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Biochemical Changes in Rice Plants Treated with Endophytic Bacteria Against Bacterial Leaf Blight

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ABSTRACT: Twenty-nine bacterial endophytes were isolated from healthy rice plants of most popular varieties *i.e.*, ADT-37, MTU-1010, BPT-5204 and RNR-15048 of the Nellore and Chittoor districts and tested for their antagonistic activity against the bacterial leaf blight (BLB) pathogen *Xanthomonas oryzae pv. oryzae* (Xoo) using the *in vitro* agar well diffusion method. Inhibition zones among the isolates ranged from 0.0 to 14.5 mm. Four isolates - EYK-3, ECP-1, ESR-5, and ECT-3 demonstrated significant antagonistic activity, with inhibition zones of 14.3 mm, 12.3 mm, 11.43 mm, and 10.53 mm, respectively, exceeding 10.0 mm, and were deemed effective antagonistic endophytic bacterial isolates. Rice plants treated with these four isolates (EYK-3, ECP-1, ECT-3, and ESR-5) showed elevated expression levels of defense-related enzymes—Peroxidase (PO), Polyphenol oxidase (PPO), and Phenylalanine ammonia lyase (PAL)—upon challenge with Xoo compared to untreated controls. Among these isolates, EYK-3 exhibited the highest expression levels of PO, PPO, and PAL. These findings suggest that the endophytic bacterial isolate EYK-3 could be effectively utilized for managing bacterial leaf blight and promoting plant growth for sustainable rice cultivation.

Keywords: Endophytic bacteria, Antagonism, *Xanthomonas oryzae* pv. *oryzae*, Plant defense enzymes.

INTRODUCTION

Rice, a staple food crop for over three billion people, faces significant threats from Bacterial Leaf Blight (BLB), caused by *Xanthomonas oryzae pv. oryzae*, a major rice disease first reported in India in Koloba district of Maharashtra by Srinivasan *et al.* (1959). While resistant genes are introgressed into high-yielding cultivars, the breakdown of this resistance and remain susceptible. Chemical control measures are not available for BLB, but still because of raise environmental concerns, prompting a shift toward eco-friendly solutions. Bacterial endophytes are emerging as promising biocontrol agents, offering sustainable plant protection with advantages over rhizospheric microbes (Hallmann, 2001). Recent studies have demonstrated that endophytic bacterial consortia, particularly those involving strains such as *Bacillus subtilis* and *Pseudomonas fluorescens*, can significantly suppress BLB and promote plant growth under greenhouse and field conditions (Choubey *et al*., 2023). Advances in molecular characterization and omics technologies have enabled the identification of novel endophyte strains capable of inducing systemic resistance and triggering defense-related enzymes in rice plants (Xin *et al*., 2022). Furthermore, the development of commercial bioformulations based on these endophytes has shown promising results in multi-location trials, highlighting their potential as sustainable alternatives for BLB management (Kumar *et al*., 2022).

MATERIAL AND METHODS

Isolation of endophytic bacteria. For isolation of endophytes, two grams of leaves were collected from healthy plants. The disinfection and isolation were performed according to Araujo *et al.* (2002).

Isolation of *Xanthomonas oryzae* pv. *oryzae*. The infected leaf sample was sterilized with one per cent Sodium hypochlorite for three minutes and then washed with sterile distilled water. Leaf bits of infected leaves after drying on sterile blotting paper were transferred onto nutrient agar (NA) medium and incubated at 28ºC for 72 h (Jabeen *et al.*, 2012). The emerging colonies which are round, convex, mucoid and yellow in colour were sub-cultured onto NA plates for pure culture.

***In vitro* evaluation of endophytic bacterial isolates against *X. oryzae* pv. *oryzae*:** Cell suspension of *Xoo* was prepared in nutrient broth to a concentration of 10^8

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 plates. After solidification, four wells were formed with the help of 6mm diameter cork borer at one cm away from either side of the petri dish and 10µl of each endophytic bacterial isolate culture suspension was poured in each well. The inoculated plates were incubated at 28±2ºC for 48 h after which the diameter of the inhibition zone (mm) was measured (Salah *et al*., 2010).

Effective antagonistic endophytic bacteria to supress BLB was evaluated in pot culture experiments under greenhouse conditions. Rice seeds (cv. NLR-34449) were surface sterilized with two per cent Sodium hypochlorite for 30 seconds, rinsed in sterile distilled water and dried overnight. The seeds were dipped in broth cultures of endophytic bacteria for 45 minutes and directly sown in pots to raise nursery. On 21st day of sowing, seedling roots were dipped endophytic bacterial suspension. On 23rd day of sowing the leaves were inoculated with bacterial leaf blight pathogen by clip inoculation method (Kauffman *et al.*, 1973). Foliar spraying of endophyte suspension was done after 24 h of clip inoculation.

Enzyme assay was carried after 24, 48, 72 and 96 hours of foliar spray of endophytic bacteria on BLB infected rice plants. 4-5 infected leaves were randomly harvested and cut into small pieces. The leaf bits were grounded into fine powder and processed immediately for enzyme. The activity of peroxidase (PO), polyphenol oxidases (PPO) and phenylalanine ammonia lyase (PAL) was determined by spectrophotometric measurement.

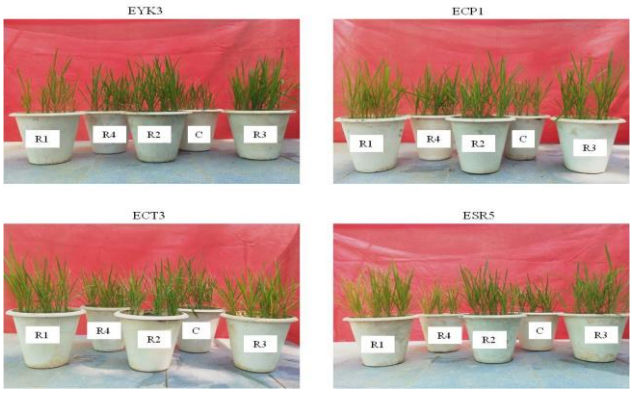

Fig. 1. Estimation of defense related enzymes in rice plants inoculated with endophytic bacteria.

Peroxidases. Fresh leaf sample of 1g was grinded with 3 ml of Sodium Phosphate buffer (pH 7.0) in pre cold mortar using pestle at 4ºC. Then the sample was centrifuged at 18,000 rpm for 15 min at 5ºC. Collect the supernatant (enzyme extract). Buffer solution of 3 ml, 0.05 ml of Guaiacol, 0.03ml of Hydrogen Peroxide and 1ml of enzyme extract were taken into a test tube and the resultant was mixed well. Preparation of solution without enzyme extract serves as a blank to calibrate the spectrophotometer. Then read absorbance (OD) at 470 nm by UV-VIS spectrophotometer using kinetics method. The change on optical density (OD) between 30 and 150 sec. at 470 nm was used to plot peroxidase activity. A change in absorbance by 0.01per minute as accepted as unit of activity. The enzyme activity was expressed as OD/min/g of fresh tissue.

Phenylalanine ammonia lyase. Apical segments of rice leaves about 3cm were harvested and washed thoroughly with deionized water. A 50 mg quantity of washed leaf tissue was then homogenized with a mortar and pestle under chilled conditions in 0.65 ml of 50mM Tris-HCl buffer (pH 8.8) containing 15mM of β-mercaptoethanol. The homogenate was centrifuged at 10,000 rpm for 20 min at 4ºC, and the supernatant was retained as the enzyme extract. 0.1 ml volume of the enzyme extract along with 1ml of the extraction buffer, 0.5 ml of 10 mML-Phenylalanine and 0.4 ml of deionized water, was incubated at 37ºC for 30 min. The reaction was terminated by the addition of 0.5 ml of 6M HCl and the product was extracted with 15 ml of ethyl acetate using a rotary evaporator. The solid residue was suspended in 3 ml of 0.05M NaOH and the Cinnamic-acid concentration was measured spectrophotometrically for the absorbance at 290 nm. One unit of PAL activity was defined as 1µ mol. of Cinnamic acid produced per minute (Ross and Sederoff 1992).

Polyphenol oxidase. The sample of 1 g was homogenized in 2 ml of 0.1M Sodium Phosphate buffer (pH 7.0) at 4ºC. The homogenate was centrifuged at 20,000 rpm for 15 min. The supernatant served as enzyme source and stored at 4ºC (Mayer *et al*., 1965). Polyphenol oxidase activity was determined as given the reaction mixture consisted of 1.5 ml of 0.1M Sodium Phosphate buffer (pH 7.0) and 200 µl of the enzyme extract. To start the reaction, 200 µl of 0.1M Catechol was added and the activity was expressed as change in absorbance at 495 nm at 30sec intervals for 3 min. The enzyme activity was expressed as OD/min/g.

RESULTS AND DISCUSSION

Isolation of endophytic bacteria. A total of 29 endophytic bacterial isolates obtained from healthy leaves of the cultivars *viz.*, ADT-37, MTU-1010, BPT-5204 and RNR-15048 which are popularly cultivated in Chittoor district.

Isolation of pathogen. Single, well separated, pin head sized, light yellow, mucoid, round and raised colonies with smooth margins were obtained on NA medium. Pathogenicity of the isolated culture was established by clip inoculation method on rice cultivar NLR-34449 under protected greenhouse conditions.

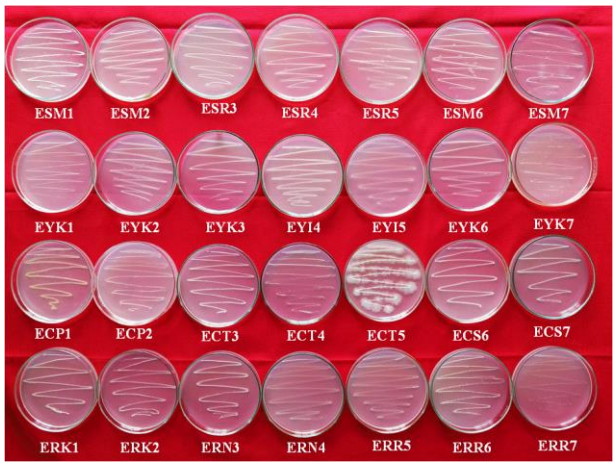

Fig. 2. Pure cultures of endophytic bacteria.

***In vitro* evaluation of endophytic bacterial isolates against *X. oryzae* pv. *oryzae*:** Twenty-nine endophytic bacterial isolates were screened against *X. oryzae* pv. *oryzae* under *in vitro* and there was a significant variation observed among the isolates of endophytic bacteria in their efficacy to inhibit the *X. oryzae* pv. *oryzae*. Among them, twenty-six isolates showed antagonistic activity while three isolates *viz.*, ESM-6, EYI-4 and ERN-4 showed zero inhibition in the screening. The inhibition diameter among the twenty-nine isolates varied from 0.0 to 14.5 mm. Highest inhibition was recorded in the isolate EYK-3 (14.5 mm) where as other endophytic bacterial isolates ECP-1, ESR- 5 and ECT-3 recorded inhibition zones of 12.3, 11.43 and 10.53 mm respectively (Fig. 3).

Biochemical changes in plants. The enzymes were estimated as the change in the absorbance based on the colour change. The levels of all the enzymes were estimated immediately after appearance first symptoms and the external foliar application of the effective endophytic bacteria. The activity of Peroxidase (PO) and Polyphenol oxidase (PPO) started to increase from 24 h after post pathogen inoculation and was maximum at 72 h after challenging with endophytic bacterial spray and thereafter declined from 96 h onwards while in control the meagre increase in PO activity was there up to 72 h and declined after 72 h post inoculation. The same trend was followed both in treatments and control like increasing enzyme activity up to 72 hours and declined after 96 hours, but the amount of expression or change in the absorbance was more in treatments than control. Among the four effective antagonistic endophytic bacterial isolates, EYK-3 showed highest levels of expression of peroxidase and polyphenol oxidase activity with 4.8-fold and 5.5-fold increase respectively with compared to control at 72 hours after inoculation (Fig. 4).

The activity of Phenylalanine ammonia lyase (PAL) increased with the challenging pathogen inoculation followed by the endophytic bacterial spray. There was a significant variation observed among the four isolates of endophytic bacteria for the PAL activity. The PAL activity was increasing from 24 hours and reached peak expression after 72 hours, while in ECT-3, ESR-5 and in control the peak activity was observed up to 48 hours and then declined from 48 hours onwards. Among the four effective antagonistic bacterial endophytes, EYK-3 recorded highest PAL activity (10.48 µmol cinnamic acid/min/g leaves) (Fig. 5).

Similarly, the activity of polyphenol oxidase started to increase from 24 h post pathogen challenging and reached peak after 72 h in all rice plants inoculated with each of the effective antagonistic bacterial endophytes *viz.*, EYK-3, ECP-1, ECT-3 and ESR-5 individually while in control there was increase in the activity up to 48h and thereafter declined from 72 h. Polyphenol oxidase activity was maximum 72 h after challenging inoculation and thereafter declined from 96 h onwards (Fig. 6).

Table 1: Bio-efficacy of endophytic bacterial isolates against X. *oryzae* pv. *oryzae* under *in vitro*.

Sr. No.	Name of the isolate	Inhibition zone of antagonistic activity (mm)*	Sr. No.	Name of the isolate	Inhibition zone of antagonistic activity (mm)*	Sr. No.	Name of the isolate	Inhibition zone of antagonistic activity (mm)*
1.	ESM-1	7.76^{f}	11.	EYI-4	0.00^{n}	21.	ECS-7	3.30^{l}
2.	ESM-2	4.63^{jk}	12.	EYI-5	6.20^{g}	22.	ERK-1	5.03^{ij}
3.	ESR-3	7.46^{f}	13.	EYK-6	4.40^{k}	23.	ERK-2	4.20^{k}
4.	ESR-4	4.43^{k}	14.	EYK-7	2.06^{m}	24.	ERN-3	2.06^{m}
5.	**ESR-5**	**11.43^{c}**	15.	**ECP-1**	**12.30^{b}**	25.	ERN-4	0.00^{n}
6.	ESM-6	0.00^{n}	16.	ECP-2	5.33^{hi}	26.	ERR-5	5.30^{hi}
7.	ESM-7	6.33^{g}	17.	**ECT-3**	**10.53^{d}**	27.	ERR-6	4.20^{k}
8.	EYK-1	4.30^{k}	18.	ECT-4	3.26^{l}	28.	ERR-7	2.10^{m}
9.	EYK-2	5.60^{h}	19.	ECT-5	8.33^{e}	29.	ERR-8	6.16^{g}
10.	**EYK-3**	**14.50^{a}**	20.	ECS-6	7.26^{h}	30.	**Control**	0.00^{n}
C.D		**0.84**						
C.V%		**9.68**						

***Mean of three replications**

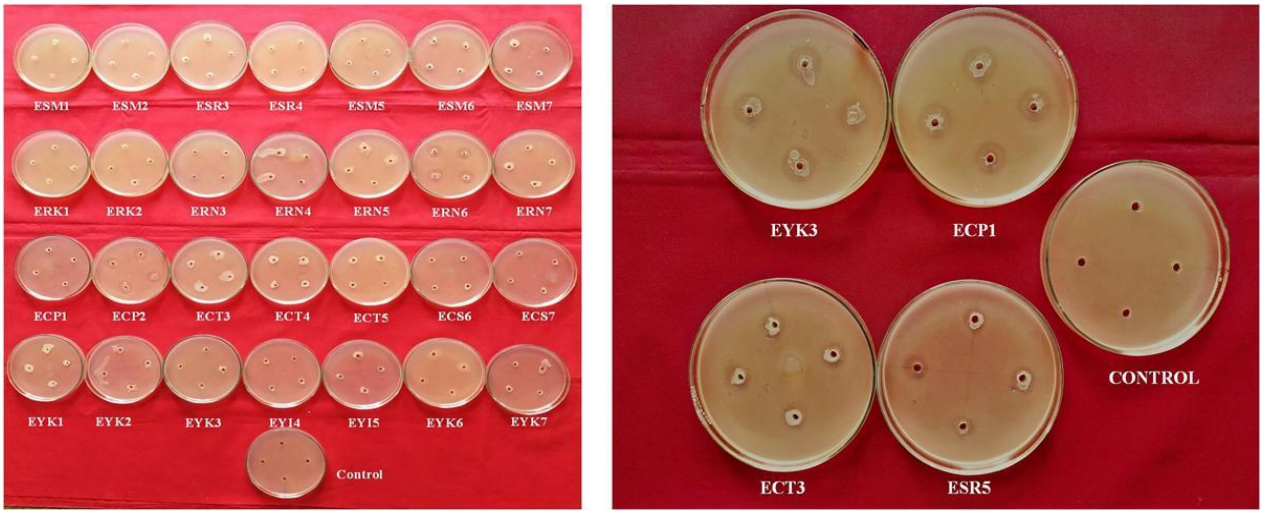

Fig. 3. Bioefficacy of endophytic bacterial isolates against BLB pathogen (*X. oryzae* pv. *oryzae*) using agar well diffusion method.

The increased levels of expression of defense related enzymes in the endophytic bacteria treated rice plants play either a direct or an indirect role in the suppression of pathogen growth. These results were in accordance with the results of Yasmin *et al*. (2016); Rasul *et al.* (2019) where they observed increased activity of defense related enzymes including Phenylalanine ammonia-lyase and Polyphenol oxidase, and Peroxidase was observed in plant inoculated with *Pseudomonas sp.* Rh323 and P-solubilizing bacteria in response to Xoo. Nagendran *et al*. (2013) reported about the PO and PPO activity in the combination treatment of seed treatment @ 4g/kg + of seedling dip @ 4g/l +soil application @500g/ha + foliar spray @ 500 g/ha with *B. Subtilis* (FZB 24) on challenging with *Xoo* at 4 days after inoculation compared to untreated plants in control. The increased activity of PO and PPO was observed only up to the third day of *Xoo* inoculation in untreated control plants and afterwards drastic reduction. Ooi *et al.* (2022) demonstrated that endophytic *Bacillus velezensis* significantly boosted PAL and PPO activity in rice, contributing to reduced bacterial blight symptoms.

Plants inoculated with efficient antagonistic endophytic bacterial consortium after challenge inoculation with the Xoo pathogen produced more defensive enzymes such PO, PPO, and PAL. The rice plants treated with endophytic bacteria have elevated defense-related enzyme activity, which either directly or indirectly inhibit pathogen growth (Swathi *et al*., 2023).

Plants are constantly involved in interactions with a wide range of bacteria. Endophytic microorganisms grow within the healthy tissues of living plants during all or part of their life cycle without causing harmful effects on the host (Hallmann *et al*., 1997; Sturz *et al*., 2000; Ray *et al*., 2017). Endophytic bacteria inhabit plant internal tissues in a similar niche as phytopathogens, and they may compete with bacterial pathogens as biocontrol agents (Berg *et al*., 2005).

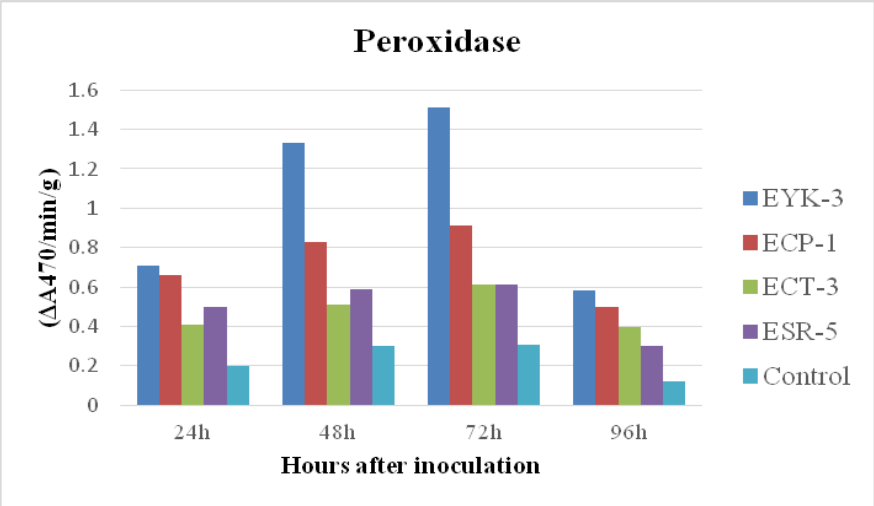

Fig. 4. Expression levels of PO activity in rice plants treated with effective antagonistic endophytic bacterial isolates and inoculated with *Xoo.*

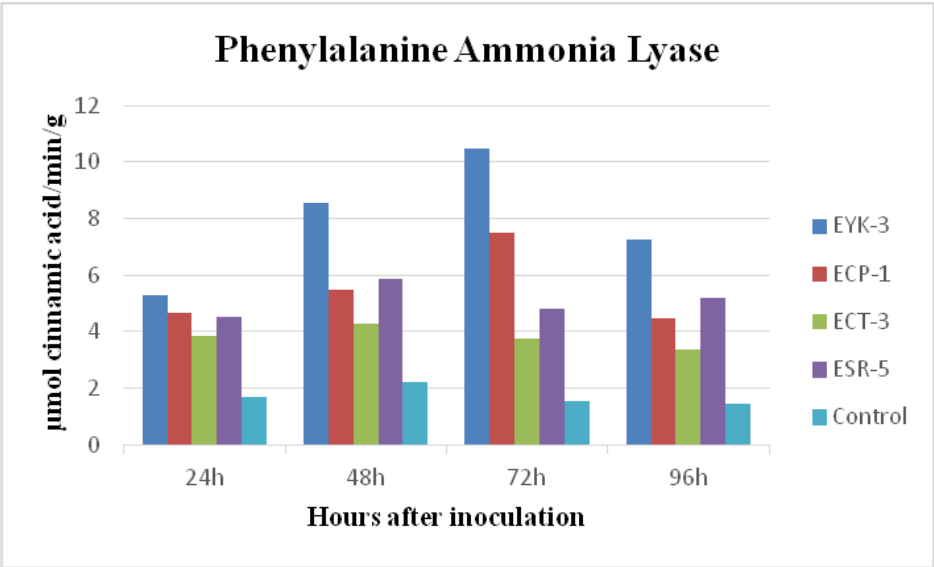

Fig. 5. Expression levels of PAL activity in rice plants treated with effective antagonistic endophytic bacterial isolates and inoculated with *Xoo*.

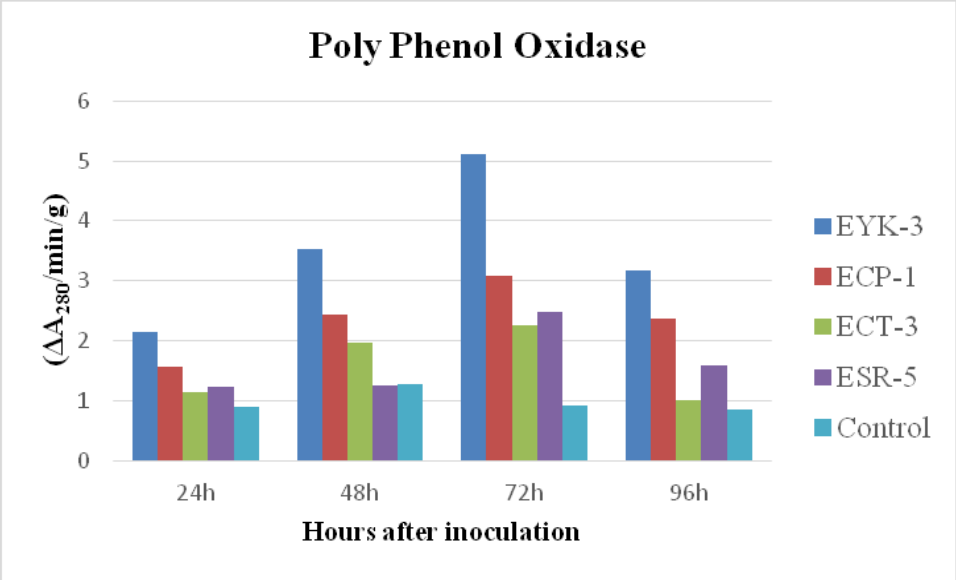

Fig. 6. Expression levels of PPO activity in rice plants treated with effective antagonistic endophytic bacterial isolates and inoculated with *Xoo*.

The increased defense related enzyme activities in the endophytic bacteria treated rice plants play either a direct or an indirect role in the suppression of pathogen growth. Increased activity of defense related enzymes including phenylalanine ammonia-lyase, polyphenol oxidase and peroxidase was observed in plant inoculated with *Pseudomonas* sp. Rh323 and P-solubilizing bacteria in response to *Xoo*. (Yasmin *et al.*, 2016; Rasul *et al.*, 2019). The enhanced induction of defence related enzymes in endophyte treated plants might have been a part of Induced systemic resistance (ISR) which eventually reduces the pathogen infection caused by *X. oryzae* pv. *oryzae* in rice up on artificial inoculation.

Biocontrol agents having plant growth promoting traits may be used in addressing the complicated and integrative phenomena of Plant disease suppression and growth promotion through plant augmentation (Yasmin *et al.*, 2016).

CONCLUSIONS

The application of endophytic bacterial isolates significantly enhanced the activity of defense-related enzymes such as PO, PPO, and PAL in rice plants, with peak expression observed at 72 hours post-pathogen inoculation. Among the isolates, EYK-3 was the most effective in inducing enzyme activity. These biochemical changes played a vital role in strengthening plant defense responses against Xoo.

FUTURE SCOPE

The potential endophytic bacterial isolate EYK-3 has to be developed into different bioformulations and to be tested under different agro-ecological regions for its effective and commercial use.

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Conflict of Interest. None.

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