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Plant Growth Promotion and Induction of Defense Response in *Crocus sativus* L. by Two Native *Bacillus* Species Against *Fusarium oxysporum* R1

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Abstract

Corm rot caused by pathogen *Fusarium oxysporum* is the most devastating disease of saffron. In the present study, previously isolated and characterized *Bacillus* sp. strain D5 (Bar D5) and *Bacillus amyloliquefaciens* W2 (Bam W2), native to *Crocus sativus,* have been compared to select the potential biostimulant for saffron cultivation. Comparative evaluation under the same conditions was done in-vitro, for various plant growth promoting (PGP) activities and in-planta for growth promotion and reduction of most devastating disease (corm rot) caused by the pathogenic fungus *Fusarium oxysporum* R1 (Fox R1). Bar D5 emerged as a clear winner in in-vitro and in-planta evaluations. In in-vitro PGP activities, Bar D5 had supremacy with phosphate solubilization > by 3.9 ± 0.04 folds, ammonia production > by 1.4 ± 0.09 folds, IAA production > by 1.5 ± 0.02 folds, amylase production > by 2.8 ± 0.1 folds and cellulose production by 1.4 ± 0.08 folds higher than Bam W2. However, siderophore and protease production were higher in Bam W2 by 1.9 ± 0.11 and 1 ± 0.06 folds, respectively, when compared to Bar D5. In pot assays, Bar D5 primed corms accumulated higher biomass and exhibited enhanced resistance by induction of various defense enzymes. The activity of defense related enzymes at 5 dpi, such as phenylalanine-ammonia lyase (PAL 1.2 ± 0.07 folds), peroxidase (PO 2 ± 0.04 folds), polyphenol oxidase (PPO 1.4 ± 0.09 folds), lipoxygenase (LOX 1.50.05 ± folds), and β,1-3 glucanase (GLU 1.6 ± 0.3 folds) was higher in Bar D5 primed and Fox R1 inoculated corms compared to Bam W2 primed and Fox R1 inoculated corms. In addition, Bar D5 helped plants to accumulate biomass better than Bam W2, both in the presence and absence of the pathogen. Bar D5 is beyond doubt the potential biostimulant for saffron cultivation.

Keywords *Crocus sativus* · Corm rot · *Fusarium oxysporum* R1 · Defense enzymes · Induced systemic resistance · *Bacillus* sp. strain D5 · *Bacillus amyloliquefaciens* W2

Introduction

Saffron is the most expensive spice of the world with extensive application in food, medicine and beverages (Menia et al. 2018; Jafari et al. 2020; Bhagat et al. 2021). Though the demand for saffron is increasing steadily, the traditional area under cultivation and production is declining concomitantly, leading to a lot of adulteration (Qadri 2017; Cardone et al. 2020). Saffron production is on a decline in Jammu and Kashmir, the only saffron cultivating union territory in India, due to various biotic and abiotic factors (Menia et al. 2018; Gupta et al. 2021). The biotic factors include various corm rot causing fungal pathogens; however rot caused by *Fusarium oxysporum* and *Fusarium solani* is the most destructive (Gupta and Vakhlu 2015; Bhagat et al. 2022). Chemical fungicides used to manage corm rot have a deleterious effects on the environment and also leads to the development of resistance phytopathogens (Burketova et al. 2015; Pandin et al. 2017); hence, there is a need to replace them with eco-friendly technology, such as the use of plant growth promoting bacteria i.e., PGPB (Mishra et al. 2018; Ramakrishna et al. 2019; Ahirwar et al. 2020). PGPB influence

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plant growth and development by various direct mechanisms such as phosphate solubilization, siderophore production, nitrogen fixation, phytohormone production (Batista et al. 2021; Ajijah et al. 2023). Indirectly PGPB protects the plant from deleterious organisms, as they have inherent antagonistic properties which include competing for food, niche or production of chemical compounds lethal for the growth of pathogens or by eliciting a defense response in plants called induced systemic resistance i.e., ISR (Shafi et al. 2017; Kour and Sodhi 2022; Selari et al. 2023).

Among all known plant growth promoting bacteria (PGPB), *Bacillus* and *Pseudomonas* spp. are most preferred (Bhagat et al. 2021). However, members of *Bacillus* are more preferred as they have the capacity to form heat resistance spores, secrete secondary metabolites (antibiotics, siderophores and volatile compounds) and cell wall degrading enzymes, such as β-1,3-glucanase and chitinase (Radhakrishna et al. 2017). *Bacillus* species also induces defense related plant genes by induction of systemic resistance (ISR), along with genetic and structural modifications of the host plant (Sharaf-Eldin et al. 2008; Shafi et al. 2017; Mahapatra et al. 2022). ISR is a phenomenon that enhances the host plant's defense by strengthening the plant cell wall, activating various signal transduction pathways and by enhancing the synthesis of plant defense enzymes when challenged by the pathogen (Sansinenea 2019; Yu et al. 2022). In plants, ISR is reported to be involved in disease suppression caused by various fungal pathogens such as *Colletotrichum truncatum, Fusarium oxysporum* f.sp. *lycopersici* (Fol), *Fusarium oxysporum* f.sp. *niveum* (Fon), *Fusarium solani*, *Xanthomonas campestris* pv. Vesicatoria, etc. (Elanchezhiyan et al. 2018; Gowtham et al. 2018; Akram et al. 2013; Jiang et al. 2019; Chandrasekaran et al. 2017). ISR is associated with increased activity of defense related enzymes such as phenylalanine ammonia lyase (PAL), peroxidase (PO), polyphenol oxidase (PPO), lipoxygenase (LOX), chitinase (Chi) and β,1-3 glucanase (GLU) (Palani et al. 2016; Elanchezhiyan et al. 2018; Jiang et al. 2019; Can et al. 2022).

All these enzymes have been reported to increase the defense of the host plant against various pathogens such as in tomato against *Fusarium oxysporum* and *Fusarium solani* (Rashid et al. 2021a, b), in bean against *Fusarium oxysporum* (Garces-Fiallos et al. 2022), in melon against *Fusarium oxysporum* f.sp. *melonis* race 1.2, (Sadeghpour et al. 2022), in pigeon pea against *Fusarium udum* (Hussain et al. 2023). PAL enzyme catalyses the committed step of the phenylpropanoid pathway resulting in the production of various secondary metabolites (Abdollahi et al. 2022). The enzyme PPO and PO provide defense barrier and resistance to host plant against pathogens by producing oxidative phenols and by detoxifying the reactive oxygen species produced during the reactive burst in plants, respectively (Garces-Fiallos et al. 2022). GLU enzyme hydrolyses the glucan which is an important structural component of fungal cell wall and LOX enzyme provides resistance to host plant by producing antifungal compounds via polyunsaturated fatty acids (PUFA) (Singh et al. 2022; Ye et al. 2023).

In the present study, a comparative evaluation of two native *Bacillus* species Bar D5 and Bam W2 has been done to select the better biostimulant of the two for field trials and subsequent commercialization.

Material and Methods

Corm Samples, Fungal and Bacterial Strains, and Culture Conditions

Saffron corms were collected in sterile polythene bags from Pampore town of Pulwama district (34.02° N longitude, 74.93° E latitude and 1574 m altitude from sea level), Jammu and Kashmir, India in July 2019. The pathogenic *Fusarium oxysporum* R1 (Fox R1) (GenBank Accession number: *KF663598*) used in the present study, has been reported earlier by our group (Gupta and Vakhlu 2015). The Fox R1 culture was cultivated on potato dextrose agar (PDB Himedia, India) at 25 °C for 7 days. Further, Fox R1 agar plug was inoculated in potato dextrose broth (PDB, Himedia) and incubated at 28 °C for 7 days at 180 rpm in incubator shaker (Scigenics, India). After 7 days the culture was filtered through five layers of muslin cloth for harvesting the spores. The filtrate was centrifuged at 10,000 g for 10 min at 4 °C and a concentration of 10^{12} spores/ml was maintained with sterile distilled.

Bacterial strains used in present study have also been characterized earlier by our group; *Bacillus* sp. strain D5 (GenBank Accession number: *KT228251*) (Magotra et al. 2021), *Bacillus amyloliquefaciens* W2 (GenBank Accession number *KF663600*) (Gupta and Vakhlu 2015). Both the cultures were maintained on nutrient agar (Himedia, India) at 37 °C for 24 h. The loop full of active culture were inoculated in nutrient broth and incubated at 37 °C for 48 h with shaking at 180 rpm till stationery phase. After 48 h, the cell count was maintained at 10^8 cells/ml (Magotra et al. 2021).

In-Vitro Plant Growth Promotion (PGP) Activities

Phosphate solubilization

The phosphate released by the isolates was quantified by chlorostannous reduced molybdophosphoric method (Thakur et al. 2019). 0.5 ml of *Bacillus* culture with 1×10^8 cells/ml spore concentration was inoculated in National Botanical Research Institute's phosphate growth medium (NBRIP) broth and incubated at 37 °C for 48 h in shaking incubator at 180 rpm. The cultures were centrifuged at

10,000 rpm at 25 °C for 15 min and supernatant was collected. 100 µl of the supernatant was added to 10 ml of chloromolybdic reagent (15 g $(NH_4)_2MoO_4$ in 400 ml distilled water and 342 ml of concentrated HCl, diluted up to 1 L) and then diluted up to 40 ml by the addition of distilled water. 5–6 drops of chlorostannous acid reagent (10 g $SnCl_2.2H_2O$ in 25 ml concentrated HCl) was added and mixed properly. *Bacillus* converts phosphate in the complex form as orthophosphate which reacts with chloromolybedic reagent and forms phosphomolybedic acid complex during the reaction which gives blue colour as a result of formation of KH_2PO_4. The blue colour formed was measured at 660 nm against blank in UV/visible spectrophotometer (Thermo Scientific™ GENESYS™ 10S UV–Vis Spectrophotometer) using KH_2PO_4 standard curve (Taktek et al. 2017).

Siderophore Production

The quantification of siderophore produced was measured by chrome azurol S (CAS) shuttle assay by inoculating 0.5 ml (1×10^8 cells/ml) of *Bacillus* culture into Luria–Bertani (LB) broth medium (Arora and Verma 2017) and inoculated for 24 h. Cultures were centrifuged at 10,000 rpm at 25 °C for 15 min. 1 ml of the supernatant was added into 1 ml CAS solution and incubated at 25 °C for 5 min. 10 µl of shuttle solution (0.2 M 5-sulfosalicylic acid) was added to it. Siderophore produced by the *Bacillus* scavenge iron from Fe-CAS complex and subsequently CAS dye is released into the medium (Pahari et al. 2017).

Siderophore content as % was calculated as:

$$\text{Siderophore } \% = \frac{(\text{Ar} - \text{As})}{\text{Ar}} \times 100$$

Ar = Absorbance of reference at 630 nm (Uninoculated culture medium with CAS solution and shuttle solution)
As = Absorbance of test sample at 630 nm.

Indole-3-Acetic Acid Quantification

The amount of IAA produced by bacteria was quantified using Salkowski's method (Glickmann and Dessaux 1995). 0.5 ml (1×10^8 cells/ml) of bacterial culture was inoculated into Luria–Bertani (LB) broth containing tryptophan (100 µg/ml) and incubated for 48 h at 37 °C. After incubation cultures were centrifuged at 12,000 rpm for 5 min at 25 °C. 2 ml of Salkowski's reagent was added to the 1 ml supernatant, ferric chloride ions in the salkowski's reagent reacts with indole moiety present in the supernatant and pink colored was produced that was measured at 535 nm. The concentration of Indole-3-acetic acid (IAA) was obtained from IAA standard curve (Walitang et al. 2017).

Ammonia Production

Bacillus isolates were tested for the production of ammonia using peptone water medium. 0.5 ml (1×10^8 cells/ml) of bacterial cultures was inoculated into peptone water (10 ml) and incubated at 37 °C for 36 h. After incubation, cultures were centrifuged for 20 min at 1800 rpm at 4 °C. 1 ml of Nessler's reagent was added to 9 ml of supernatant, iodide ions present in Nessler's reagent react with ammonia under alkaline conditions and colored complex is formed that was measured at 530 nm. The concentration of ammonia produced was determined by $(NH_4)_2SO_4$ standard curve by varying the known concentration of substrate (Sahoo and Chaudhuri 2019).

Amylase, Cellulase and Protease Activity

1% skim milk powder (Asha and Palaniswamy 2018), 1% soluble starch (Luang-In et al. 2019) and 1% Carboxy Methyl Cellulose (CMC) (Croos et al. 2019), was added to LB media for the production of protease, amylase, and cellulase, respectively. *Bacillus* cultures were inoculated in protease, amylase, and cellulase production media and incubated at 37 °C for 24 h at 180 rpm. Supernatant from all the cultures were collected by centrifugation at 5000 rpm for 15 min at 4 °C.

Amylase assay was performed by using 1% starch solution in 0.1 M Citrate buffer (pH 5) and 100 µl supernatant at 45 °C for 10 min. The produced reducing sugars were quantified by adding 3 ml of 3,5-dinitrosalicyclic acid (DNS) and heated for 15 min in boiling water bath. 1 ml of 40% potassium sodium tartarate (Rochelle salt) was added and final volume was raised up to 10 ml by adding distilled water. Reducing sugars produced were measured at 575 nm. The concentration was measured in reference to standard curve made by varying the concentrations of glucose. One unit of amylase was defined as the amount of enzyme required to release 1 µmol of sugar in 1 min (Kalyani and Rajesh 2018).

Endo-β-1,4-glucanase activity of cellulase enzyme was determined by DNS (3,5-dinitrosalicylic acid) method. 1% CMC substrate solution was prepared in citrate buffer 1 N (pH 5.0). 100 µl of supernatant, 1 ml citrate buffer and 1 ml CMC solution were mixed and incubated for 30 min at 45 °C. DNS was added to stop the reaction; solution was placed in boiling water for 10 min, and then cooled for color stabilization. Glucose released during reaction was measured at 540 nm. One unit of cellulase activity was the amount of enzyme required to produce 1 µmol of glucose per minute (Viswanath et al. 2018).

Protease assay was performed using casein as a substrate prepared in carbonate-bicarbonate buffer 20 mM (pH 10). 0.25 ml of supernatant (cell free extract) was added to 0.5 ml of buffer containing 0.5% substrate and incubated for 30 min

at 37 °C. After incubation 0.75 ml of 10% trichloroacetic acid was added and incubated at 25 °C for 30 min to terminate the reaction. Supernatant was collected by centrifugation for 10 min at 12,000 rpm 0.6 ml of 1 N NaOH was added to 0.6 ml of the supernatant and incubated at 25 °C for 15 min. Absorbance was measured at 450 nm. One unit of protease activity was defined as the amount of enzyme which produces 0.001 changes in absorbance by hydrolyzing casein per mg of substrate per minute under assay conditions (Lim et al. 2019).

In-Planta Pot Assays for Growth Parameters

Bioformulation preparation was done by the method described by Magotra et al. (2021). In brief, nutrient broth of Bam W2 and Bar D5 containing (10^8 cells ml^{-1}) individually, were mixed with autoclaved calcium carbonate in the ratio of 1:2 (v/w) and dried for 4 days. Finally 1% sterile carboxymethyl cellulose (CMC) was added to it. Slurry for priming of the corms was prepared by mixing 100 g dried bioformulation in 100 ml sterile distilled water. Priming of the corms was done by dipping corms in the slurry for 5 min, and then air drying them for 5 h before sowing in open in the laboratory. Pot trials were laid in complete randomized block design (CRBD) method. Following treatments were given to 6 corm sets with 10 corms in each set:

C-Mock primed corms (primed with slurry of calcium carbonate and CMC in distilled water) planted in sterile soil
B1-Bam W2 primed corms planted in sterile soil
B2-Bar D5 primed corms planted in sterile soil
CF-Mock primed corms planted in Fox R1 inoculated soil
T1-Bam W2 primed corms planted in Fox R1 inoculated soil
T2-Bar D5 primed corms planted in Fox R1 inoculated soil

Inoculation with Fox R1 was done by mixing 1 ml of 10^{12} ml^{-1} Fox R1 spores to 200 g of autoclaved sand: soil mixture (1:1 w/w) soil that makes final spore count in soil to 5×10^{10} spores g^{-1} of soil. 10 pots were laid for each experiment with 1 corms/pot and in total, 60 pots were laid for the 6 treatments. After planting the corms in pots, pots were incubated in plant growth chamber, under 16 h light/8 h dark cycle at 26 ± 2 °C with 80% relative humidity for 30 days.

After 30 days, all the corms along with the shoot and roots developed in this period were taken out and evaluated for various growth parameters and disease severity index (DSI). DSI was calculated in each treatment by the method (Gupta and Vakhlu 2015) and disease reduction percentage (DRP) by the formula:

$$\mathrm{DRP} = \frac{\mathrm{DSI\ of\ CF} - \mathrm{DSI\ of\ T}}{\mathrm{DSI\ of\ CF}} \times 100$$

CF-corms planted in Fox R1 (10^{12} spores ml^{-1}) inoculated soil and T (Corms primed with respective bacterial bioformulation and planted in Fox R1 inoculated soil).

In-Planta Estimation of Induction of Defense Related Enzymes

A separate experiment was conducted for evaluation of induction of defense related enzymes in corms. In saffron, *Fusarium oxysporum* penetrates the roots and corms only through wounds or injury (Bhagat et al. 2022; Mansotra et al. 2023). So, for the induction of defense mechanism of saffron plant, injury was given using sterile tip in order to mimic natural wounds. A total of 150 corms were taken and divided in to three sets (S1, S2, and S3; 50 corms/set). S1-Mock primed corms, S2-Bam W2 primed corms and S3-Bar D5 primed corms (Fig. 1). A total of 150 pots were

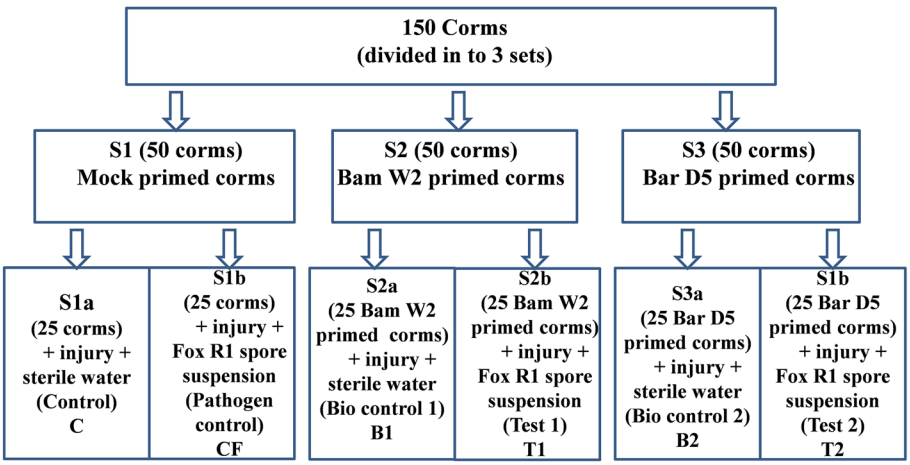

Fig. 1 Experiment layout for the in-planta interaction of *Bacillus* species (Bar D5 and Bam W2) and pathogen (Fox R1) in saffron corms. Bar D5-*Bacillus* sp. strain D5, Bam W2-*Bacillus amyloliquefaciens* W2 and Fox R1-*Fusarium oxysporum* R1

laid maintaining 1 corm/pot. All the 150 corms were grown for a period of 30 days so that corms get primed in the presence of Bar D5 and Bam W2 before inoculating them with pathogen Fox R1. Corms were sown in autoclaved sand: soil mixture (1:1 w/w) in pots and were incubated in plant growth chamber, under 16 h light/8 h dark cycle at 26 ± 2 °C with 80% relative humidity for 30 days. After 30 days of incubation, all the corms were taken out and 50 corms of each set was further divided into two sets (25 corms in each set) so in total six sub sets (S1a, S1b, S2a, S2b, S3a, S3b) were made. All the corms were injured with sterilized needle (3 × 1 cm) and placed in their respective pots. The pots of sub sets (S1b, S2d and S3b) were inoculated with Fox R1 and inoculation was done by mixing 1 ml of 10^{12} ml^{-1} Fox R1 spores to 200 g of soil that makes final spore count in soil to 5×10^{10} spores g^{-1} of soil as mentioned earlier. The remaining subsets (S1a, S2a, and S3a) were inoculated with 1 ml of sterile distilled water that served as control for each treatment, respectively. The details of the different subsets have been shown in Fig. 1. Plantlets at 1, 3, 5, 8, and 11 days post inoculation from all the treatments were taken out and washed under running tape water for 15 min to remove soil particles adhered to the corm surface, leaves and roots followed by tunic removal. The clean corms were then used for the following tests:

In-Planta Inhibition of Fox R1

Fox R1 load was determined in different treatments; CF, T1, and T2 (Fig. 1) and inhibition % was calculated in presence of both Bam W2 and Bar D5. Two different methods were used for determining the antifungal potential (inhibition %) of Bar D5 and Bam W2 against Fox R1.

1. *Visual symptoms*: In this method, a lesion diameter was measured (mm) at 1, 3, 5, 8, 11 dpi and inhibition percentage was calculated as:

$$\frac{\text{Diameter of CF lesion} - \text{Diameter of T lesion}}{\text{Diameter of CF lesion}} \times 100 = \text{Inhibition percentage}$$

CF-Fox R1 inoculated corms, T (Bam W2 and Bar D5 primed and Fox R1 inoculated corms)

2. *Fox R1 load*: Fox R1 load was determined in CF, T1, and T2 (Fig. 1) at day 1, 3, 5, 8, 11 dpi by calculating the colony forming units (CFU). *Fusarium* specific Komada medium was used for the culturing of Fox R1 from infected part of corm (Komada 1975). 100 mg of the tissue was taken and washed with sterile distilled water under sterile conditions. Then it was crushed using sterile pestle mortar and 1 ml of distilled water was added to the crushed tissue. Serial dilution of the suspension was prepared and 10^{-2} dilution was spread on the media plates and incubated at 28 °C for 5 days. After 5 days, colonies were counted and the load was determined. Inhibition percentage was calculated as

$$\frac{\text{Load in CF} - \text{Load in T}}{\text{Load in CF}} \times 100 = \text{Inhibition percentage}$$

All the experiments were replicated independently three times at different time intervals.

Dry Matter Accumulation

Washed corms from different treatments were taken and air dried for 1 h at 25 °C. Fresh weight (FW) of each corm was determined. Corms were dried in the hot air oven at 60 °C for 48 h (Gaspar et al. 2017). Dry weight (DW) was determined. The dry matter percentage (DM %) was calculated using formula:

$$\text{DM\%} = \frac{\text{DW}}{\text{FW}} \times 100$$

Defense Enzyme Activity

Crude protein was extracted by the method developed by Abdel-Monaim et al. (2012) with some modifications. 1 g of fresh corm tissue at 1, 3, 5, 8, 11 dpi was ground with liquid nitrogen in a mortar and pestle at 25 °C. The resulting powder was added to 50 mM ice-cold potassium phosphate buffer (pH 6.8) (4 ml) containing 1 mM ethylene diamine tetra-acetate (EDTA), 1% polyvinylpyrrolidone (PVP) and 1 M NaCl. The solution was centrifuged at 8000 rpm for 25 min at 4 °C. The supernatant was used for activity of defense enzymes.

Phenylalanine-Ammonia Lyase (PAL)

PAL activity was measured according to the method of Astaneh et al. (2018). The reaction mixture consists of 4 ml of 100 mM Tris–HCL buffer (pH 8.8), 0.2 ml of 40 mM L-phenylalanine and 0.2 ml crude enzyme extract. PAL activity leads to release of *trans*-cinnamic acid by its activity on phenylalanine and was measured at 290 nm after 5 min of start of reaction. 10,900 M^{-1} cm^{-1} was employed as molar extinction coefficient for enzyme activity.

Peroxidase (PO)

PO activity was determined according to Uarrota et al. (2016) with some modifications. The reaction mixture was prepared by adding 25 µl of crude enzyme extract, 2 ml of solution containing 1% guaiacol, 1% hydrogen peroxide, and

50 mM potassium phosphate buffer (pH 6.8). PO activity leads to release of oxidized guaiacol from guaiacol and oxygen, which was measured after 15 min of start of reaction at 436 nm and 26.6 mM^{-1} cm^{-1} employing molar extinction coefficients (e) for enzyme activity calculation.

Polyphenol Oxidase (PPO)

PPO activity was determined by method given by Nguyen et al. (2018). The reaction mixture was prepared by adding 50 µl of crude protein extract, 3 ml of solution containing 100 mM potassium phosphate buffer (pH 6.8) and catechol (25 mM). PPO activity leads to release of ortho-quinones from catechol that was measured at 410 nm after 10 min of start of reaction at 30 °C and 1300 M^{-1} cm^{-1} was employed as molar extinction coefficient (e) for enzyme quantification.

Lipoxygenase (LOX)

LOX activity was determined according to Garcia et al. (2017). The reaction mixture was prepared by adding 3 ml of 50 mM sodium phosphate buffer pH 6.0, 60 µl of 10 mM linoleic acid, and 30 µl of crude protein extract. LOX activity leads to release of hydroxyperoxides from linoleic acid was measured at 234 nm after 10 min of start of reaction at min at 25 °C and 25,000 M^{-1} cm^{-1} was employed as molar extinction coefficient (e) for quantification of enzyme activity.

β,1-3 Glucanase (GLU)

GLU activity was determined by Laminarin-DNS method (Gowtham et al. 2018) using laminarin (Sigma-Aldrich) as a substrate. The reaction mixture was prepared by adding 62.5 µl of crude protein extract and 62.5 µl of 4% laminarin prepared in 50 mM sodium acetate buffer (pH 5.0) and incubated at 37 °C for 1 h. Reaction was stopped by the addition of 375 µl of DNS (dinitrosalicyclic acid) reagent and the mixture was incubated at 40 °C for 10 min then 4.5 ml of distilled water was added. The glucose released from the laminarin was measured at 540 nm. Enzyme activity was quantified as mg équivalent of glucose per min per gram of fresh weight and expresed as IU/g of fresh tissue.

RNA Isolation and cDNA Preparation

Total RNA was isolated from 100 mg of corm tissue at 5 dpi using TRIZOL reagent (Invitrogen; Cat No. 15596026) according to the manufacturer's protocol with some modifications. RNA samples were treated with DNase (Promega, USA Cat No. M6101) to remove DNA contamination. The purity and concentration of the total RNA was determined using spectrophometer (Thermo Scientific™ GENESYS™ 10S UV–Vis Spectrophotometer). RNA sample with ratio 1.8–2.0 was used for further study. cDNA was synthesized from 10 µg of the total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied biosystems Cat No. 4368814) following the manufacturer's protocol and stored at − 20 °C for further use. Gene specific primers for RT-PCR (Table 1) were designed by using Primer Express (v3.0) software (Applied Biosystems®, USA) based on the sequences retrieved from National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov).

Quantitative Real Time PCR (qPCR)

The RT-PCR was performed in 8-well strips using SYBR Green-based assay (Thermo-Scientific, Cat No. 4309155) on 7500 Real Time PCR System (Applied Biosystems® model). The 10 µl reaction mixture consisted of SYBR Green Master Mix (5 µl), cDNA template (1 µl) and gene specific primers (0.5 µM each; 0.5 µl forward primer and 0.5 µl reverse primer). The PCR program followed was; for holding stage 95 °C for 10 min, followed by 40 cycles of amplification with following thermal cycle: denaturation at 95 °C for 15 s, annealing at 60 °C for 60 s, and extension at 72 °C for 30 s. The relative quantification was done by $2^{-\Delta\Delta CT}$

Table 1 Gene specific primers used in present study for quantitative RT-PCR expression

Gene	Gene ID	Reference organism	Primer sequences (5′–3′)
PAL	EX147644.1	*Crocus sativus*	F CTGATCGACGTCTCGAGGAAC
			R TCACCGGATTTGCCAGAAACT
PO	EX148603	*Crocus sativus*	F TCCGTGGATTTATCGCCGAG
			R GTAGCATCAGGTAGACGCCC
GLU	BM005643	*Crocus sativus*	F CTCAGCTGTCACAAACCGGA
			R CCTGCACACGTGTAAACCTT
LOX	102598541	*Solanum tuberosum*	F TGCAGGGGAATCGCTGAAAA
			R GGTGGTGGCGATTTGGACTA
PPO	606710	*Solanum lycopersicum*	F CAGCAGGCCAATGTTCACTG
			R CTCATCGAAGACAACCTCAAG

method (Pfaffl 2006) and saffron tubulin gene was taken as an internal control for normalization.

Statistical Analysis

The experiment for plant growth promotion and disease severity was conducted using 10 biological replicates. All other experiments were conducted using five biological replicates and each biological replicate was evaluated based on three technical replicates. Results were expressed as mean ± standard deviation. Data were statistically analyzed by ANOVA using IBM SPSS statistics version 26. The Multiple Duncan range test was performed for analyzing differences between mean values at significant level ($p < 0.05$). All the experiments were replicated independently three times at three different time intervals.

Results

Comparison of In-Vitro Plant Growth Promotion Activities of Two *Bacillus* Species

Bar D5 and Bam W2 were evaluated for PGP activities under same conditions. Both produced phosphatase, siderophores, indole acetic acid, ammonia, protease, amylase and cellulase in-vitro; but production was higher in Bar D5, except for siderophores that was higher in Bam W2 (Table 2). Phosphate solubilization by Bar D5 was 482.68 µg ml^{-1}, IAA production 213.15 µg ml^{-1}, ammonia production 3.9 mg ml^{-1}, amylase activity 30.8 IU ml^{-1} and cellulase activity was 42.3 IU ml^{-1}. Whereas in Bam W2, phosphate solubilization was 22.31 µg ml^{-1}, IAA production 139.84 µg ml^{-1}, ammonia production 2.8 mg ml^{-1}, amylase activity 11.2 IU ml^{-1} and cellulase activity was 30.0 IU ml^{-1}. Siderophore production by Bam W2 was 30.1 µg ml^{-1} that is 1.5 times (56%) more than Bar D5. Protease enzyme activity was almost equal i.e., 5.6 IU ml^{-1} in Bar D5 and 5.7 IU ml^{-1} in Bam W2 (Table 2).

Growth Parameters

The effects of Bam W2 and Bar D5 on the growth parameters of saffron plant in different treatments were monitored after 30 days of incubation. The results have been tabulated in Table 3. Both Bam W2 and Bar D5 increased the shoot number (1.3 ± 0.56, 1.6 ± 0.75 folds), shoot length (1.1 ± 0.03, 1.2 ± 0.07 folds), root number (1.0 ± 0.03, 1.2 ± 0.05 folds), root length (1.0 ± 0.02, 1.1 ± 0.05 folds), number of leaves (1 ± 0.08, 1.2 ± 0.03 folds), shoot dry matter percentage (1.0 ± 0.01, 1.2 ± 0.04 folds), and root dry matter percentage (1.0 ± 0.05, 1.3 ± 0.07 folds) as compared to untreated control (C). In pathogen treated corms (CF), the shoot number decreased by 0.7 ± 0.01 folds, shoot length by 0.9 ± 0.02 folds, root number by 1.0 ± 0.06 folds, root length by 1.0 ± 0.03 folds, number of leaves by 0.9 ± 0.08 folds, shoot dry matter percentage by 0.9 ± 0.09 folds, and root dry matter percentage by 0.8 ± 0.02 folds compared to untreated corms (C) (Table 3). The results indicated that both the strains promoted the growth of the saffron plant though Bar D5 showed better results as compared to Bam W2. As expected, in the presence of pathogen plant growth was reduced (Table 3).

Disease Severity Index and Disease Reduction Percentage

Disease severity index and disease reduction percentage was calculated in all the treatments after 30 days post inoculation and has been tabulated in Table 4. Bar D5 and Bam W2 significantly reduced the disease severity in plants (T2 by 72.4% and T1 by 55.1%) as compared to pathogen control CF (Table 4). These results indicated that Bar D5 was more efficient as compared to Bam W2, in reducing the corm rot symptoms.

In-Planta Inhibition of Fox R1

Severe corm rot symptoms were observed in corms inoculated with Fox R1 (CF). Lesion diameter kept on increasing from 1 to 11 dpi i.e., maximum symptoms was observed at 11 dpi. In T1 (corms primed with Bam W2 and inoculated with Fox R1) diameter of lesion was less compared to CF. However, in T2 (corms primed with Bar D5 and inoculated with Fox R1) less symptoms of corm rot (less lesion diameter) were observed compared to CF and T1. In control, corms inoculated with sterile distilled water, no lesions were observed. The inhibition % has been calculated and tabulated in Table 5. The maximum lesion diameter in all the treatments were at 11 dpi but maximum inhibition was at 5 dpi in both T1 (40.8%) and T2 (60.9%) compared to CF (Table 5). Similar pattern of results were obtained in case of Fox R1 load determined by CFU method. The maximum load was at 11 dpi but maximum inhibition was at 5 dpi in T1 (Bam W2 treated), it was 48.7% and T2 (Bar D5 treated) it was 70.5% compared to CF, and then decrease in inhibition rate was observed. Although, the pattern of inhibition was same in both the assays, the actual value of inhibition % varied in both (Table 5).

Table 2 Quantitative evaluation of in-vitro plant growth promoting activities of *Bacillus* sp. strain D5 and *B. amyloliquefaciens* W2 and in other reported *Bacillus* species

Bacteria	Phosphate solubilization (µg/ml)	Siderophore production (%)	Ammonia production (mg/ml)	IAA production (µg/ml)	Amylase production (IU/ml)	Cellulase production (IU/ml)	Protease production (IU/ml)	Source	Reference
Bacillus sp. strain D5	482.68 ± 0.30	30.1 ± 0.08	3.9 ± 0.060	213.15 ± 0.4	30.8 ± 0.54	42.3 ± 0.15	5.6 ± 0.004	Saffron cormosphere	Present study
B. amyloliquefaciens W2	122.31 ± 0.1	56.0 ± 0.1	2.8 ± 0.005	139.8 ± 0.08	11.2 ± 0.33	30.0 ± 0.1	5.7 ± 0.24	Saffron field bulk soil	Present study
B. amyloliquefaciens S-134	–	–	–	25.9 ± 0.60	–	–	–	*Acacia Arabica* rhizosphere	Raheem et al. (2018)
B. aryabhattai MCC3374	–	✓	✓	166 ± 0.050	–	–	–	Rice rhizosphere	Ghosh et al. (2018)
B. aryabhattai SRB02	–	–	–	✓	–	–	–	Soybean rhizosphere	Park et al. (2017)
B. cereus	✓	✓	✓	82 ± 0.25	✓	–	–	*Aegle marmelos* rhizosphere	NR and Kulkarni (2018)
B. licheniformis AI20	–	–	–	–	166.5 ± 0.4	–	–	Exogenous (Soil sample)	Abdel-Fattah et al. (2012)
B. licheniformis	–	–	–	–	100 ± 0.22	–	–	Exogenous (Soil sample)	Roy and Mukherjee (2013)
B. methylotrophicus (PM19)	✓	–	✓	97 ± 0.004	–	–	✓	Sugarcane rhizosphere	Din et al. (2019)
B. pumilis	✓	✓	✓	126 ± 0.26	✓	–	–	*Aegle marmelos* rhizosphere	NR and Kulkarni (2018)
B. siamensis (PM13)	✓	✓	✓	81 ± 0.40	✓	✓	✓	Sugarcane rhizosphere	Din et al. (2019)
B. siamensis RS8	309.6	✓	✓	120.3 ± 0.20	–	–	–	Tomato rhizosphere	Ramavath et al. (2019)
Bacillus spp. KR-8104	–	–	–	–	3.82 ± 0.05	–	–	Potato rhizosphere	Hashemi et al. (2013)
Bacillus spp. (PM15)	✓	✓	✓	77 ± 0.24	✓	✓	✓	Sugarcane rhizosphere	Din et al. (2019)
Bacillus spp PSB 6	✓	✓	–	24.40 ± 0.50	–	–	–	Maize rhizosphere	Babu et al. (2017)
B. subtilis	–	–	–	–	–	–	✓	Exogenous (Soil sample)	Pant et al. (2015)
B. subtilis PH	S I 2.8 ± 0.2	–	–	–	–	–	–	Tomato rhizosphere	Mohamed et al. (2018)

Table 2 (continued)

Bacteria	Phosphate solubilization (µg/ml)	Siderophore production (%)	Ammonia production (mg/ml)	IAA production (µg/ml)	Amylase production (IU/ml)	Cellulase production (IU/ml)	Protease production (IU/ml)	Source	Reference
B. subtilis subsp stercoris	271.6 ± 0.40	✓	✓	124.2 ± 0.30	–	–	–	Tomato rhizosphere	Ramavath et al. (2019)
*B. subtilis*UO-01,	–	–	–	–	–	–	9.35 ± 0.12	Biotechnology Center, University of Oriente Cuba	Blanco et al. (2016)
B. tequilensis MS3,	257.8 ± 0.60	✓	✓	117.1 ± 0.20	–	–	–	Tomato rhizosphere	Ramavath et al. (2019)
B. velezensis MS20	276.5 ± 0.4	✓	✓	121.3 ± 0.10	–	–	–	Tomato rhizosphere	Ramavath et al. (2019)

✓ Represents the isolate positive for particular activity

Table 3 Effect of *Bacillus* species (Bar D5 and Bam W2) on the growth of saffron in presence and absence of pathogen (*Fusraium oxysporum* R1) in pot assays

S. no.	Growth parameters	Treatments						$F_{(5,24)}$ value	*p* value
		C (mock primed corms)	B1 (Bam W2 primed corms planted in sterile soil)	B2 (Bar D5 primed corms planted in sterile soil)	CF (mock primed corms planted in Fox R1 inoculated soil)	T1 (Bam W2 primed corms planted in Fox R1 inoculated soil)	T2 (Bar D5 primed corms planted in Fox R1 inoculated soil)		
1	Shoot number	1.8 ± 0.44 ab	2.4 ± 0.54 bc	3 ± 0.70 c	1.2 ± 0.44 a	2 ± 0.70 b	2.4 ± 0.54 bc	5.680	0.001
2	Shoot length (cm)	18.3 ± 0.41 b	20.3 ± 0.60 c	22.2 ± 0.95 d	17.3 ± 0.40 a	19.1 ± 0.49 b	21.5 ± 0.43 d	51.831	0.000
3	Root number	91.2 ± 1.09 ab	95 ± 1.22 cd	105 ± 2.38 e	89.6 ± 1.14 a	93.2 ± 1.90 bc	96 ± 1.80 d	53.920	0.000
4	Root length (cm)	8.82 ± 0.71 ab	9.28 ± 0.36 bc	9.82 ± 0.40 c	8.5 ± 0.0.34 a	8.7 ± 0.38 ab	9 ± 0.61 ab	4.884	0.003
5	Number of leaves	7 ± 0.70 ac	7.2 ± 1.09 ac	8.40 ± .54 c	6.2 ± 0.44 a	7.2 ± 0.83 b	7.6 ± 0.54 b	6.416	0.001
6	Shoot dry matter (%)	30.2 ± 0.68 b	32.9 ± 0.70 c	38.5 ± 0.47 d	26.4 ± 0.42 a	30.6 ± 0.80 b	33.6 ± 0.63 c	234.866	0.000
7	Root dry matter (%)	31.2 ± 0.69 b	34.3 ± 1.09 d	39.3 ± 0.6 e	24.4 ± 0.67 a	32.1 ± 0.56 b	33.1 ± 0.46 c	204.900	0.000

Values are expressed as (mean ± SD, $n = 10$). *Bacillus amyloliquefaciens* W2 (Bam W2) and *Bacillus* sp. strain D5 (Bar D5) primed corms were grown in Fox R1 inoculated soil for 30 days in pots. ANOVA was performed at significant level ($p < 0.05$). Means with same superscript letters (within same row) indicate no significant difference between according to the multiple Duncan test ($p < 0.05$)

Dry Matter Accumulation in Corms

Corms were evaluated for the accumulation of dry matter in the presence and absence of *Bacillus* species and pathogen up to 11 dpi. At 0 dpi, *Bacillus* primed corms (S2 and S3) accumulated 1.17 ± 0.05 and 1.23 ± 0.02 fold more dry matter, respectively, compared to mock primed corms (S1). At 1, 3, 5, 8, and 11 dpi, maximum biomass accumulation were in B2 (Bar D5) and B1 (Bam W2) primed corms followed by T2 and T1 (Bar D5 and Bam W2 primed and Fox R1 inoculated corms) compared to untreated corms (Fig. 2), whereas in CF there was significant folds decrease in biomass i.e., 1.07 ± 0.08 (1 dpi), 1.09 ± 0.03 (3 dpi), 1.12 ± 0.06 (5 dpi), 1.13 ± 0.05 (8 dpi), 1.15 ± 0.07 (11 dpi) compared to C (Fig. 2).

Table 4 Disease severity index and Disease reduction percentage of corm rot symptoms caused by *Fusarium oxysporum* R1 in saffron plant with different treatments in pot assays

Treatments	Disease severity index	Disease reduction percentage
C (corms planted in sterile soil)	0.2	–
B1 (Bam W2 treated corms)	0.1	–
B2 (Bar D5 treated corms)	0.1	–
CF (Fox R1 treated corms)	2.9	–
T1 (Bam W2 + Fox R1 treated corms)	1.3	55.1%
T2 (Bar D5 + Fox R1 treated corms)	0.8	72.4%

Values are expressed as (mean ± SD, $n = 10$). ANOVA was performed at significant level ($p < 0.05$). Bam W2 and Bar D5 primed corms were grown in Fox R1 inoculated soil for 30 days in pots. After 30 days, Disease severity index and Disease reduction percentage was calculated using formula mentioned in the text

Plant Defense Related Enzymes Activity

The activity of plant defense related enzymes PAL, PO, PPO, LOX, and GLU was quantified in corm tissues of saffron plant, as per experimental design shown in Fig. 1 at 1, 3, 5, 8, and 11 dpi. The activities of all defense enzymes increased rapidly after inoculation of Fox R1 and maximum activities were observed at 5 dpi in all the treatments (Fig. 3a–e, Table 6) then it was found to be decreased at 8 and 11 dpi. Among different treatments, T2 showed the maximum activities of all the tested enzymes at 5 dpi i.e., PAL expression (2.8 ± 0.66 folds), PO (6 ± 0.47 folds), PPO (5 ± 1.1 folds), LOX (3.4 ± 0.71 folds), and GLU (3.4 ± 0.31 folds) increase as compared to C; whereas in T1, PAL (2.2 ± 0.37 folds), PO (2.58 ± 0.20 folds), PPO (3.5 ± 0.57 folds), LOX (2.2 ± 0.41 folds), and GLU (2.1 ± 0.58 folds), increase compared to C. To summarize, induction of all defense enzymes estimated that the activity was highest in T2 (Bar D5 + Fox R1), followed by T1 (Bam W2 + Fox R1) and last in CF (corms challenged with Fox R1 alone) and C (basal activity in corms without infection) (Fig. 3a–e).

Plant Defense Enzyme Related Genes Expression

Differential expression of genes, encoding above defense related enzymes were studied by qPCR at 5 days post inoculation (5 dpi) as the maximum enzyme activity was observed at 5 dpi. The pattern of expression of these genes was in tune with the enzyme production. Among all the treatments the maximum expression was observed in T2 (Bar D5 + Fox R1) i.e., priming with Bar D5 significantly increased the transcription of defense related enzymes when challenged with Fox R1. Peroxidase gene was highest expressed gene with 10.1 ± 0.95 folds increase, followed by PPO (8.1 ± 0.62 folds), GLU (4.9 ± 0.30 folds), PAL (4.5 ± 0.11 folds) and LOX (3.5 ± 0.32 folds) as compared to C (mock primed corms). In T1 (Bam W2 + Fox R1) as well, peroxidase was highest expressed gene with 5.6 ± 0.32 fold increase followed by PPO with 5 ± 0.43 folds, GLU with 3.4 ± 0.35 folds, PAL with 3.6 ± 0.2 folds, and LOX with 3 ± 0.62 folds increase as compared to C (mock primed corms). In CF (corm challenged with pathogen) the activity fold increase was 4.1 ± 0.3 folds in PO, 3.4 ± 0.35 folds in PPO, 2.3 ± 0.26 folds GLU in 3.1 ± 0.41 folds in PAL and 2.6 ± 0.87 folds in LOX as compared to C (Fig. 4). A comparison of fold change of activity and expression of all the 5 enzymes at 5 dpi has been tabulated in Table 7.

Discussion

Plant growth promoting bacteria (PGPB) can promote plant growth directly and/or indirectly (Batista et al. 2021; Danesh et al. 2021). The direct promotion is by phosphate solubilization, nitrogen fixation, siderophore production, enhancement of root proliferation for mineral uptake from soil and by the production of various phytohormones (Enebe and Babalola 2019; Bhattacharyya et al. 2020). Among rhizospheric bacteria, *Bacillus* species are the most common and commercialized PGPB. They are also most commercialized biological control agents, against various phytopathogens, due to their direct and indirect role in plant growth promotion and defense (Shafi et al. 2017; Timmusk et al. 2017). In addition, *Bacillus* species also possesses faster replication rate, root colonization ability and longer field life, on account of the spore formation (Aloo et al. 2019). Direct biological control ability of *Bacillus* species is a result of production of various antibiotics and extracellular enzymes that directly inhibit the growth of pathogens and indirect biocontrol activity is by induction of host plant defense system (Sahu et al. 2019; Bhusal and Mmbaga 2020; Vishwanathan et al. 2020). *Bacillus* species has been previously reported as PGPB in saffron (Gupta and Vakhlu 2015; Kour et al. 2018; Magotra et al. 2021), tomato (Abdallah et al. 2018; Masmoudi et al. 2021), sugarcane (Xia et al. 2020), rice (Kumar et al. 2021), wheat (Rashid et al. 2021a, b), maize (De Sousa et al. 2021) etc.

Native *Bacillus* sp. strain D5 isolated from cormosphere (corm sheath) of saffron corm (Magotra et al. 2021) and *B. amyloliquefaciens* W2 from bulk soil of saffron, have been isolated and characterized as PGPB earlier by our group (Gupta and Vakhlu 2015). These *Bacillus* species solubilize phosphate, produce siderophores, ammonia and various other enzymes in-vitro (Table 2). Amongst the two *Bacillus* strains, Bar D5 exhibited higher in-vitro PGP activities, except for siderophore production, that was 1.9-folds higher in Bam W2. Various *Bacillus* species are reported to possess these activities that have been tabulated in Table 2. The Phosphate solubilization activity of bacteria (PGPB) results

Table 5 In-planta inhibition of Fox R1 by Bam W2 and Bar D5

Days post inoculation ⟹	Mean lesion diameter (mm) in different treatments									
	1 dpi		3 dpi		5 dpi		8 dpi		11 dpi	
	Lesion diameter	Inhibition %	Lesion diameter	Inhibition %	Lesion diameter	Inhibition %	Lesion diameter	Inhibition %	Lesion diameter	Inhibition %
Corms inoculated with Fox R1 (CF)	6.5 ± 0.51 a	0	12.6 ± 0.41 a	0	16.4 ± 0.41 a	0	19.8 ± 0.57 a	0	25.5 ± 0.50 a	0
Corms primed with Bam W2 and inoculated with Fox R1 (T1)	4.4 ± 0.42 b	31.1 ± 1.97	7.7 ± 0.23 b	38.8 ± 3.01	9.7 ± 0.57 b	40.8 ± 2.79	13.6 ± 0.41 b	30.3 ± 2.03	19.8 ± 0.44 b	22.3 ± 1.96
Corms primed with Bar D5 and inoculated with Fox R1 (T2)	3.3 ± 0.27 c	44.9 ± 2.44	5.6 ± 0.41 c	55.6 ± 2.09	6.4 ± 0.41 c	60.9 ± 2.04	11.7 ± 0.59 c	40.9 ± 3.7	15.9 ± 0.22 c	37.6 ± 1.85
Days post inoculation ⟹	*Fusarium oxysporum* R1 load (10^5 CFU/g of tissue) in different treatments									
	1 dpi		3 dpi		5 dpi		8 dpi		11 dpi	
	Load (CFU/g) of tissue	Inhibition %	Load (CFU/g) of tissue	Inhibition %	Load (CFU/g) of tissue	Inhibition %	Load (CFU/g) of tissue	Inhibition %	Load (CFU/g) of tissue	Inhibition %
Corms inoculated with Fox R1 (CF)	0.41 ± 0.1 a	0	1.58 ± 0.08 a	0	3.94 ± 0.12 a	0	6.14 ± 0.15 a	0	7.14 ± 0.07 a	0
Corms primed with Bam W2 and inoculated with Fox R1 (T1)	0.24 ± 0.06 b	34.6 ± 2.98	0.92 ± 0.07 b	41 + 1.90	2.02 ± 0.11 b	48.7 ± 2.03	3.44 ± 0.15 b	43.9 ± 2.31	4.48 ± 0.28 b	37.2 ± 1.82
Corms primed with Bar D5 and inoculated with Fox R1 (T2)	0.2 ± 0.07 b	49.5 ± 1.11	0.42 ± 0.04 c	73.4 ± 2.8	1.16 ± 0.06 c	70.5 ± 1.37	2.08 ± 0.14 c	66.1 ± 1.67	3.08 ± 0.08 c	56.8 ± 0.94

Values are expressed as (mean ± SD, $n = 5$). ANOVA was performed at significant level ($p < 0.05$). Means with same superscript letters (within same column) indicate no significant difference between according to the multiple Duncan test ($p < 0.05$)

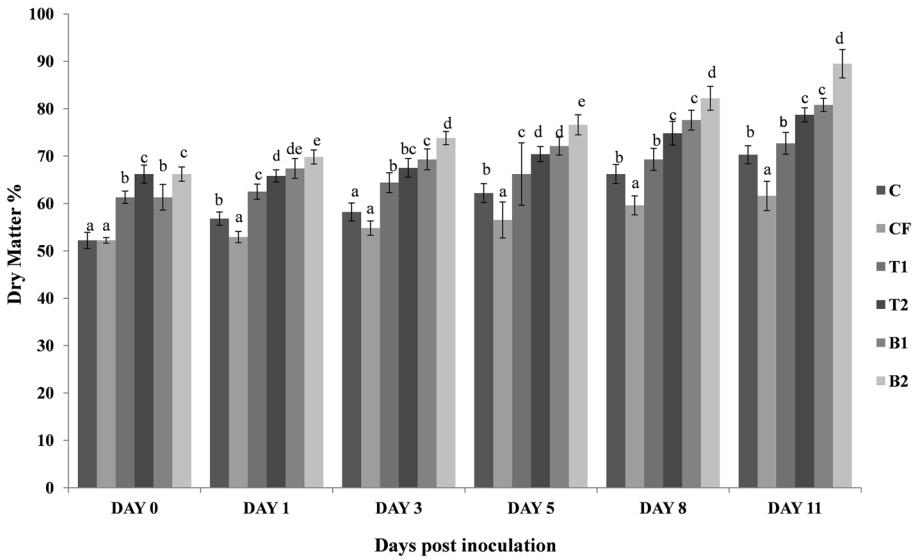

Fig. 2 The dry matter percentage accumulation in saffron corms with different treatments at different days post inoculations (1, 3, 5, 8 and 11 days post inoculation). C-Mock primed corms, CF-Fox R1 inoculated corms, T1-Bam W2 primed corms and Fox R1 inoculated corms, T2-Bar D5 primed and Fox R1 inoculated corms, B1-Bam W2 primed corms only and B2-Bar D5 primed corms only. Bar D5-*Bacillus* sp. strain D5, Bam W2-*Bacillus amyloliquefaciens* W2, and Fox R1-*Fusarium oxysporum* R1. Error bars represent the standard deviation (SD). ANOVA was performed at significant level ($p < 0.05$). Means with same superscript letters (within each day) indicate no significant difference between according to the multiple Duncan test ($p < 0.05$)

in the uptake of phosphorous from the soil that is an important element for the growth of plants (Asril et al. 2021). Siderophores are the low molecular weight iron chelating compounds that help in the uptake of iron from the soil that is vital for plant growth and renders them less available to the pathogenic organism present in the soil (Sultana et al. 2021). Plant growth promoting bacteria are known to produce many plant hormones that play major role in the many developmental stages, of the plant's life cycle. In the present study, both the *Bacillus* species produced IAA that is important for plant growth and development and modifies root structure for better absorption of nutrients (Ismail et al. 2021). Ammonia production is another strategy used by PGPB that helps in increasing the fresh weight of the plant as well as the shoots and roots (Ismail et al. 2021).

In pot assays, as well, both the *Bacillus* species significantly increased the growth of the saffron corms, as compared to untreated corms. In addition, both reduce the disease severity, as compared to pathogen control (Tables 3, 4). Both the *Bacillus* inoculated plants had growth due to the PGP properties possessed by Bar D5 and Bam W2, as they make the unavailable nutrients present in the soil available to plants. As reported earlier, Bam W2 significantly inhibits increased the growth of Fox R1 by 40% in in-vitro plate assays and disease by 57.1% in pot assays (Gupta and Vakhlu 2015). Bar D5 significantly increased the growth of the saffron plant in pot trials compared to untreated control and also reduced the disease incidence by 71.4% compared to pathogen control (Magotra et al. 2021). Sharaf-Eldin et al. (2008) have reported that the commercially available *Bacillus subtilis* (FZB24) significantly increased the leaf length, stigma weight, crocin, picrocrocin, and safranal content in saffron and reduced the sprouting time in saffron corms, as compared to untreated control (Sharaf-Eldin 2008). *B. aryabhattai* SRB02 from the soyabean rhizosphere, is reported to enhance root and shoot length of the plant, in pots by the production of various phytohormones (Park et al. 2017). Abdallah et al. (2018) reported *Bacillus amyloliquefaciens* subsp. *Plantarum* 32A significantly increased the germination level and growth of the tomato plant and reduced the symptoms of crown gall disease in the root of the tomato plant, in pot assays. *Bacillus xiamenensis* PM14 from sugarcane rhizosphere increased the fresh weight (30%), plant length (5%), cane length (27.5%), root length (37%), and reduced red rot disease incidence, in greenhouse experiments (Xia et al. 2020). In the present study, Bam W2 and Bar D5 significantly reduced the disease severity in the saffron plant (T1 by 55.1% and T2 by 72.4%) as compared to pathogen control (CF). Similar to the present study, Gupta et al. (2021) have reported that different biocontrol agents such as *Tricoderma asperellium* (68%), *Bacillus subtilis* (42.9%), *Bacillus pumilus* (20.6%), and *Bacillus straptosphericus* (14.2%) inhibits the growth of *Fusarium oxysporum* and reduce the disease incidence of corm rot in saffron.

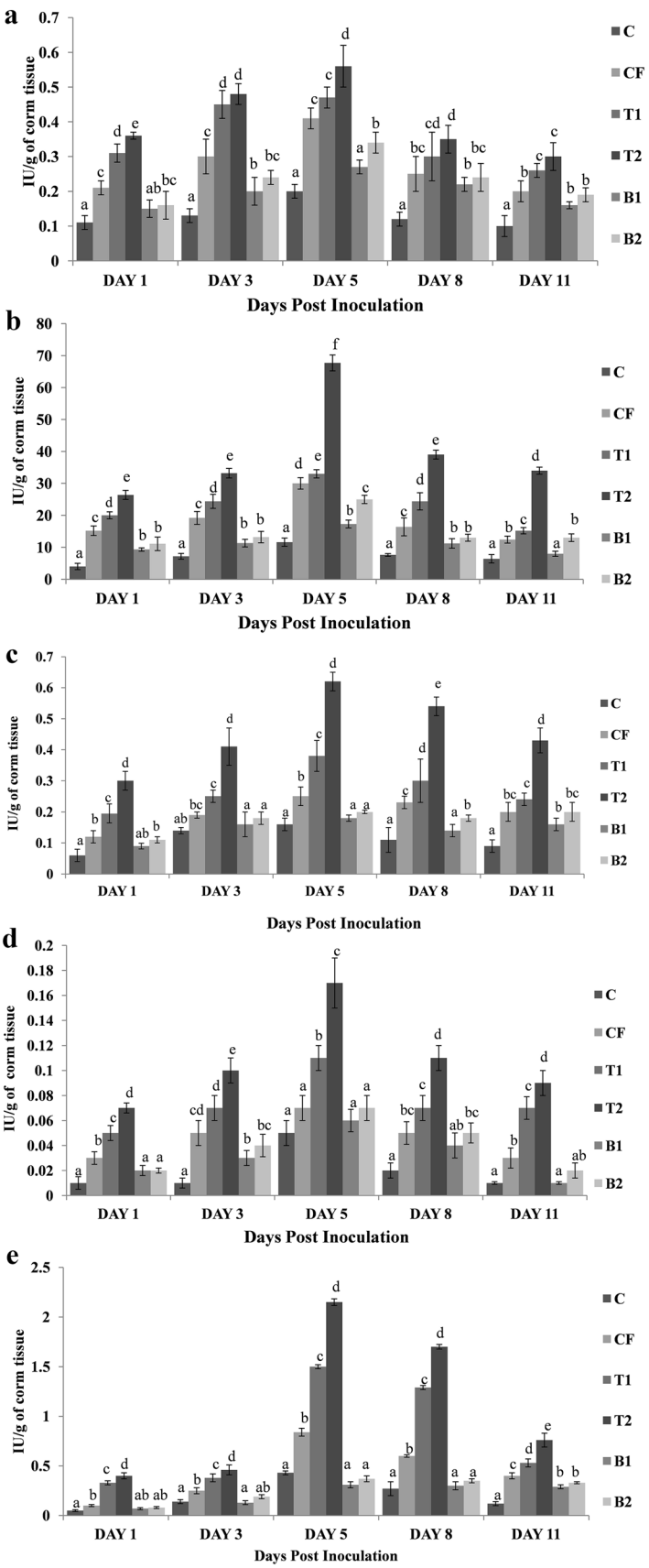

Fig. 3 The activities of different defense related enzymes. **a** Phenylalanine ammonia lyase (PAL), **b** peroxidase (PO), **c** β-1,3 glucanase (GLU), **d** Lipoxygenase (LOX), **e** polyphenol oxidase (PPO) in saffron corm with different treatments at different days post inoculations (1, 3, 5, 8, and 11 days post inoculation). C-Mock primed corms, CF-Fox R1 inoculated corms, T1-Bam W2 primed and Fox R1 inoculated corms, T2-Bar D5 primed and Fox R1 inoculated corms, B1-Bam W2 primed corms only and B2-Bar D5 primed corms only. Bar D5-*Bacillus* sp. strain D5, Bam W2-*Bacillus amyloliquefaciens* W2 and Fox R1-*Fusarium oxysporum* R1. Error bars represent the standard deviation (SD). ANOVA was performed at significant level ($p < 0.05$). Means with same superscript letters (within each day) indicate no significant difference between according to the multiple Duncan test ($p < 0.05$)

Table 6 In-planta induction of defense enzymes [fold increase compared to untreated corms (control)] and maximum activity (dpi) of Bar D5 and Bam W2 and other reported *Bacillus* species

Bacteria	Defense enzyme	Fold increase	Maximum activity at day	Pathogen	Plant	References
Bacillus amyloliquefaciens W2	PAL	2	5	*Fusarium oxysporum* R1	Saffron	Present study
	PO	3				
	PPO	3.4				
	LOX	1.2				
	GLU	1.5				
Bacillus sp. strain D5	PAL	2.5	5	*Fusarium oxysporum* R1	Saffron	Present study
	PO	6				
	PPO	5				
	LOX	2				
	GLU	2				
B. amyloliquefaciens	PAL	2	3	*Colletotrichum truncatum*	Chilli	Gowtham et al. (2018)
	PPO					
	PO					
	GLU					
B. amyloliquefaciens (FZB24)	PAL	3	9	*Fusarium* wilt	Tomato	Elanchezhiyan et al. (2018)
	PPO	3				
	PO	4				
	SOD	2				
	CAT	4				
Bio consortia	PAL		5	*Fusarium solani*	Mulberry	Palani et al. (2016)
	PO					
	PPO					
B. subtilis B579	PAL	2	5	*Fusarium oxysporum*	Cucumber	Chen et al. (2010)
	POX	3				
	PPO	3				
B. subtilis CBR05	PAL	93.9%	5	*Xanthomonas campestris* pv. vesicatoria	Tomato	Chandrasekaran et al. (2017)
	GLU	42.5%				
B. thuringiensis 199	PAL	1.8	5	*Fusarium oxysporum* f.sp. *lycopersici*	Tomato	Akram et al. (2013)
	PO	1.4				
	PPO	1.3				
B. velezensis F21	CAT	4	3	*Fusarium oxysporum* f.sp. *niveum* (Fon)	Watermelon	Jiang et al. (2019)
	PO	3	7			
	SOD	4	7			

To evaluate the potential of Bar D5 over Bam W2 as a biostimulant, the experiments were conducted in-planta in pot assays. One of the direct ways to monitor plant growth is to measure the effect of the biostimulant on biomass accumulation. Various species of *Bacillus* are known to increase the dry matter in crop plants (Kang et al. 2014). Though the experiments were initiated with corms of similar weight (~ 10 g) after 30 days of incubation with both the biostimulants, there was a clear indication of an increase in biomass in corms primed with Bar D5 and Bam W2 as compared to mock primed corms. Bam W2 (B1) and Bar D5 (B2) primed corms had maximum biomass accumulation in comparison to mock primed corms (C) (Fig. 2). However, Bam W2 and Bar D5 primed corms after challenged with Fox R1 (T1 and T2) accumulated less biomass than B1 and B2 but more than mock primed corms (C) and pathogen inoculated corms (CF) up to 11 dpi (Fig. 2). The order of biomass accumulation among different treatments was B2 > B1 > T2 > T1 > C > CF. This result indicates that even after infection both the biostimulant help plant to accumulate biomass. It is known fact that as pathogen invades the plant, it channels its energy/resources towards defense, similar results were observed in present case as well. Before infection with the pathogenic Fox R1, the primed corms with Bar D5 and Bam W2 had similar biomass on day zero but at 11 dpi, the

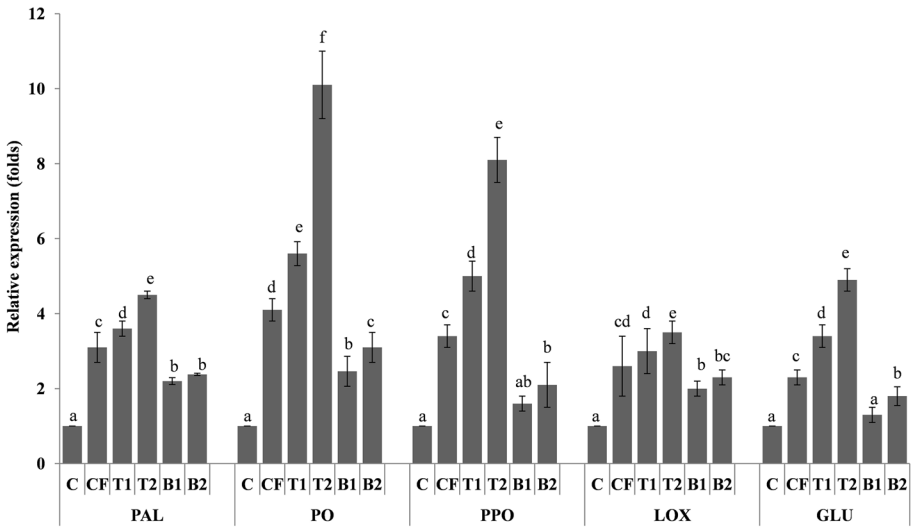

Fig. 4 Quantitative reverse transcriptase PCR expression of defense related genes, Phenylalanine ammonia lyase (PAL), Peroxidase (PO), Polyphenol oxidase (PPO), Lipoxygenase (LOX), β-1,3 glucanase (GLU) in saffron corm at day 5 post inoculation in different treatments. C-Mock primed corms, CF-Fox R1 inoculated corms, T1-Bam W2 primed and Fox R1 inoculated corms, T2-Bar D5 primed and Fox R1 inoculated corms, B1-Bam W2 primed corms only and B2-Bar D5 primed corms only. Bar D5-*Bacillus* sp. strain D5, Bam W2-*Bacillus amyloliquefaciens* W2 and Fox R1-*Fusarium oxysporum* R1. Error bars represent the standard deviation (SD). ANOVA was performed at significant level ($p < 0.05$). Means with same superscript letters (within each enzyme) indicate no significant difference between according to the multiple Duncan test ($P < 0.05$)

Table 7 Comparative fold of defense related activities and gene expression of different enzymes in saffron corms treated with bacilli challenged with pathogen *Fusarium oxysporum* R1

Defense enzyme/gene	Enzyme activity Fold increase compared to untreated corms (C)	Gene expression Fold increase in expression compare to untreated corms (C)	Fold change Gene expression: enzyme activity
PAL			
BarD5 + Fox R1 (T2)	2.5	4.5	1.8
BamW2 + Fox R1(T1)	2	3.6	1.8
PO			
BarD5 + Fox R1(T2)	6	10.1	0.59
BamW2 + Fox R1(T1)	3	5.6	1.8
PPO			
BarD5 + Fox R1(T2)	5	8.1	0.61
BamW2 + Fox R1(T1)	3.4	5	1.4
LOX			
BarD5 + Fox R1(T2)	2	3.5	0.51
BamW2 + Fox R1(T1)	1.2	3	2.5
GLU			
BarD5 + Fox R1(T2)	2	4.9	2.45
BamW2 + Fox R1(T1)	1.5	3.4	2.26

accumulation was less in pathogen infected corms as compared to the corms that were not infected (Fig. 2). The decrease in biomass accumulation in bacilli primed, pathogen inoculated corms could be a result of stress caused by the pathogen. It was concluded that both Bar D5 and Bam W2 stimulate biomass accumulation, however upon infection, the accumulation is decreased relatively. Further, pathogen severely affects the biomass accumulation as uninfected corm accumulates more biomass in comparison to infected corm (Fig. 2). Similarly, *Bacillus subtilis* FZB24® has been reported to significantly increase the biomass and yield in the saffron plant (Sharaf-Eldin et al. 2008), though in this study no disease or pathogen was studied. *B. simplex* has been reported to increased the yield of kiwifruit (Erturk et al. 2010), *B. subtilis ALB629* enhances the dry matter in cacao (Falcäo et al. 2014) and

B. megaterium mj1212 increased the fresh weight in mustard plants (Kang et al. 2014).

In the present study, both Bar D5 and Bam W2 induced production of defense related enzymes by increased expression of the genes encoding these enzymes in the presence of Fox R1 (Figs. 3a–e, 4). The maximum activity of defense related enzymes was in T2 (Bar D5 primed and Fox R1 inoculated corms) followed by T1 (Bam W2 primed and Fox R1 inoculated corms) as compared to pathogen inoculated corm (CF) and mock primed corm (C) at 5 dpi. Biocontrol agents inhibit the pathogen attack either by direct inhibition, resource scavenging or by induced systemic resistance (Charpe et al. 2019; Palani et al. 2016). ISR is the response of a plant to pathogen invasion, by enhanced production of defense related enzymes, that is augmented many fold by PGPB (Borris et al. 2019; Chandrashekaran et al. 2017). These PGPB makes host plant more tolerant to pathogens attack. Except for *B. amyloliquefaciens* (FZB24) against *Fusarium* wilt in tomato plant (Elanchezhiyan et al. 2018) and *B. velezensis* F21 in watermelon against *Fusarium oxysporum* f. sp. niveum (Fon) (Jiang et al. 2019), most of the studies have reported maximum activity of defense related enzymes at 5 dpi (Table 6). The production of defense enzymes by Bar D5 was more than Bam W2 (Fig. 3a–e). These defense enzymes are effective in controlling the disease cause by various pathogens. These enzymes are induced by *Bacillus* species in various plants but their production increases after challenge with pathogens (Table 6). These enzymes are present in plants at the basal level, at a given point of time but get enhanced on interaction with both pathogen and/or biocontrol (Chandrashekaran and Chun 2016). However, different kind of responses has been reported in different plant. The comprehensive data has been tabulated in Table 6.

PAL is an enzyme of the phenylpropanoid pathway that converts L-phenylalanine to trans-cinnamic acid contributing to the production of secondary metabolites, including lignins, flavonoids, coumarins and jasmonate resulting in plant cell wall lignifications (Astaneh et al. 2018; Garces-Fiallos et al. 2022). Similarly, PPOs produce quinones from ortho-diphenolic compounds that are highly reactive and by using molecular oxygen they cross link the proteins in the cell, creating a protein deficient environment for the pathogens (Taranto et al. 2017). Likewise, reactive oxygen species produced by plants as first line of defense against pathogens and are detoxified by enzyme PO, which is reported to be involved in cell wall lignifications and defense against oxidative stress (Hanaka et al. 2018; Thiebaut et al. 2022). Furthermore, β-1,3-glucanases are the lytic enzymes that hydrolyze the fungal cell wall components, β-1,3-glucan (Xu et al. 2016; Ye et al. 2023). Moreover, LOXs are non-heme dioxygenases that produce unsaturated fatty acid hydroperoxides, such as oxylipins, (from polyunsaturated fatty acids by adding molecular oxygen) and oxylipins have antimicrobial activity hence are involved in plant defense (Babenko et al. 2017; Gonzalez-Gordo et al. 2022).

In the present study, control corms have some basal level of these enzymes production, but once challenged with the pathogen, the production of enzyme increased. There is a clear indication of some cross talk between all the three participants that is plant-*Bacillus*-pathogen. Similar results have also been reported by (Elanchezhiyan et al. 2018 and Jiang et al. 2019; Sadeghpour et al. 2022; Hussain et al. 2023), but the underlying molecular mechanism and signaling pathway in saffron is a matter of further investigation. One underlying response in all the cases was that biocontrol agents and pathogens induce systemic response, but the magnitude of defense response varies in different plants. In certain reports, the induction of defense genes was highest in biocontrol treated plants alone (Chandrashekaran et al. 2017; Jiang et al. 2019) and in other plants the highest activity was in combination of biocontrol and pathogen (Elanchezhiyan et al. 2018 and Gowtham et al. 2018; Naz et al. 2021). This is the first report of pathogen-biocontrol interaction and induction of defense response in saffron.

The defense enzyme production profile was complemented with analysis of real time expression of the genes encoding them, by qPCR at 5 dpi only because the defense enzyme production was observed maximum at 5 dpi. Similar to results of defense enzyme production, maximum expression was found in corms primed with Bar D5 and inoculated with Fox R1 (T2) followed by Bam W2 primed and Fox R1 inoculated corms (T1) compare to CF, C, B1 and B2. The gene expression profiles for these defense enzymes were in sync with the enzyme production profiles, though the fold increase in expression of transcripts was more than that of enzymes (Table 7). Chandrashekaran et al. (2017) have reported maximum expression at 4 dpi of β,1-3 glucanase and PAL gene, in the tomato plant treated with *B. subtilis* CBR05 and challenged with *Erwinia carotovora* sub sp. *carotovora* (Chandrashekaran et al. 2017). In another study, *B. subtilis* CBR05 significantly induced the activity and expression of GLU and PAL enzymes, in tomato plant against *Xanthomonas campestrispv versicatoria* (XCV) and maximum activity and expression were at 72 h (Chandrashekaran et al. 2017). Interestingly, among all the defense enzymes studied in the present study, peroxidase (PO) was expressed maximum both at translational and transcriptional levels. Peroxidase is reported to detoxify reactive oxygen species generated during stress, therefore reactive burst is the major defense during stress conditions in many plants (Bhattacharjee 2019), which could be the probable reason of defense against Fox R1 in *Crocus sativus* as well.

In the present study, there was significant inhibition of Fox R1 in-planta by Bam W2 and Bar D5 (Table 5). However, Bar D5 substantially reduced the load of Fox

R1 as compared to Bam W2. The maximum inhibition was observed at 5 dpi and then the inhibition rate decreased at 8 and 11 dpi. The maximum inhibition at 5 dpi can be correlated with the maximum activity of defense related enzymes at 5 dpi. In lesion diameter method the inhibition % was (40.8 ± 2.79 in T1 and 60.9 ± 2.04 in T2) and in CFU method the inhibition % was (48.7 ± 2.03 in T1 and 70.5 ± 1.37 in T2) (Table 5). As the activity of enzymes decreased, there was a sudden rise in Fox R1 load. Similarly, bacterium *Rhodopseudomonas palustris* strain GJ-22 in-planta inhibited the growth of pathogen *Phytophthora Infestans* causing late blight in potato by 75% at 5 dpi (Zhang et al. 2020). Murolo et al. (2019) have checked the effect of three bioformulation in-planta against *Cryphonectria parasitica*, causing chestnut blight disease in sweet chestnut. In the co-inoculation method, *B. subtilis* reduced the necrotic areas by > 70% and in dual inoculations, *B. subtilis* reduced the size of cankers on chestnut stems (29–67%), *Trichoderma* spp. (36–65%) and *Glomus* spp. (31%–63%) at 15 dpi (Murolo et al. 2019). In the present study, it can be concluded that based on in-vitro PGP activities, pot assays for plant growth promotion, induction of defense related enzyme activity and expression and inhibition of Fox R1, Bar D5 has the potential to be used as a commercial PGP and biocontrol agent against *Fusarium oxysporum* R1 pathogen causing corm rot of saffron. However, commercialization of this *Bacillus* strain as a biostimulant or biocontrol will need standardization of formation methods for its large scale production and toxicological studies to certify it as safe to use.

Conclusion

Since replacement and/or complementation of chemical fertilizers by biological agents is the need of the hour, and in the case of saffron chemical augmentation is not effective anymore, as the yield is on decline year after year. Despite the availability of chemical fungicides, corm rot caused by *Fusarium oxysporum* is a major threat to saffron production world over. Based on comparison of in-vitro activity, in-planta pot assay for growth parameters, dry matter accumulation, defense enzyme production and expression of the corresponding genes, Bar D5 comes out to be the most suited biostimulant, in comparison to Bam W2. It has a clear advantage for its use as a bioinoculant for *Crocus sativus* due to its nativeness and its biocontrol activity through ISR. However, the actual molecular cross talk between the *Bacillus*-corm-pathogen and its signaling needs further investigation. Consequently, it may be evaluated for plant growth promotion and induction of systemic resistance against fungal pathogens, in other plants as well.

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Author Contributions TA and JV conceived and designed research. TA and NB conducted the experiments. TA, NB and JV wrote the manuscript. NB had done the statistical analysis of data. SM isolated and characterized the Bar D5. JV supervised and arranged the funding for the research. All authors read and approved the manuscript.

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

Abdallah DB, Frikha-Gargouri O, Tounsi S (2018) Rizhospheric competence, plant growth promotion and biocontrol efficacy of *Bacillus amyloliquefaciens* subsp. plantarum strain 32a. Biol Control 124:61–67

Abdel-Fattah YR, Soliman NA, El-Toukhy NM, El-Gendi H, Ahmed RS (2012) Production, purification, and characterization of thermostable α-amylase produced by *Bacillus licheniformis* isolate AI20. J Chem 2013:1

Abdel-Monaim MF, Ismail ME, Morsy KM (2012) Induction of systemic resistance in soybean plants against *Fusarium* wilts disease by seed treatment with benzothiadiazole and humic acid. Afr J Biotechnol 11:2454–2465

Abdollahi MR, Kianersi F, Moosavi SS, Dastan D, Asadi S (2022) Identification and expression analysis of two genes involved in the biosynthesis of t-anethole in fennel (Foeniculum vulgare Mill.) and their up-regulation in leaves in response to methyl jasmonate treatments. J Plant Growth Regul 2022:1–12

Ahirwar NK, Singh R, Chaurasia S, Chandra R, Ramana S (2020) Effective role of beneficial microbes in achieving the sustainable agriculture and eco-friendly environment development goals: a review. Front Microbiol 5:111–123

Ajijah N, Fiodor A, Pandey AK, Rana A, Pranaw K (2023) Plant growth-promoting bacteria (PGPB) with biofilm-forming ability: a multifaceted agent for sustainable agriculture. Diversity 15:112

Akram W, Mahboob A, Javed AA (2013) *Bacillus thuringiensis* strain 199 can induce systemic resistance in tomato against *Fusarium* wilt. Eur J Microbiol Immunol 3:275–280

Aloo BN, Makumba BA, Mbega ER (2019) The potential of Bacilli rhizobacteria for sustainable crop production and environmental sustainability. Microbiol Res 219:26–39

Arora NK, Verma M (2017) Modified microplate method for rapid and efficient estimation of siderophore produced by bacteria. 3 Biotech 7(6):1–9

Asha B, Palaniswamy M (2018) Optimization of alkaline protease production by *Bacillus cereus* FT 1 isolated from soil. J App Pharm Sci 8(02):119–127

Asril M, Lisafitri Y, Niswati A, Dirmawati SR, Rismawati R, Rini I, Oktaviani I (2021) Assessment of phosphate solubilization and indole acetic acid production of phosphate solubilizing bacteria isolated from acid soils, Lampung, Indonesia. Adv Biol Sci Res 14:469–477

Astaneh RK, Bolandnazar S, Nahandi FZ, Oustan S (2018) Effect of selenium application on phenylalanine ammonia-lyase (PAL) activity, phenol leakage and total phenolic content in garlic (*Allium sativum* L.) under NaCl stress. Inf Process Agr 5:339–344

Babenko LM, Shcherbatiuk MM, Skaterna TD, Kosakivska IV (2017) Lipoxygenases and their metabolites in formation of plant stress tolerance. Ukr Biochem J 89:5–21

Babu SV, Triveni S, Reddy RS, Sathyanarayana J (2017) Screening of maize Rhizosperic phosphate solubilizing isolates for plant growth promoting characteristics. Int J Curr Microbiol App Sci 6:2090–2101

Batista BD, Bonatelli ML, Quecine MC (2021) Fast screening of bacteria for plant growth promoting traits. In: The plant microbiome. Humana, New York, pp 61–75

Bhagat N, Sharma S, Ambardar S, Raj S, Trakroo D, Horacek M, Vakhlu J (2021) Microbiome fingerprint as biomarker for geographical origin and heredity in *Crocus sativus*: a Feasibility Study. Front Sustain Food Syst 5:688393

Bhagat N, Magotra S, Gupta R, Sharma S, Verma S, Verma PK, Vakhlu J (2022) Invasion and colonization of pathogenic *Fusarium oxysporum* R1 in *Crocus sativus* L. during corm rot disease progression. J Fungi 8:1246

Bhattacharjee S (2019) ROS in aging and senescence. Reactive Oxygen Species Plant Biol 1:65–79

Bhattacharyya C, Banerjee S, Acharya U, Mitra A, Mallick I, Haldar A, Ghosh A (2020) Evaluation of plant growth promotion properties and induction of antioxidative defense mechanism by tea rhizobacteria of Darjeeling India. Sci Rep 10:1–19

Bhusal B, Mmbaga MT (2020) Biological control of Phytophthora blight and growth promotion in sweet pepper by *Bacillus* species. Biol Control 150:104373

Blanco AS, Durive OP, Pérez SB, Montes ZD, Guerra NP (2016) Simultaneous production of amylases and proteases by *Bacillus subtilis* in brewery wastes. Braz J Microbiol 47:665–674

Borriss R, Wu H, Gao X (2019) Secondary metabolites of the plant growth promoting model rhizobacterium *Bacillus velezensis* FZB42 are involved in direct suppression of plant pathogens and in stimulation of plant-induced systemic resistance. In: Secondary metabolites of plant growth promoting rhizomicroorganisms, vol. 1. Springer, Singapore, pp 147–168

Burketova L, Trda L, Ott PG, Valentova O (2015) Bio-based resistance inducers for sustainable plant protection against pathogens. Biotechnol Adv 33:994–1004

Can H, Kal U, Kayak N, Dal Y, Turkmen O (2022) Use of microbial inoculants against biotic stress in vegetable crops: physiological and molecular aspect. In Sustainable horticulture. Academic Press, London, pp 263–332

Cardone L, Castronuovo D, Perniola M, Cicco N, Candido V (2020) Saffron (*Crocus sativus* L.), the king of spices: an overview. Sci Hortic 272:109560

Chandrasekaran M, Chun SC (2016) Expression of PR-protein genes and induction of defense-related enzymes by *Bacillus subtilis* CBR05 in tomato (*Solanum lycopersicum*) plants challenged with *Erwinia carotovora* subsp. carotovora. Biosci Biotechnol Biochem 80:2277–2283

Chandrasekaran M, Belachew ST, Yoon E, Chun SC (2017) Expression of β-1,3-glucanase (GLU) and phenylalanine ammonia-lyase (PAL) genes and their enzymes in tomato plants induced after treatment with *Bacillus subtilis* CBR05 against *Xanthomonas campestris* pv. vesicatoria. J Gen Plant Pathol 83:7–13

Charpe AM (2019) Free-living PGPRs in biotic stress management. Plant Growth Promot Rhizobacteria Sustain Stress Manag 1:275–324

Chen F, Wang ZY, Luo J, Yang X, Wang X (2010) Quantitative changes of plant defense enzymes and phytohormone in biocontrol of cucumber *Fusarium* wilt by *Bacillus subtilis* B579. World J Microbiol Biotechnol 26:675–684

Croos AMB, Rajendran S, Ranganathan K (2019) Isolation of a cellulase producing *Bacillus cereus* from cow dung and determination of the kinetic properties of the crude enzyme. J Natl Sci Found 47(2):1

Danesh YR, Demir S, Najafi S, Sagun Ç (2021) The role of plant-growth promoting bacteria in sustainable plant disease management. In: Biopesticides in organic farming. CRC Press, London, pp 65–70

de Sousa SM, de Oliveira CA, Andrade DL, de Carvalho CG, Ribeiro VP, Pastina MM, Gomes EA (2021) Tropical *Bacillus* strains inoculation enhances maize root surface area, dry weight, nutrient uptake and grain yield. J Plant Growth Regul 40:867–877

Din BU, Sarfraz S, Xia Y, Kamran MA, Javed MT, Sultan T, Chaudhary HJ (2019) Mechanistic elucidation of germination potential and growth of wheat inoculated with exopolysaccharide and ACC-deaminase producing *Bacillus* strains under induced salinity stress. Ecotoxicol Eenviron Saf 183:109466

Elanchezhiyan K, Keerthana U, Nagendran K, Prabhukarthikeyan SR, Prabakar K, Raguchander T, Karthikeyan G (2018) Multifaceted benefits of *Bacillus amyloliquefaciens* strain FBZ24 in the management of wilt disease in tomato caused by *Fusarium oxysporum* f. sp. *lycopersici*. Physiol Mol Plant Pathol 103:92–101

Enebe MC, Babalola OO (2019) The impact of microbes in the orchestration of plants' resistance to biotic stress: a disease management approach. Appl Microbiol Biotechnol 103:9–25

Erturk Y, Ercisli S, Haznedar A, Cakmakci R (2010) Effects of plant growth promoting rhizobacteria (PGPR) on rooting and root growth of kiwifruit (*Actinidia deliciosa*) stem cuttings. Biol Res 43:91–98

Falcäo LL, Silva-Werneck JO, Vilarinho BR, da Silva JP, Pomella AWV, Marcellino LH (2014) Antimicrobial and plant growth-promoting properties of the cacao endophyte *Bacillus subtilis* ALB 629. J Appl Microbiol 116:584–1592

Garcés-Fiallos FR, de Quadros FM, Ferreira C, de Borba MC, Bouzon ZL, Barcelos-Oliveira JL, Stadnik MJ (2022) Changes in xylem morphology and activity of defense-related enzymes are associated with bean resistance during *Fusarium oxysporum* colonization. Protoplasma 259:717–729

Garcia ML, Ferreira NLO, Lima R, Arruda M, Da EC (2017) Standardized rosemary extract induces host plant defenses and suppresses rice leaf blast. J Med Plants 5:39–45

Gaspar AP, Laboski CA, Naeve SL, Conley SP (2017) Dry matter and nitrogen uptake, partitioning, and removal across a wide range of soybean seed yield levels. Crop Sci 57:2170–2182

Ghosh SK, Mitra PK, Maiti TK, Pramanik KS, De TK (2018) The role of arsenic resistant *Bacillus aryabhattai* MCC3374 in promotion of rice seedlings growth and alleviation of arsenic phytotoxicity. Chemosphere 211:407–419

Glickmann E, Dessaux Y (1995) A critical examination of the specificity of the Salkowski reagent for indolic compounds produced by phytopathogenic bacteria. Appl Environ Microbiol 61:793–796

González-Gordo S, Canas A, Munoz-Vargas MA, Palma JM, Corpas FJ (2022) Lipoxygenase (LOX) in sweet and hot pepper (*Capsicum annuum* L.) fruits during ripening and under an enriched nitric oxide (NO) gas atmosphere. Int J Mol Sci 23:15211

Gowtham HG, Murali M, Singh SB, Lakshmeesha TR, Murthy KN, Amruthesh KN, Niranjana SR (2018) Plant growth promoting rhizobacteria-*Bacillus amyloliquefaciens* improves plant growth and induces resistance in chilli against anthracnose disease. Biol Control 126:209–217

Gupta R, Vakhlu J (2015) Native *Bacillus amyloliquefaciens* W2 as a potential biocontrol for *Fusarium oxysporum* R1 causing corm rot of *Crocus sativus*. Eur J Plant Pathol 143:123–131

Gupta V, Sharma A, Rai PK, Gupta SK, Singh B, Sharma SK, Sharma R (2021) Corm rot of saffron: epidemiology and management. Agronomy 11:339

Hanaka A, Lechowski L, Mroczek-Zdyrska M, Strubińska J (2018) Oxidative enzymes activity during abiotic and biotic stresses in *Zea mays* leaves and roots exposed to Cu methyl jasmonate and *Trigonotylus caelestialium*. Physiol Mol Biol Plants 24:1–5

Hashemi M, Mousavi SM, Razavi SH, Shojaosadati SA (2013) Comparison of submerged and solid state fermentation systems effects on the catalytic activity of *Bacillus* sp. KR-8104 α-amylase at different pH and temperatures. Ind Crops Prod 43:61–667

Hussain K, Jaweed TH, Kamble AC (2023) Modulation of phenylpropanoid and lignin biosynthetic pathway is crucial for conferring resistance in pigeon pea against *Fusarium wilt*. Gene 851:146994

Ismail MA, Amin MA, Eid AM, Hassan SED, Mahgoub HA, Lashin I, Fouda A (2021) Comparative study between exogenously applied plant growth hormones versus metabolites of microbial endophytes as plant growth-promoting for *Phaseolus vulgaris* L. Cells 10(5):1059

Jafari SM, Tsimidou MZ, Rajabi H, Kyriakoudi A (2020) Bioactive ingredients of saffron: extraction analysis applications. In: Saffron, vol 1. Woodhead Publishing, London, pp 261–290

Jiang C, Guo J, Yao X, Mi D, Li Z, Yang B, Zheng Y (2019) Comparative transcriptome analysis reveals the biocontrol mechanism of *Bacillus velezensis* F21 against *Fusarium* wilt on watermelon. Front Microbiol 10:652

Kalyani G, Rajesh EM (2018) Production and purification of amylase from *Bacillus subtilis* isolated from soil. Int J Eng Manag Res 8:246–254

Kang SM, Radhakrishnan R, You YH, Joo GJ, Lee IJ, Lee KE, Kim JH (2014) Phosphate solubilizing *Bacillus megaterium* mj1212 regulates endogenous plant carbohydrates and amino acids contents to promote mustard plant growth. Ind J Microbiol 54:427–433

Kaur M, Sodhi HS (2022) Implication of microbial signals: plant communication. In: Plant–microbe interactions. CRC Press, London, pp 41–57

Komada H (1975) Development of a selective medium for quantitative isolation of Fusarium oxysporum from natural soil. J Plant Prot Res 8:114–124

Kumar A, Singh S, Mukherjee A, Rastogi RP, Verma JP (2021) Salt-tolerant plant growth-promoting *Bacillus pumilus* strain JPVS11 to enhance plant growth attributes of rice and improve soil health under salinity stress. Microbiol Res 242:126616

Lim YH, Foo HL, Loh TC, Mohamad R, Abdullah N (2019) Comparative studies of versatile extracellular proteolytic activities of lactic acid bacteria and their potential for extracellular amino acid productions as feed supplements. J Anim Sci Biotechnol 10(1):1–13

Luang-In V, Yotchaisarn M, Saengha W, Udomwong P, Deeseenthum S, Maneewan K (2019) Isolation and identification of amylase-producing bacteria from soil in Nasinuan Community Forest, Maha Sarakham, Thailand. Biomed Pharmacol 12(3):1061–1068

Magotra S, Bhagat N, Ambardar S, Ali T, Hurek BR, Hurek T, Vakhlu J (2021) Field evaluation of PGP *Bacillus* sp. strain D5 native to *Crocus sativus*, in traditional and non traditional areas, and mining of PGP genes from its genome. Sci Rep 11:5454

Mahapatra S, Chakraborty S, Samanta M, Das S, Islam T (2022) Current understanding and future directions of biocontrol of plant diseases by *Bacillus* spp., with special reference to induced systemic resistance. In: Bacilli in agrobiotechnology: plant stress tolerance, bioremediation, and bioprospecting. Springer, Cham, pp 127–150

Mansotra R, Ali T, Bhagat N, Vakhlu J (2023) Injury and not the pathogen is the primary cause of corm rot in *Crocus sativus* (saffron). Front Plant Sci 14:1074185

Masmoudi F, Tounsi S, Dunlap CA, Trigui M (2021) Halotolerant *Bacillus spizizenii* FMH45 promoting growth, physiological, and antioxidant parameters of tomato plants exposed to salt stress. Plant Cell Rep 2021:1–15

Menia M, Iqbal S, Zahida R, Tahir S, Kanth RH, Saad AA, Hussian A (2018) Production technology of saffron for enhancing productivity. J Pharmacognos Phytochem 7:1033–1039

Mishra RK, Bohra A, Kamaal N, Kumar K, Gandhi K, Sujayanand GK, Mishra M (2018) Utilization of biopesticides as sustainable solutions for management of pests in legume crops: achievements and prospects. Egypt J Biol Pest Co 28:3

Mohamed EA, Farag AG, Youssef SA (2018) Phosphate solubilization by *Bacillus subtilis* and *Serratia marcescens* isolated from tomato plant rhizosphere. J Environ Prot 9:266–277

Murolo S, Concas J, Romanazzi G (2019) Use of biocontrol agents as potential tools in the management of chestnut blight. Biol Control 132:102–109

Naz R, Bano A, Nosheen A, Yasmin H, Keyani R, Shah STA, Roberts TH (2021) Induction of defense-related enzymes and enhanced disease resistance in maize against *Fusarium verticillioides* by seed treatment with *Jacaranda mimosifolia* formulations. Sci Rep 11(1):1–15

Nguyen KK, Cuellar C, Mavi PS, LeDuc D, Bañuelos G, Sommerhalter M (2018) Two poplar hybrid clones differ in phenolic antioxidant levels and polyphenol oxidase activity in response to high salt and boron irrigation. J Agric Food Chem 66:7256–7264

Nr D, Kulkarni SW (2018) Screening of rhizomicroflora isolated from the rhizosphere of aegle marmelos for multiple plant growth promoting and antimicrobial activities. World J Pharm Res 7:824–838

Pahari A, Mishra BB (2017) Characterization of siderophore producing Rhizobacteria and Its effect on growth performance of different vegetables. Int J Curr Microbiol App Sci 6:1398–1405

Palani NA, Seethapathy PA, Jeyaraman RA, Kathaperumal AR, Shanmugam VA (2016) Systemic elicitation of defense related enzymes suppressing Fusarium wilt in mulberry (*Morus* spp.). Afr J Microbiol Res 10:813–819

Pandin C, Le Coq D, Canette A, Aymerich S, Briandet R (2017) Should the biofilm mode of life be taken into consideration for microbial biocontrol agents? Microb Biotechnol 10:719–734

Pant G, Prakash A, Pavani JVP, Bera S, Deviram GVNS, Kumar A, Prasuna RG (2015) Production optimization and partial purification of protease from *Bacillus subtilis*. J Taibah Uni Sci 9:50–55

Park YG, Mun BG, Kang SM, Hussain A, Shahzad R, Seo CW, Lee IJ (2017) *Bacillus aryabhattai* SRB02 tolerates oxidative and nitrosative stress and promotes the growth of soybean by modulating the production of phytohormones. PLoS ONE 12:e0173203

Pfaffl MW (2006) Relative quantification. Real Time PCR 63:63–82

Qadri B (2017) Saffron adulteration: lets bunk the saffron junk. Indian J Hortic 7:212–215

Radhakrishnan R, Hashem A, Abd-Allah EF (2017) *Bacillus:* a biological tool for crop improvement through bio-molecular changes in adverse environments. Front Physiol 8:667

Raheem A, Shaposhnikov A, Belimov AA, Dodd IC, Ali B (2018) Auxin production by rhizobacteria was associated with improved yield of wheat (*Triticum aestivum* L.) under drought stress. Arch Agron Soil Sci 64:574–587

Ramakrishna W, Yadav R, Li K (2019) Plant growth promoting bacteria in agriculture: two sides of a coin. Appl Soil Ecol 138:10–18

Ramavath K, Hameeda B, Reddy G (2019) Enhancement of plant growth in tomato by inoculation with plant growth promoting *Bacillus* spp. World 7:69–75

Rashid TS, Qadir SA, Awla HK (2021a) Induction of defence related enzymes and biocontrol efficacy of *Trichoderma harzianum* in tomato plants infected with *Fusarium oxysporum* and *Fusarium solani*. Acta Agric Slov 117:1–6

Rashid U, Yasmin H, Hassan MN, Naz R, Nosheen A, Sajjad M, Ahmad P (2021b) Drought-tolerant *Bacillus megaterium* isolated from semi-arid conditions induces systemic tolerance of wheat under drought conditions. Plant Cell Rep 2021:1–21

Roy JK, Mukherjee AK (2013) Applications of a high maltose forming thermo-stable α-amylase from an extremely alkalophilic *Bacillus licheniformis* strain AS08E in food and laundry detergent industries. Biochem Eng J 77:220–230

Sadeghpour N, Asadi-Gharneh HA, Nasr-Esfahani M, Khankahdani HH, Golabadi M (2022) Antioxidant enzymes associated with resistance to *Fusarium oxysporum* f. sp. melonis race 1.2 in melon. Physiol Mol Plant Pathol 121:10180

Sahoo B, Chaudhuri S (2019) Screening of lindane degrading bacteria isolated from soil for their plant growth promoting attributes. Environ Sustain 2:97–106

Sahu PK, Singh S, Gupta A, Singh UB, Brahmaprakash GP, Saxena AK (2019) Antagonistic potential of bacterial endophytes and induction of systemic resistance against collar rot pathogen *Sclerotium rolfsii* in tomato. Biol Control 137:104014

Sansinenea E (2019) *Bacillus* spp: as plant growth-promoting bacteria. In: Secondary metabolites of plant growth promoting rhizomicroorganisms, vol 1. Springer, Singapore, pp 225–237

Selari PJRG, Tsui S, de Almeida TT, Olchanheski LR, Dourado MN (2023) Biological control of phytopathogenic fungi: mechanisms and potentials. Agric Biocatal 2023:3–39

Shafi J, Tian H, Ji M (2017) *Bacillus* species as versatile weapons for plant pathogens: a review. Biotechnol Biotechnol Equip 31:446–459

Sharaf-Eldin M, Elkholy S, Fernández JA, Junge H, Cheetham R, Guardiola J, Weathers P (2008) *Bacillus subtilis* FZB24® affects flower quantity and quality of saffron (*Crocus sativus*). Planta Med 74:1316–1320

Singh P, Arif Y, Miszczuk E, Bajguz A, Hayat S (2022) Specific roles of lipoxygenases in development and responses to stress in plants. Plants 11:979

Sultana S, Alam S, Karim MM (2021) Screening of siderophore-producing salt-tolerant rhizobacteria suitable for supporting plant growth in saline soils with iron limitation. J Agric Food Inf 4:100150

Taktek S, St-Arnaud M, Piché Y, Fortin JA, Antoun H (2017) Igneous phosphate rock solubilization by biofilm-forming mycorrhizobacteria and hyphobacteria associated with Rhizoglomus irregulare DAOM 197198. Mycorrhiza 27:13–22

Taranto F, Pasqualone A, Mangini G, Tripodi P, Miazzi M, Pavan S, Montemurro C (2017) Polyphenol oxidases in crops: biochemical physiological and genetic aspects. Int J Mol Sci 18:377

Thakur A, Verma S, Reddy VP, Sharma D (2019) Hypersensitive responses in plants. Agric Rev 40:113–120

Thiebaut F, Urquiaga MCDO, Rosman AC, da Silva ML, Hemerly AS (2022) The impact of non-nodulating diazotrophic bacteria in agriculture: understanding the molecular mechanisms that benefit crops. Int J Mol Sci 23:11301

Timmusk S, Behers L, Muthoni J, Muraya A, Aronsson AC (2017) Perspectives and challenges of microbial application for crop improvement. Front Plant Sci 8:49

Uarrota VG, Moresco R et al (2016) The role of ascorbate peroxidase guaiacol peroxidase and polysaccharides in cassava (*Manihot esculenta Crantz*) roots under postharvest physiological deterioration. Food Chem 197:737–746

Vishwanathan K, Zienkiewicz K, Liu Y, Janz D, Feussner I, Polle A, Haney CH (2020) Ectomycorrhizal fungi induce systemic resistance against insects on a nonmycorrhizal plant in a CERK1-dependent manner. New Phytol 228:728–740

Viswanath V, Beena AK, Silva JD (2018) Characterization of a cellulase producing *Pseudomonas fluorescens* isolated from agricultural waste. Biotechnol Res 4:109–113

Walitang DI, Kim K, Madhaiyan M, Kim YK, Kang Y, Sa T (2017) Characterizing endophytic competence and plant growth promotion of bacterial endophytes inhabiting the seed endosphere of Rice. BMC Microbiol 17:209

Xia Y, Farooq MA, Javed MT, Kamran MA, Mukhtar T, Ali J, Chaudhary HJ (2020) Multi-stress tolerant PGPR *Bacillus xiamenensis* PM14 activating sugarcane (*Saccharum officinarum* L.) red rot disease resistance. Plant Physiol Biochem 151:640–649

Xu A, Zhan JC, Huang WD (2016) Combined elicitation of chitosan and ultraviolet C enhanced stilbene production and expression of chitinase and β-1,3-glucanase in *Vitis vinifera* cell suspension cultures. Plant Cell Tissue Organ Culture (PCTOC) 124:105–117

Ye X, Xu C, Xie T, Zhang Y, Zhao Y, Xia C, Cui Z (2023) Myxobacterial outer membrane β-1,6-glucanase induced the cell death of *Fusarium oxysporum* by destroying the cell wall integrity. Appl Environ Microbiol 2023:e01236-22

Yu Y, Gui Y, Li Z, Jiang C, Guo J, Niu D (2022) Induced systemic resistance for improving plant immunity by beneficial microbes. Plants 11:386

Zhang X, Li X, Zhang Y, Chen Y, Tan X, Su P, Liu Y (2020) Integrated control of potato late blight with a combination of the photosynthetic bacterium *Rhodopseudomonas palustris* strain GJ-22 and fungicides. Biocontrol 65:635–645