate PO and PPO in rice plants inoculated with *Xoo*. Increased PO and PPO activity was observed in the combination treatment of seed treatment @ 4g/kg + seedling dip @ 4g/l + soil application @ 500g/ha + foliar spray @ 500g/ha with *B. subtilis* (FZB24) inoculated with *Xoo* compared to untreated control plants. The activity was found to increase at 4 days after inoculation and, thereafter, it was declined. In contrast, the increased activity of PO and PPO was observed only up to the third day of *Xoo* inoculation in untreated control plants and, thereafter, a drastic reduction in enzyme activity was documented (Figure 1 and 2). Similarly, the assay of PAL from the *B. subtilis* (FZB24) treated plants inoculated with Xoo showed an enhanced activity compared to untreated control plants (Figure 5).

**Accumulation of Total Phenols:** The application of *B. subtilis* (FZB24), as seed treatment @ 4g/kg + seedling dip @ 4g/l + soil application @ 500g/ha + foliar spray @ 500g/ha resulted in higher accumulation of total phenols in rice leaves challenged with *Xoo* (Figure 6). In contrast, the inoculated and uninoculted healthy control plants showed lower amount of accumulation of phenols than bacterized plants. A higher phenol accumulation was observed on 4th day of inoculation in above treatment.

**Native PAGE Analysis of Defence Enzymes:** The native PAGE analysis of enzyme extract from *B. subtilis* (FZB24) treated as seed treatment @ 4g/kg + seedling dip @ 4g/l + soil application @ 500g/ha + foliar spray @ 500g/ha rice plants inoculated with *Xoo* expressed four isoforms PO1, PO2, PO3 and PO4, whereas in non-bacterised plants, only two isoforms PO1 and PO4 were observed. (Figure 3).

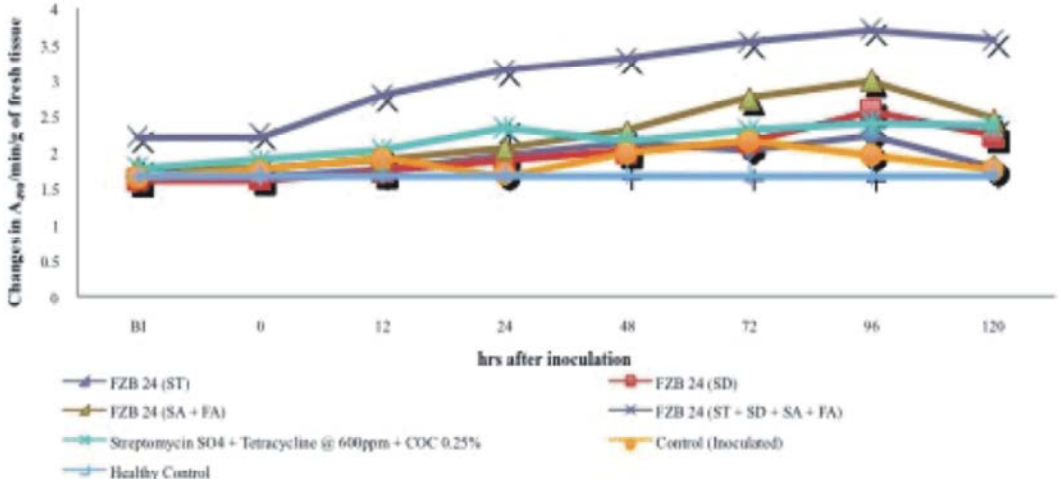


Fig. 1: Induction of peroxidase activity in rice plants upon treated with endophytic *B. subtilis* (FZB24) challengaed with *X. oryzae* pv. *oryzae* under glasshouse cindition

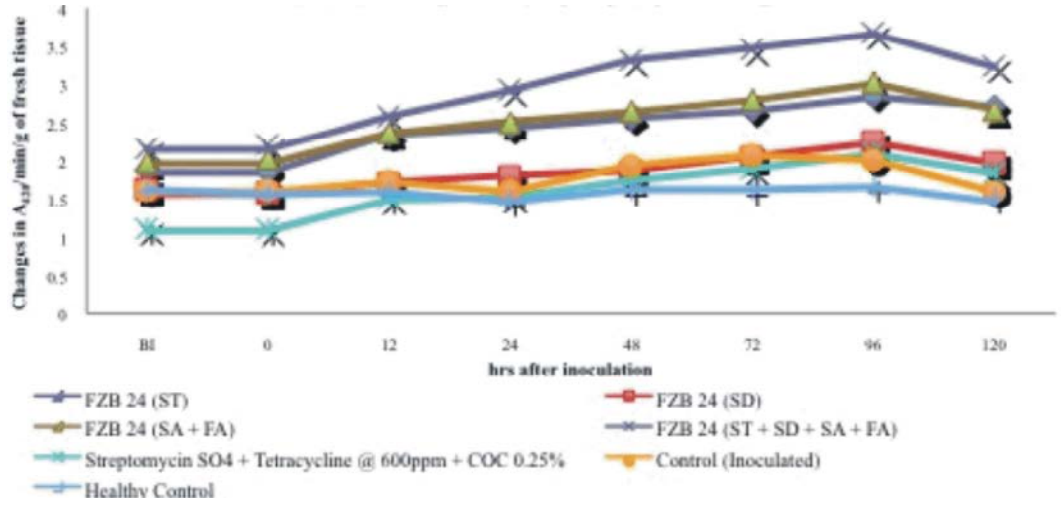


Fig. 2: Induction of Polyphenol exidase activity in rice plants upon treated with emlophytic *B. subtilis* (FZB24) challenged with *X. oryzae* pv. *oryzae* under glasshouse condition

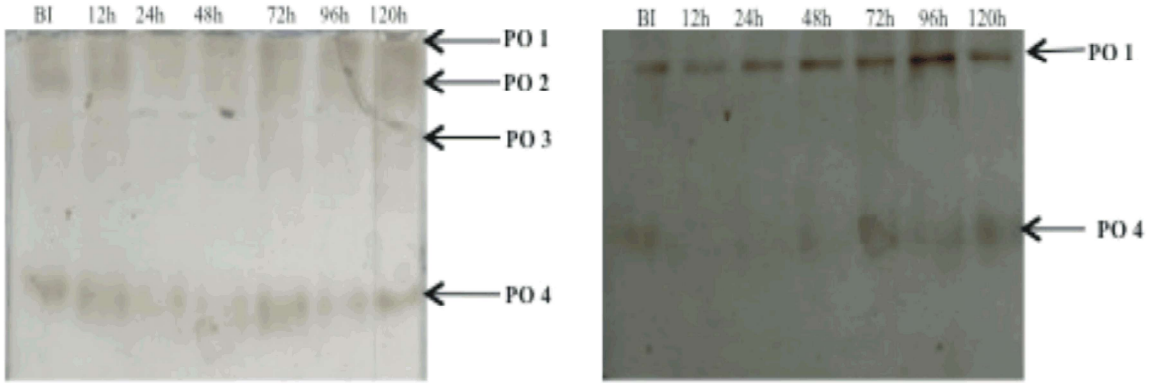


Fig. 3: Expression of PO isoforms in rice plants upon treatment with FZB24 and untreated control challenged with *X. Oryzae* pv. *Oryzae* under glasshouse conditions

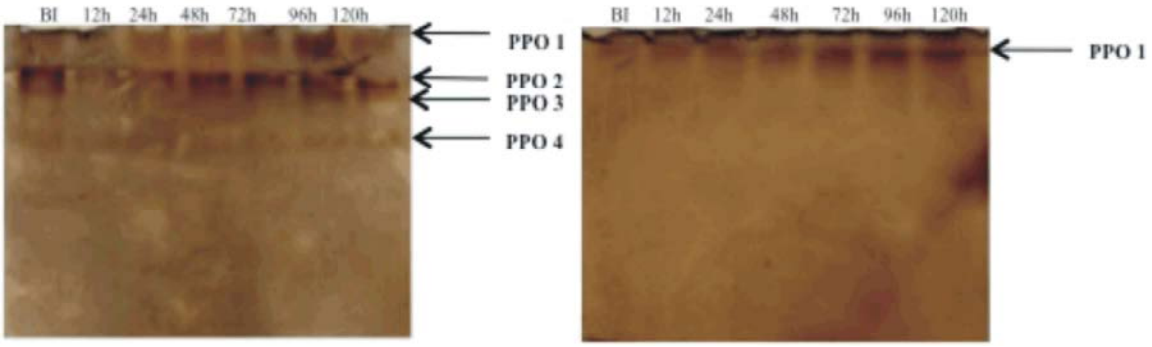


Fig. 4: Expression of PPO isoforms in rice plants upon treatment with FZB24 and untreated control challenged with *X. Oryzae* pv. *Oryzae* under glasshouse conditions

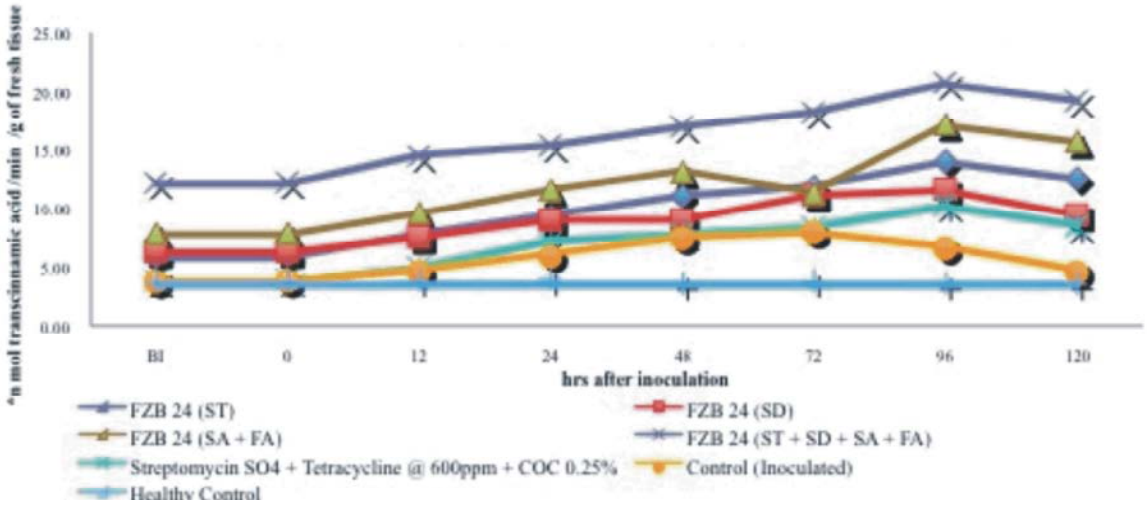


Fig. 5: Induction of Phenylalanine Ammonia Lyase in rice plants upon treated with *B. Subtilis* (FZB24) challenged with *X. Aryzae* pv. *Oryzae* under glasshouse condition

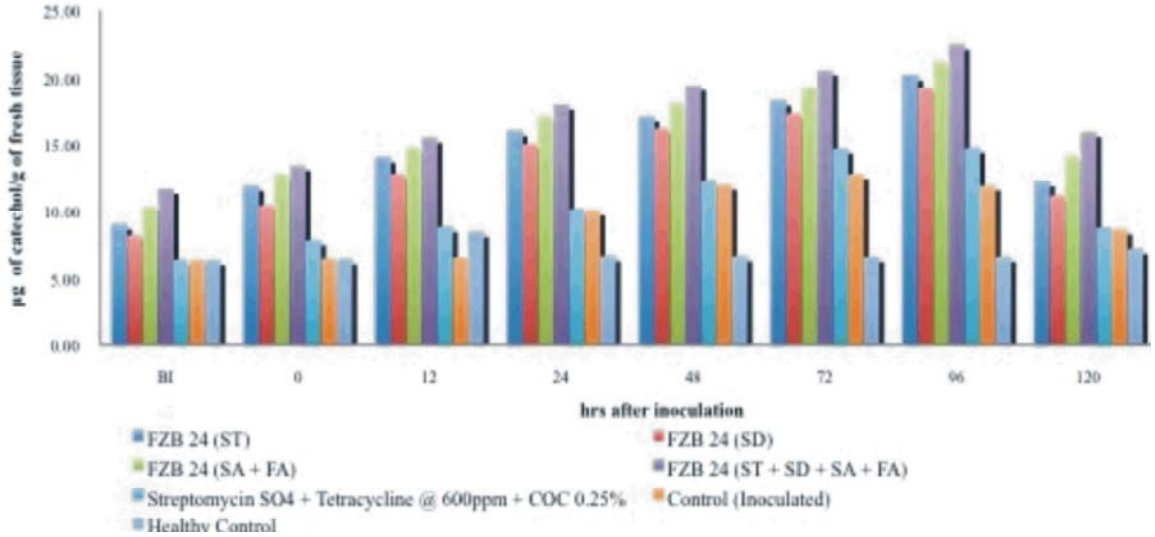


Fig. 6: Accumulation of total phenols activity in rice plants upon treated withendophytic *B.subtilis* (FZB24) challenged with *X. Oryzae* pv. *Oryzae* under glasshouse condition

Table 3: Effect of *B. subtilis* (FZB24) treatments on the plant growth parameters and bacterial leaf blight intensity of rice (ADT39) under Field conditions

| | | Growth parametrs* | | Disease intensity* | | Yield* | |
|---|---|---|---|---|---|---|---|
| S.No | Treatment | Plant Height 90 DAT (cm) | No. of Tillers 60 DAT | PDI | % reduction over control | Grain (kg/ha) | Straw (kg/ha) |
| $T_1$ | Seed treat with FZB24@2g/kg | 81.36$^{j}$ | 16.89$^{e}$ | 10.110$^{e}$ (18.53) | 48.99 | 4078$^{j}$ | 6358$^{i}$ |
| $T_2$ | Seed treat with FZB24@4g/kg | 82.58$^{b}$ | 17.52$^{b}$ | 7.580$^{g}$ (15.97) | 61.76 | 4570$^{b}$ | 6813$^{c}$ |
| $T_3$ | Seedling dip with FZB24@2g/l | 80.29$^{k}$ | 15.79$^{i}$ | 14.210$^{b}$(22.13) | 28.30 | 4042$^{k}$ | 6192$^{j}$ |
| $T_4$ | Seedling dip with FZB24@4g/l | 81.93$^{e}$ | 15.78$^{j}$ | 10.110$^{e}$(18.53) | 48.99 | 4208$^{h}$ | 6438$^{h}$ |
| $T_5$ | Soil + Foliar app with FZB24@250g each /ha | 81.85$^{g}$ | 16.11$^{h}$ | 9.670$^{d}$ (18.11) | 51.21 | 4276$^{f}$ | 6561$^{g}$ |
| $T_6$ | Soil + Foliar app with FZB24@500g each /ha | 81.48$^{h}$ | 15.51$^{k}$ | 9.220$^{f}$(17.66) | 53.48 | 4438$^{e}$ | 6773$^{d}$ |
| $T_7$ | T3 + Foliar app with FZB24@250g/ha | 81.46$^{i}$ | 16.61$^{f}$ | 9.460$^{e}$ (17.9) | 52.27 | 4220$^{g}$ | 6605$^{e}$ |
| $T_8$ | T4 + Foliar app with FZB24@500g/ha | 81.93$^{f}$ | 17.16$^{c}$ | 5.780$^{h}$(13.9) | 70.84 | 4485$^{c}$ | 6841$^{b}$ |
| $T_9$ | T1 + T3 + Soil application @250g/ha + Foliar spray @ 250g each /ha with FZB24@30 DAT | 82.20$^{d}$ | 16.93$^{d}$ | 5.720$^{i}$ (13.83) | 71.14 | 4456$^{d}$ | 6602$^{f}$ |
| $T_{10}$ | T2 + T4 + Soil application @500g/ha + Foliar spray @ 500g each /ha with FZB24@30 DAT | 83.14$^{a}$ | 18.48$^{a}$ | 2.800$^{j}$ (9.62) | 85.87 | 5025$^{a}$ | 7052$^{a}$ |
| $T_{11}$ | Streptomycin sulphate @600ppm + COC 0.25% | 82.51$^{c}$ | 16.30$^{g}$ | 2.070$^{k}$ (8.26) | 89.56 | 4081$^{i}$ | 5977$^{k}$ |
| $T_{12}$ | Control | 73.67$^{l}$ | 14.35$^{l}$ | 19.820$^{a}$(26.42) | 0.00 | 3720$^{l}$ | 5445$^{l}$ |

* Mean of three replications.

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

Values in parentheses are arcsine-transformed values

Similarly, four isoforms of PPO1, PPO2, PPO3 and PPO4 were observed in *B. subtilis* (FZB24) treated as seed treatment @ 4g/kg + seedling dip @ 4g/l + soil application @ 500g/ha + foliar spray @ 500g/ha rice plants inoculated with *Xoo* while in the controls, only one isoform was noticed (Figure 4).

**Efficacy of *B. subtilis* var. Amyloliquefaciens (FZB 24) Against BLB under Field Conditions:** Two different field trials conducted at different locations and seasons revealed that, among different treatments, application of *B. subtilis* (FZB24) bio-formulation as seed treatment @ 4g/kg + seedling dip @ 4g/l + soil application @ 500g/ha + foliar spray @ 500g/ha has recorded a lowest PDI of 2.80% with a per cent reduction of 85.87% over control plots. The yield attributing parameters *viz.,* plant height and number of active tillers were recorded in both the field trials. The plots treated with the treatment combination of seed treatment @ 4g/kg + seedling dip @ 4g/l + soil application @ 500g/ha + foliar spray @ 500g/ha with *B. subtilis* (FZB24) recorded higher plant height of 83.14 cm on 90 days after transplanting with maximum number of tillers as much as 18.48 on 60 days after transplanting where as untreated control plants recorded the plant height of 73.67 cm with an average minimum number of tillers of 14.35. In addition to disease control, above treatment recorded a 35% increase in yield over untreated controls, whereas in the chemical treatment, an increase of only 9.7% were recorded in both the trials (Table 3) through the severity is less.

## DISCUSSION

Endophytic bacteria colonize an ecological niche similar to that of plant pathogens, especially vascular wilt pathogens. Exploiting an additional microbial habitat for biocontrol purposes might enhance overall biocontrol efficacy and increase consistency in performance, since the endophytic agent could avoid unfavourable conditions prevailing in the soil environment by entering and localizing in the intercellular spaces of the epidermal cells of root tissues. Endophytic bacteria have shown significant control of diseases such as *Fusarium vasinfectum* in cotton (Chen *et al*. [37]; Van Buren *et al*. [38]), *Verticillium albo-atrum*, *Rhizoctonia solani* and *Clavibacter michiganesis* subsp, *sepedonicum* in potato (Nowak *et al*. [39]), *Sclerotium rolfsii* in bean (Pleban *et al*. [40]), *Rhizoctonia solani, Pythium myriotylum, Gauemannomyces graminis* and *Helerobasidium annosum* in rice (Mukhopadhyay *et al*. [41]) and *Fusarium moniliformae* in maize (Hinton and Bacon, [42]).

The development of biocontrol strategies using endophytes is an emerging area in crop protection to reduce the damage caused by plant pathogens in economically important crops. Forty endophytes were isolated from different plant sources. Similarly, endophytic bacterial isolates of *Bacillus* (39%), *Pseudomonas* (27.6%), *Corynebacterium* (16.7%), *Actinomyces* (11.1%) and *Staphylococcus* (5.6%) with enzymatic activity in solid media isolated from *Jacaranda decurrens* (a medicinal plant) were reported (Carrim *et al.* [43]).

In the present study, endophytic bacterium, *B. subtilis* (FZB24) was found to increase the vigour index of rice seedlings significantly and effectively inhibited the growth of *Xoo in vitro.* Similar report was given by Rajendran *et al*. [44] that endophytic Bacillus (EPC 5) inhibited the growth of *Ganoderma lucidum* in coconut. The endophytic *Bacillus* spp. CY22 isolated from balloon flower produced iturin A with antifungal activity against *Rhizoctonia solani, Pythium ultimum* and *Fusarium oxysporum* (Cho *et al.* [45]).

In addition to biological control, endophytic bacteria improved plant growth in different crops like potato (Sturz, [46]) and rice (Hurek *et al*. [47]). Hallmann *et al*. [48] speculated that the observed plant growth promotion in different crops might have been caused by enhanced plant mineral uptake and improved plant water relationships associated with the colonization of endophytic strains. Some strains of *Pseudomonas, Enterobacter*, *Staphylococcus, Azotobacter* and *Azospirillum* produce plant growth regulators such as ethylene, auxins or cytokinins and have, therefore, been considered as causal agents for altering plant growth and development. In addition to a direct mechanism for growth promotion, plant growth promotion is also thought to be due to the suppression of deleterious microflora by introduced endophyte (Kloepper *et al*. [49]; Leifert *et al*. [50]). The beneficial effects of bacterial endophytes, however, vary and appear to operate through similar mechanisms as described for PGPR (Kloepper *et al*. [49]; Hoflich *et al*. [51]). However, because of the different habitats colonized, endophytes offer another tool for developing biological control strategies. By integrating the use of bacterial endophytes with rhizosphere antagonists, a holistic biological control system could be developed that works against the pathogens.

The selection of bacterial endophytes for the management of bacterial leaf blight of rice was done based on the consideration of criteria such as growth promotion, antagonistic activity against *X. oryzae* pv. *oryzae,* antibiotics and ACC deaminase production. Considering all the above criteria *Bacillus subtilis* strain FZB24 was selected to assess its performance for the management of bacterial leaf blight of rice both under glasshouse and field conditions. It was applied to the rice plants by different methods of application such as seed treatment, seedling dip, soil application and foliar application at two different concentrations. It was also tried in combination of different methods of application.

It was found that *B. subtilis* (FZB24) has performed well in treatment combination *viz*., seed treatment @ 4g/kg + seedling dip @4g/l + soil application @500g/ha + foliar application @500g/ha both under glasshouse and field conditions. This treatment recorded more plant height with more number of tillers compared to untreated control plants. Upon artificial inoculation of *X. oryzae* pv. *oryzae,* the endophytes treated (seed treatment @ 4g/kg + seedling dip @4g/l + soil application @ 500g/ha + foliar application @500g/ha on 30 days after transplanting) plants recorded a significantly lower disease severity of bacterial leaf blight compared to untreated control plants. This finding is on par with findings of (Melnick *et al*. [52]), that significant reductions of disease severity on cacao leaf disks challenged with *Phytophthora capsici* were recorded following colonization with endophytic *Bacillus,* BT8. Also Salah *et al.* [53] has found that *B. subtilis* B49 found to be the most effective in inhibiting the growth of *Xanthomonas axonopodis* pv. *malvacearum in vitro* among 93 isolates of rhizobacteria and also found to effectively control the bacterial blight of cotton both under greenhouse and field conditions.

Kloepper *et al*. [54] found that five of six rhizobacteria, which induced systemic resistance in cucumber, exhibited both external and internal colonization. In addition, *Pseudomonas fluorescens* 89B-27 and *Serratia marcescens* 90-166 induced resistance in cucumber to *Pseudomonas syringae* pv. *lachrymans* as well as to the fungal pathogens, *F. oxysporum* f. sp. *cucumerinum* and *Colletotrichum orbiculare* (Liu *et al*. [54]). In the present study, rice plants treated with the bioformulation containing endophytic bacteria *Bacillus subtilis* (FZB24) with the treatment combination of seed treatment @ 4g/kg + seedling dip @ 4g/l + soil application @ 500g/ha + foliar spray @ 500g/ha and challenged with *Xoo* showed higher induction of PO, PPO and PAL. The timing and expression patterns of the defense mechanisms are important for the suppression of pathogen. Higher level expression of defense related proteins and timely accumulation of chemicals at the infection site certainly prevent the colonization of pathogen in rice seedlings. The maximum accumulation of PO, PPO and PAL was observed on 4 days of challenge inoculation with *Xoo* after it starts declining.

Similarly, the activities of PO, PPO and PAL were found to be higher after two days of challenge inoculation after that it was declined in the cotton plants treated with endophytic *Bacillus* EPCO 16 and EPCO 102 upon challenge inoculation with *X. axonopodis* pv. *malvacerum* (Rajendran *et al.* [55]). Thus, enhanced induction of defence related enzymes in endophyte treated plants might have been a part of ISR which eventually reduced

the pathogen infection caused by *X. oryzae* pv. *oryzae* upon artificial inoculation under glasshouse conditions. In the present study, higher levels of phenolics occurred in rice plants treated with endophytes against *Xoo.* Benhamou *et al.* [56] reported that the endophytic bacterium *Serratia plymuthica* raised levels of phenolics in cucumber roots, affording protection against *Pythium ultimum.* Also Rajendran *et al*. [55] reported that endophytic bacillus EPCO 16 and EPCO 102 increased the accumulation of phenolics in cotton plant upon challenged inoculation with *Xam*. Further, biocontrol strains stimulate the activities of defence enzymes PO, PPO and PAL in plants that could be involved in the synthesis of phytoalexins (Chen *et al*. [57]; van Loon and Bakker [58]).

In the present study, incidence of bacterial leaf blight under natural condition is found to be less in Bacillus (FZB 24) treated plots than the untreated plots. Incidence was much lower in the treatment combination of seed treatment @ 4g/kg + seedling dip @4g/l + soil application @500g/ha + foliar application @500g/ha on 30 days after transplanting. Also treated plots showed increased plant height with maximum number of tillers and recorded higher grain and straw yield compared to untreated control plots. Similar result was observed in the work of Dunne *et al*. [59], that strain mixtures of DAPG producer *P. fluorescens* F113 and a proteolytic rhizobacterium enhanced the plant growth in terms of increased seedling vigour and grain yield.

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