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Bacillus siamensis and *Bacillus amyloliquefaciens*: exploring plant growth-promoting features for sustainable agricultural applications

Bacillus siamensis i *Bacillus amyloliquefaciens*: istraživanje karakteristika koje podstiču rast biljaka za održive poljoprivredne primene

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ABSTRACT

The growing global demand for food production has led to the intensified use of chemical pesticides and mineral fertilisers to maximise crop yields. However, the adverse impact of these chemicals on soil, human health, and crop quality has driven research into sustainable alternatives, such as Plant Growth-Promoting Rhizobacteria (PGPR). This study evaluated the plant growth-promoting (PGP) mechanisms of *Bacillus siamensis* 32 DfEM and *Bacillus amyloliquefaciens* 4I through *in vitro* PGP assays, enzymatic profiling (API ZYM), and a confrontation test against *Botrytis cinerea*, *Fusarium oxysporum*, *Alternaria* sp., and *Macrophomina* sp. Enzymatic analysis revealed that *B. amyloliquefaciens* 4I produced six enzymes, while *B. siamensis* 32 DfEM produced four, including phosphatases, esterases and glycosidases. A zone of inhibition indicated that both strains exhibited a certain level of antifungal activity (30.97% ≤ IR ≤ 50.96%) against the fungal phytopathogens. PGP profiling showed that both isolates could produce ammonia and siderophores. They could neither solubilize inorganic P or Zn on the plate assay nor produce IAA and cellulase. These findings indicate that *B. siamensis* 32 DfEM and *B. amyloliquefaciens* 4I possess multiple PGP traits that qualify them as functional PGPR; however, the exhibited traits suggest that they are better suited for biocontrol applications rather than biofertilization. Therefore, it is recommended that additional mechanisms employed by these strains be investigated in greater detail to validate their potential for application as biofungicides for the sustainable and diversified promotion of plant growth.

Keywords: *Bacillus amyloliquefaciens*, *Bacillus siamensis*, biocontrol, biofertilization, PGPR, sustainable agriculture

SAŽETAK

Rastuća potražnja za proizvodnjom hrane širom sveta ubrzala je upotrebu hemijskih pesticida i mineralnih đubriva radi maksimizacije prinosa useva. Negativan uticaj ovih hemikalija na zemljište, ljudsko zdravlje i kvalitet useva podstakao je istraživanja održivih alternativa, poput rizobakterija koje podstiču rast biljaka (PGPR). Ova studija je procenila mehanizme podsticanja rasta biljaka (PGP) kod *Bacillus siamensis* 32 DfEM i *Bacillus amyloliquefaciens* 4I kroz *in vitro* PGP testove, enzimatsko profilisanje (API ZYM) i test konfrontacije protiv *Botrytis cinerea*, *Fusarium oxysporum*, *Alternaria* sp. i *Macrophomina* sp. Enzimatska analiza je pokazala da je *B. amyloliquefaciens* 4I proizvodio šest enzima, dok je *B. siamensis* 32 DfEM proizvodio četiri, uključujući fosfataze, esteraze i glikozidaze. Zona inhibicije pokazala je da su oba soja ispoljila određeni nivo antifungalne aktivnosti (30,97% ≤ IR ≤ 50,96%) protiv biljnih fitopatogena. PGP profilisanje je pokazalo da oba izolata mogu da proizvode amonijak i siderofore. Međutim, nisu mogli da solubilizuju neorganski P ili Zn u testovima na ploči, niti da proizvode IAA i celulazu. Ovi nalazi ukazuju da *B. siamensis* 32 DfEM i *B. amyloliquefaciens* 4I poseduju više PGP svojstava koja ih kvalifikuju kao funkcionalne PGPR, ali ispoljena svojstva sugerišu da su pogodniji za biokontrolne primene nego za biofertilizaciju. Stoga se preporučuje dalje istraživanje dodatnih mehanizama koje ovi sojevi koriste kako bi se potvrdio njihov potencijal za primenu kao biofungicidi za održivo i diverzifikovano unapređenje rasta biljaka.

Ključne reči: *Bacillus amyloliquefaciens*, *Bacillus siamensis*, biokontrola, biofertilizacija, PGPR, Održiva poljoprivreda

INTRODUCTION

Traditionally, subsistence farming was practised to ensure survival by producing adequate food for local consumption. However, the rapid growth of the global population, projected to reach 9 billion by 2050, has drastically increased the demand for food production (Shah et al., 2021; Luo et al., 2022). As a result, agricultural systems have transitioned toward more intensive production methods, resulting in significant improvements in crop yields. Nevertheless, these advancements have come at the cost of environmental degradation, soil health deterioration, and the loss of biodiversity (Luo et al., 2022). The global decline in crop growth, quality, and yield can be attributed to the depletion of essential mineral nutrients like nitrogen, phosphorus, and potassium in the soil, biotic stressors such as bacterial and fungal pathogens, and abiotic stress factors like salinity, drought, and heavy metal contamination (Shahzad et al., 2019; Luo et al., 2022). For instance, the fungal pathogen *Colletotrichum truncatum*, which causes anthracnose disease in chili (*Capsicum annuum* L.), is capable of reducing global chili yields by up to 50% (Than et al., 2008). Similarly, *Fusarium* wilt disease threatens cucumber (*Cucumis sativus* L.), while various abiotic stressors contribute to reduced soil fertility and crop productivity (Han et al., 2019).

Over the past few decades, conventional approaches, such as the widespread use of synthetic fertilizers and chemical pesticides, have been employed to address these challenges (Luo et al., 2022). However, the prolonged use of these inputs has proven both economically costly and environmentally unsustainable, exacerbating long-term issues like soil degradation and water contamination. The urgent need to develop nutrient-efficient agricultural systems while reducing reliance on agrochemicals has driven a search for more sustainable alternatives (Shahzad et al., 2019; Luo et al., 2022). One promising solution lies in the adoption of natural resource management strategies, including the use of Plant Growth-Promoting Rhizobacteria (PGPR). These beneficial soil microorganisms promote plant growth through the production of phytohormones (phytostimulation), improvement of nutrient uptake (biofertilization), and pathogen defence (biocontrol). PGPR play a vital role in supporting plant health and productivity, making them a critical component of sustainable agriculture (Agbodjato et al., 2015).

Among the diverse species of PGPR, *Bacillus siamensis* and *Bacillus amyloliquefaciens* have attracted considerable attention due to their ability to produce a variety of bioactive compounds, including metabolites with antibiotic properties that suppress pathogenic microbes, as well as compounds that stimulate plant growth (Luo et al., 2022). Despite extensive research on the plant growth-promoting capabilities of these species (Islam et al., 2019; Awan et al., 2020; Shen et al., 2022; Asari et al., 2016; Jamal et al., 2018; Gowtham et al., 2018), the specific biochemical mechanisms and comparative efficacy of *Bacillus siamensis* and *Bacillus amyloliquefaciens* strains remain underexplored. There is a paucity of detailed biochemical characterizations that elucidate the unique enzymatic profiles and antifungal mechanisms distinguishing these strains.

The present study evaluated the plant growth-promoting potential of two bacterial strains: *B. siamensis* 32 DfEM and *B. amyloliquefaciens* 4I. The objective was the biochemical characterization of these strains and the detection of certain traits that contribute to the stimulation of plant growth, potentially indicating their suitability as biofertilizers and/or biocontrol agents. By exploring the mechanisms through which these bacteria promote plant growth, this research contributes to the development of sustainable and eco-friendly farming systems, which can reduce the environmental impact of intensive agriculture while maintaining or improving crop yields.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Bacillus siamensis 32 DfEM and *Bacillus amyloliquefaciens* 4I were obtained from the collection of the Department of Environmental Microbiology, Faculty of Agricul-

ture (Belgrade, Serbia). Cultures were stored in nutrient broth supplemented with 25% glycerol at -80 °C until use.

In vitro plant growth-promoting (PGP) characterization of isolates

Phosphate and Zinc solubilization

The ability of isolates to dissolve inorganic phosphates was tested on National Botanical Research Institute's phosphate (NBRIP) medium [10 g glucose, 5 g $MgCl_2.6H_2O$, 0.25 g $MgSO_4.7H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$, 5 g $Ca_3(PO_4)_2$, 20 g agar and 1000 mL deionized water] (Nautiyal, 1999) incubated for 14 days at 30 °C, while zinc solubilization was assessed using tris-mineral agar [10 g D-glucose; 1.0 g $(NH_4)_2SO_4$; 0.2 g KCl; 0.1 g K_2HPO_4; 0.2 g $MgSO_4$; 1.244 g ZnO (0.1% Zn) in 1000 ml deionized water] incubated at 30 °C for 48 hours (Eshaghi et al., 2019). The tests were carried out in triplicate, and the presence of a halo zone around the colonies after the incubation period indicated solubilization.

Ammonia production

Ammonia production was tested by inoculating fresh cultures with 10 ml of peptone water and incubating at 30 °C for 72 hours. After the incubation period, 0.5 ml of Nessler's reagent was added to each tube, and the development of a brown/yellow color compared to the control indicated a positive reaction (Alotaibi et al., 2022).

Cellulase activity

Cellulase activity was determined on carboxymethylcellulose agar (CMC) (Romsaiyud et al., 2009). After a 3-day incubation period at 28 °C, 5 ml of 0.1% M Congo red solution was added to the agar plates, and after 15 minutes, the plates were washed with 1% M NaCl. The presence of clear zones around the colonies indicated cellulase production.

Siderophores production

Siderophore production was qualitatively assessed using Chrome Azurol Sulfonate (CAS) agar, developed by Schwyn and Neilands (1987), following the adapted protocol of Lakshmanan et al. (2015). Additionally, the overlaid CAS (O-CAS) method by Pérez-Miranda et al. (2007) was employed to circumvent the growth inhibition of Gram-positive bacteria often caused by HDTMA. The development of a yellow-orange halo around colonies after 24 h indicated positive siderophore production. Quantitative measurements of halo diameter (in centimetres) were recorded using a calibrated ruler to facilitate the comparative efficacy of this trait between the strains.

Auxin (indole-3-acetic acid, IAA) production

Fresh cultures of *B. siamensis* 32 DfEM and *B. amyloliquefaciens* 4I were inoculated into 5 mL of M9 medium (200 mL M9 salt solution, 2 mL of 1 M $MgSO_4$, 20 mL of 20% glucose, 0.1 mL of 1 M $CaCl_2$, deionized water) and incubated at 30 °C for 24 h. The inoculum (0.5 mL) was transferred to 9 mL of M9 medium supplemented with 100 µg/mL tryptophan and incubated on an orbital shaker for 72 h/30 °C/150 rpm (Ika, Lithuania). After incubation, 2 mL of the suspension was centrifuged (6000 × g, 15 min) (Mini Spin, Eppendorf, Germany), and 1 mL of the supernatant was mixed with 2 mL of Salkowski reagent. The mixture was vortexed and incubated for 25 min at room temperature. The IAA concentration was measured by reading the absorbance at 540 nm and comparing it with a standard curve of commercial IAA (Sigma-Aldrich, USA).

Enzymatic profile

The activities of 19 enzymes involved in the main biogeochemical cycles of nutrients: carbon (β-glucosidase, α-glucosidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-mannosidase, α-fucosidase, esterase lipase, and lipase), phosphorus (acid and alkaline phosphomonoesterases and naphthol-AS-BI-phosphohydrolase) and nitrogen (protease, leucine-arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin and N-acetyl-glucosaminidase), were determined using the API ZYM test (bioMérieux, France) according to the manufacturer's instructions. Enzyme activity was assessed semi-quantitatively using the API ZYM color chart.

Antagonism bioassay

The antagonistic activity of *B. siamensis* 32 DfEM and *B. amyloliquefaciens* 4I was assessed using a dual-culture confrontation test against *Botrytis cinerea*, *Fusarium oxysporum*, *Alternaria* sp., and *Macrophomina* sp. Potato dextrose agar (PDA) plates were inoculated with a 5 × 5 mm slice of 7-day-old fungal culture on one side and a bacterial culture line 3 cm away on the other side. Treatments were performed in triplicate, with control plates containing only the fungal culture in the center. Plates were sealed with parafilm and incubated at 25 ± 2 °C until the control fungus reached the edge of the plate. After incubation, the presence/absence of inhibition zones was recorded, and the inhibition rate (%) of mycelial growth was calculated using the formula (Gowtham et al., 2018):

$$IR\ (\%) = [(DC - DT) / DT] \times 100$$

where:

- IR - Inhibition rate
- DC - Diameter of fungal colony in control plates
- DT - Mean diameter of fungal colony in treatment plates.

Based on inhibition rates, the degree of antagonistic activity was categorized as follows (Sookchaoy et al., 2009):
[% > 75] – very high, [% = 61 – 75] – high, [% = 51 – 60] – moderate, and [% < 51] – low.

RESULTS

In vitro PGP characterization

Isolates of *B. siamensis* 32 DfEM and *B. amyloliquefaciens* 4I were subjected to a series of tests in order to determine the presence of traits that are involved in promoting plant growth. Table 1 shows the results of PGP characterization.

In this study, both *B. siamensis* 32 DfEM and *B. amyloliquefaciens* 4I could produce ammonia and siderophores. Quantitative analysis revealed that *B. amyloliquefaciens* 4I produced significantly larger halo zones (0.5 cm to 0.8 cm) compared to *B. siamensis* 32 DfEM (0.1 cm to 0.2 cm), indicating a higher efficiency in siderophore synthesis. No significant solubilization of inorganic phosphorus or zinc was observed for either strain, nor was there evidence of indole-3-acetic acid (IAA) or cellulase production under the tested conditions (Table 1).

Table 1. PGP characteristics of the bacterial strains

PGP characteristics	*Bacillus siamensis* 32 DfEM	*Bacillus amyloliquefaciens* 4I
Phosphate solubilization	—	—
Zinc solubilization	—	—
Ammonia production	+	+
Cellulase activity	—	—
Siderophore production	+	+
Auxin (IAA) production	—	—

— indicates absence of trait, + indicates presence of trait.

Enzyme profile

Semi-quantitative evaluation of extracellular enzyme activities of *B. siamensis* 32 DfEM and *B. amyloliquefaciens* 4I using the API ZYM test revealed distinct enzymatic profiles for both strains (Table 2). It was confirmed that *B. amyloliquefaciens* 4I can produce six types of enzymes (alkaline phosphatase, esterase, esterase lipase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, and α-glucosidase) (Table 2). In contrast, *B. siamensis* 32 DfEM showed the ability to produce C4 esterase, esterase lipase, naphthol-AS-BI-phosphohydrolase and β-glucosidase (Table 2).

Table 2. Enzymatic activity of bacterial strains

		Enzyme	
Enzyme	Substrate	*B. siamensis* 32 DfEM	*B. amyloliquefaciens* 4I
Alkaline phosphatase	2-naphthyl phosphate	—	+
Esterase (C4)	2-naphthyl butyrate	+	+
Esterase Lipase (C8)	2-naphthyl caprylate	+	+
Lipase (C14)	2-naphthyl myristate	—	—
Leucine arylamidase	L-leucyl-2-naphthylamide	—	—
Valine arylamidase	L-valyl-2-naphthylamide	—	—
Cystine arylamidase	L-cystyl-2-naphthylamide	—	—
Trypsin	N-benzoyl-DL-arginine-2-naphthylamide	—	—
α-chymotrypsin	N-glutaryl-phenylalanine-2-naphthylamide	—	—
Acid phosphatase	2-naphthyl phosphate	—	+
Naphthol-AS-BI-phosphohydrolase	Naphthol-AS-BI-phosphate	+	+
α-galactosidase	6-Br-2-naphthyl-αD-galactopyranoside	—	—
β-galactosidase	2-naphthyl-βD-galactopyranoside	—	—
β-glucuronidase	Naphthol-AS-BI-βD-glucuronide	—	—
α-glucosidase	2-naphthyl-αD-glucopyranoside	—	+
β-glucosidase	6-Br-2-naphthyl-βD-glucopyranoside	+	—
N-acetyl-β-glucosaminidase	1-naphthyl-N-acetyl-βD-glucosaminide	—	—
α-mannosidase	6-Br-2-naphthyl-αD-mannopyranoside	—	—
α-fucosidase	2-naphthyl-αL-fucopyranoside	—	—

— indicates negative reaction, + indicates positive reaction.

Antagonism bioassay

The confrontation test used to evaluate the antagonistic potential of the studied bacterial strains revealed that both isolates exhibited inhibitory activity against *B. cinerea*, *F. oxysporum*, *Alternaria* sp. and *Macrophomina* sp., as evidenced by the presence of an inhibition zone on the PDA medium (Figure 1). Inhibition rate (IR) ranged from 30.97% to 50.48% for *B. siamensis* 32 DfEM, and 46.03% - 50.96% for *B. amyloliquefaciens* 4I. Both strains showed the highest inhibition against *Macrophomina* sp. (Figure 2). Isolate *B. siamensis* 32 DfEM showed greater antagonistic activity against *B. cinerea* and *Alternaria* sp. while *B. amyloliquefaciens* 4I showed higher antagonistic activity against *F. oxysporum* and *Macrophomina* sp. (Figure 2). Although the bacterial isolates showed low antagonistic activity (IR<51%) against all phytopathogens, the zone of inhibition in each plate was well-defined (Figure 1).

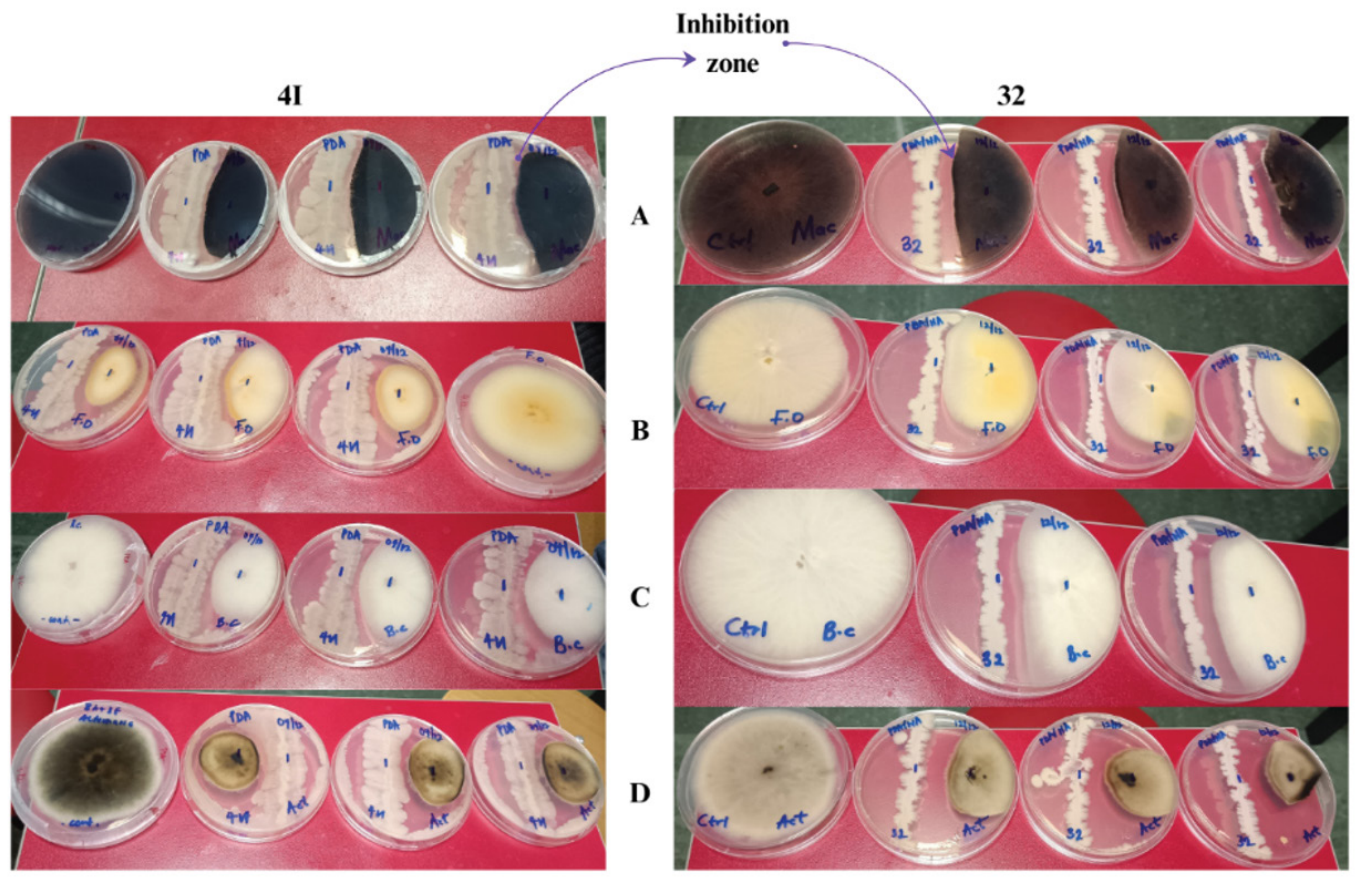


Figure 1. Confrontation test between bacterial isolates (*B. amyloliquefaciens* 4I - left and *B. siamensis* 32 DfEM - right) and phytopathogenic fungi (A – D represent PDA plates containing *Macrophomina* sp., *Fusarium oxysporum*, *Botrytis cinerea*, and *Alternaria* sp.)

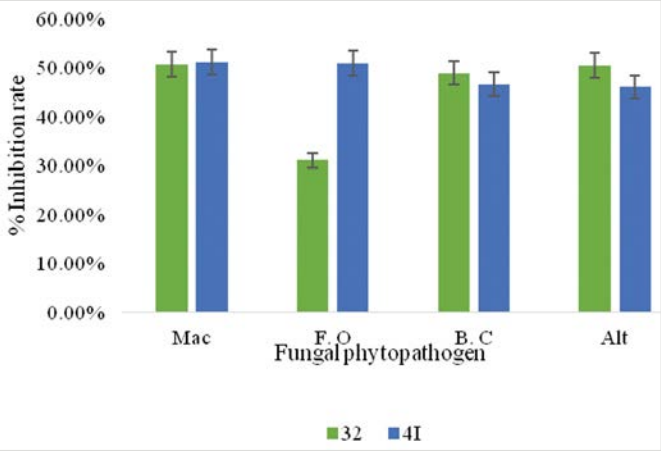


Figure 2. Inhibition rates (mean ± S.E %) against mycelia of phytopathogens (Mac - *Macrophomina* sp., F.O - *Fusarium oxysporum*, B.C - *Botrytis cinerea*, and Alt - *Alternaria* sp., 32 DfEM – *B. siamensis*, 4I – *B. amyloliquefaciens*)

Zones of inhibition, as a clear indication of the mechanism of antagonistic action, were measured, and Table 3 shows the mean values of the distances established between bacterial isolates and phytopathogens. The zone of inhibition ranged from 0.12 cm (*F. oxysporum*) to 1 cm (*Alternaria* sp.) for *B. siamensis* 32 DfEM and 0.3 cm (*B. cinerea*) to 0.46 cm (*Alternaria* sp.) for *B. amyloliquefaciens* 4I. In comparison to *B. amyloliquefaciens* 4I, *B. siamensis* 32 DfEM demonstrated a larger zone of inhibition in confrontation with all phytopathogens except *F. oxysporum*. In addition to the inhibition zones observed during the confrontation test, alterations in mycelial pigmentation were recorded on certain culture plates. In particular, changes in mycelial coloration were noted in the regions adjacent to the bacterial zone when *B. amyloliquefaciens* 4I was co-cultured with *Alternaria* sp. and *Fusarium oxysporum*. Similarly, a comparable pigmentation change was observed in the confrontation between *Alternaria* sp. and *B. siamensis* 32 DfEM (Figure 1).

Table 3. Zone of inhibition (cm) in the confrontation test

	Macrophomina sp.	*Fusarium oxysporum*	*Botrytis cinerea*	*Alternaria* sp.
B. siamensis 32 DfEM	0.77 ± 0.11	0.12 ± 0.03	0.87 ± 0.06	1.00 ± 0.26
B. amyloliquefaciens 4I	0.40 ± 0.14	0.38 ± 0.03	0.30 ± 0.00	0.46 ± 0.11

Values shown are means ± S.D of three replicates.

DISCUSSION

In vitro PGP characterization

Ammonia production is a key trait of many PGPR; hence, the ability of *B. siamensis* 32 DfEM and *B. amyloliquefaciens* 4I to produce ammonia underscores their role in enhancing nitrogen availability within the rhizosphere (Bhattacharyya et al., 2020). Nitrogen is an essential macronutrient required for the synthesis of proteins, nucleic acids, and other cellular components, making it vital for plant growth and development (Agbodjato et al., 2015). The ammonia production observed, indicated by the brown/yellow coloration upon addition of Nessler's reagent, aligns with findings of Agbodjato et al. (2015), who reported that approximately 80% of plant-promoting *Bacillus* species exhibit this trait. Ammonia production was observed in several *B. amyloliquefaciens* isolates (Cherif-Silini et al., 2016), *B. siamensis* (Oubaha et al., 2024), *B. aryabhattai* (Ramesh et al., 2014), and *B. megaterium* (Alotaibi et al., 2022), which is in corroboration with the findings of this study. In addition to the biofertilization role played by this PGPR mechanism, soil ammonia accumulation can indirectly suppress the growth of several phytopathogens by creating unfavorable alkaline conditions (pH 9 – 9.5) (Gohil et al., 2022). This dual role of ammonia as both a nutrient source and a biocontrol agent highlights the importance of this trait and the multifaceted approach of these ammonia-producing strains in sustainable agriculture.

Siderophores are small organic molecules produced by microorganisms under conditions of iron deficiency to improve Fe absorption (Saeed et al., 2021). Iron is a crucial micronutrient for plants, involved in processes such as chlorophyll synthesis and electron transport during photosynthesis (Bishnoi, 2015). However, the bioavailability of iron in most soils is usually low because it is predominantly present in its insoluble form (Fe^{3+}) (Luo et al., 2022), especially in the rhizosphere/root region, where plants and microbes compete for Fe (Saeed et al., 2021). PGPR-produced siderophores can bind to insoluble iron to form soluble complexes (Fe-phytosiderophore complex), which can be absorbed by plants to promote growth (Luo et al., 2022). The siderophore-producing abilities of *B. siamensis* 32 DfEM and *B. amyloliquefaciens* 4I in the present study were validated by the yellowish halo zones formed as a result of the removal of iron from $FeCl_3$-enriched CAS media. This finding is in agreement with those of previous studies obtained for different strains of *B. amyloliquefaciens* (Sharma et al., 2013; Abdallah et al., 2018; Jamal et al., 2018), *B. siamensis* (Shen et al., 2022; Oubaha et al., 2024), *B. velezensis* (Bhattacharyya et al., 2020; Fiuza et al., 2024), and *Pseudomonas* sp. JEC4 (Waday et al., 2022), *B. inaquosorum* ST7 (Lee et al., 2023) and *B. cereus* P8 (Sherpa et al., 2021). However, it contradicts the findings of Alotaibi et al. (2022), who found no siderophore-producing activity in *B. marisflavi* ET25 and *B. megaterium* WT10 isolated from the rhizosphere of Eleocharis and Salix plants, respectively.

Variation in halo zone diameter within and between colonies of *B. siamensis* 32 DfEM and *B. amyloliquefaciens* 4I can be attributed to certain factors such as the intrinsic capacity of the strains, iron content of the medium, pH, temperature, cell biomass, and organic acids (Cherif-Silini et al., 2016; Utama et al., 2021). By facilitating iron uptake, PGPR-produced siderophores not only promote plant growth but also contribute to biocontrol by limiting the availability of iron to pathogenic microorganisms, which rely on iron for their growth and virulence (Bishnoi, 2015; Saeed et al., 2021). The significantly larger siderophore halo in *B. amyloliquefaciens* 4I suggests a high-

er capacity for iron chelation, which could translate to superior competitive exclusion of pathogenic microbes. This trait of siderophore production by PGPR is critical for conferring the survival fitness to colonize plant roots and compete for iron with other microorganisms in the micro-ecological zone of the rhizosphere (Bishnoi, 2015; Bhattacharyya et al., 2020).

Phosphorus (P) is a critical nutrient for plant growth, but its availability in soils is limited, with only 0.1% of total soil P accessible to plants (Bishnoi, 2015; Saeed et al., 2021). In agricultural ecosystems, phosphate-solubilizing bacteria (PSB) play a critical role in making phosphorus biologically available by secreting low molecular weight organic acids (e.g., oxalic, malic, fumaric, and gluconic acids) and other compounds that chelate cations and lower the pH, facilitating its solubilization (Abdallah et al., 2018; Saeed et al., 2021; Luo et al., 2022). In the present study, *B. siamensis* 32 DfEM and *B. amyloliquefaciens* 4I did not demonstrate the ability to solubilize inorganic phosphate on NBRIP medium. However, it is well-established that not all PGPR exhibit phosphate-solubilizing abilities, which aligns with findings of Jabiri et al. (2023), who reported that certain isolates of *B. amyloliquefaciens* lacked this trait. According to Nautiyal (1999), the low diffusion rates of organic acids on solid media could prevent the formation of clear halo zones, even when the bacteria are capable of solubilizing phosphorus in liquid media. Cherif-Silini et al. (2016) also reported similar findings, where *Bacillus* strains did not show solubilization in solid media but exhibited efficient phosphate solubilization in liquid cultures. This suggests that the solubilization potential of *B. siamensis* 32 DfEM and *B. amyloliquefaciens* 4I could be better assessed in liquid media, which were not tested in this study.

Zinc (Zn) is another critical micronutrient required for various plant physiological functions, including enzyme activation, protein synthesis, and regulation of growth hormones (Kamran et al., 2017). Similar to phosphate solubilization, the ability of PGPR to solubilize zinc is an important trait for enhancing plant nutrition in zinc-deficient soils. The inability of *B. siamensis* 32 DfEM and *B. amyloliquefaciens* 4I to solubilize zinc oxide (ZnO) on tris-mineral agar medium is consistent with the findings of Majeed et al. (2015) and Kamran et al. (2017) that reported a lack of solubilization activity on solid media, potentially due to similar limitations in acid diffusion as observed with phosphorus (Nautiyal, 1999; Sharma et al., 2013).

The ability of rhizobacterial isolates to produce cellulase indicates their potential to compromise the structural integrity of fungal cell walls, thereby inhibiting pathogen growth and preventing disease (Jabiri et al., 2023). The inability of *B. siamensis* 32 DfEM and *B. amyloliquefaciens* 4I to produce cellulase may suggest that degradation of the cellulose content of the fungal cell wall is not one of their biocontrol mechanisms. In line with this, Jabiri et al. (2023) and Islam et al. (2019) described *B. amyloliquefaciens* and *B. siamensis*, respectively, without cellulolytic abilities on CMC media. Similarly, *B. velezensis* AB230, AB237 and *B. subtilis* AB267 did not produce cellulase on M9 agar medium supplemented with carboxymethylcellulose (CMC) (Bhattacharyya et al., 2020). In contrast to this, several *Bacillus* strains, including *B. amyloliquefaciens* SS35 (Singh et al., 2013), *B. amyloliquefaciens* D1B3 (Bouzaiene et al., 2023), *B. subtilis* ST8, and *B. vallismortis* SP4 (Lee et al., 2023) could produce cellulase. The absence of cellulase activity in our strains, as opposed to other *Bacillus* strains, suggests alternative pathways of pathogen suppression, potentially through the production of siderophores, ammonia, or other antifungal compounds that contribute to the observed inhibition zones *in vitro*. This differentiation not only enhances understanding of strain-specific functionalities but also informs the selection and application of PGPR based on targeted agricultural needs and pathogen profiles.

The most commonly studied phytohormone produced by PGPR is indole-3-acetic acid (IAA), the most physiologically potent auxin (Javoreková et al., 2020). IAA production by PGPR can enhance root elongation and lateral root formation (Vacheron et al., 2013; Saeed et al., 2021), improving the plant's ability to absorb water and nutrients. Both bacterial strains (*B. siamensis* 32 DfEM and *B. amyloliquefaciens* 4I) in this study did not show IAA synthesis when tested *in vitro* on M9 medi-

um supplemented with 100 µg/ml tryptophan. Similar conclusions were reached by Jabiri et al. (2023), Alotaibi et al. (2022), and Lee et al. (2023), who did not detect IAA production in *B. amyloliquefaciens* M2-3, *B. megaterium* WT10, and *B. subtilis* ST8, respectively. In addition to tryptophan dose, IAA synthesis by PGPR depends on substrate availability, culture conditions, growth stage and species/strain (Cherif-Silini et al., 2016; Bashir et al., 2023). In contrast to the results of the current study, several studies have reported the synthesis of IAA by *Bacillus* species at different concentrations: *B. siamensis* BW - 59.69 µg/ml (Oubaha et al., 2024), *B. amyloliquefaciens* UCMB5113 (Asari et al., 2017), *B. amyloliquefaciens* 32a (Abdallah et al., 2018), *Bacillus cereus* P8 - 66.5 µg/ml (Sherpa et al., 2021).

Enzyme activity of strains using API ZYM

The API ZYM test detected the ability of *B. amyloliquefaciens* 4I to produce enzymes belonging to the group of phosphatases (alkaline phosphatase, acid phosphatase and naphthol-AS-BI-phosphohydrolase), esterases (C4 esterase and C 8 esterase lipase) and glycosyl hydrolase (α- glucosidase). Meanwhile, *B. siamensis* 32 DfEM demonstrated the ability to produce naphthol-AS-BI-phosphohydrolase, C4 esterase, C8 esterase lipase, and β-glucosidase (Table 2). This finding is comparable to the findings of Lee et al. (2023), who observed equivalent exoenzyme activity in *B. inaquosorum* ST7, *B. subtilis* ST8, and *B. vallismortis* SP4. In contrast, Buntić et al. (2019) reported that most *Bacillus* strains isolated from the rhizosphere of *Medicago sativa* exhibited aminopeptidase and protease activities.

The phosphatase and phosphohydrolase activities observed in this study suggest that both strains may enhance phosphorus availability to plants by facilitating the mineralization of organic phosphorus compounds (Przemieniecki et al., 2015). According to Ngalimat et al. (2021), esterase and lipase are hydrolytic enzymes involved in the suppression of soil-borne pathogenic infections; therefore, the production of these ester hydrolases by *B. amyloliquefaciens* 4I and *B. siamensis* 32 DfEM indicates their potential for antagonistic activity against phytopathogens. Additionally, glycosidases such as α-glucosidase and β-glucosidase are essential in maintaining soil quality and improving plant growth (Ngalimat et al., 2021). The enzyme profile of *B. amyloliquefaciens* 4I, which exhibited higher enzymatic activity compared to *B. siamensis* 32 DfEM, suggests that this strain may be particularly effective in promoting plant growth, both directly and indirectly, through nutrient mineralization and pathogen suppression.

Antifungal activity of PGPR strains

Plant diseases account for approximately one-third of global crop yield losses (Bishnoi, 2015), making the suppression of pathogens a key mechanism in plant growth promotion by PGPR. Both *B. siamensis* 32 DfEM and *B. amyloliquefaciens* 4I demonstrated antifungal activity against *B. cinerea*, *F. oxysporum*, *Alternaria* sp., and *Macrophomina* sp. This direct antagonistic effect aligns with the well-established biocontrol potential of *Bacillus* species. For example, a survey of the genome of *B. siamensis* MG64 revealed that it contains abundant antimicrobial gene clusters, including non-ribosomal peptide synthetase (brevicidin, auriprocin, tyrocidin, petrobactin and bogorol), polyketide synthetase and bacteriocin (Li et al., 2020). In addition, *B. amyloliquefaciens* subsp. *plantarum* dedicates about 10% of its genome to producing antimicrobial compounds, making it a highly efficient biocontrol agent (Abdallah et al., 2018). The presence of inhibition zones, detected in confrontation tests conducted within the study (Figure 1; Table 3), confirms the ability of *B. siamensis* 32 DfEM and *B. amyloliquefaciens* 4I to produce antimicrobial substances that prevent the proliferation of fungal mycelium. Numerous past studies have confirmed the *in vitro* inhibitory activities of both *B. amyloliquefaciens* and *B. siamensis* against various phytopathogens (Sharma et al., 2013; Asari et al., 2016; Cherif-Silini et al., 2016; Islam et al., 2016; Abdallah et al., 2018; Shen et al., 2022; Lee et al., 2023; Hussain et al., 2024).

The capacity of *B. siamensis* and *B. amyloliquefaciens* to produce a wide range of structurally diverse secondary metabolites, including cyclic lipopeptides (surfactins, fengycin, iturin A and bacillomycin D), which function as

antifungal antibiotics, may represent a potential mechanism of action of the strains investigated in this study (Saeed et al., 2021; Luo et al., 2022; Hussain et al., 2024). Apart from the release of diffusible metabolites on agar, the antagonistic effect can occur through volatile compounds (VOCs) such as ammonia, HCN, acetoin, 2,3-butanediol, etc. For example, Asari et al. (2016) showed that VOCs from strains of *B. amyloliquefaciens* subsp. *plantarum* inhibited the growth of phytopathogens *in vitro*. The ability of the strains to produce siderophores may be another mechanism of antagonistic action.

The aforementioned antimicrobial compounds produced by *B. siamensis* and *B. amyloliquefaciens* can disrupt pathogen cell membranes, lead to potassium ion loss, and inhibit both mycelial growth and spore germination (Hyder et al., 2020; Jabiri et al., 2023). The inhibition rates for both isolates in this study ranged from 30.97% to 50.96%. These results are generally higher compared to the research of Cherif-Silini et al., 2016; Gowtham et al., 2018; Utama et al., 2021; and Fiuza et al., 2024, who recorded the percentage of inhibition ranging from 4.3% to 51.78%, but lower than the results obtained by Islam et al. (2016) and Jabiri et al. (2023) in the range of 55% to 90.08%. Despite the promising antagonistic activity demonstrated by these isolates, effective root colonization is crucial for biocontrol efficacy in field applications (Luo et al., 2022). Many PGPR strains that perform well *in vitro* may not exhibit the same effectiveness in the field due to challenges related to root colonization and adaptation to non-laboratory conditions. Therefore, further research is needed to evaluate root colonization, survival, and proliferation of these strains under more complex environmental conditions.

CONCLUSION

This study provides a comprehensive biochemical and enzymatic characterization of *Bacillus siamensis* 32 DfEM and *Bacillus amyloliquefaciens* 4I, elucidating their multifaceted roles in plant growth promotion and pathogen suppression. *In vitro* screening demonstrated that both strains produce ammonia and siderophores, suggesting their potential to enhance the availability of essential nutrients such as N and Fe for plants, while simultaneously creating unfavorable conditions for pathogen proliferation. Detection of phosphorous and ester hydrolase activity using API ZYM further demonstrated the probable involvement of the isolates in organic P mineralization (biofertilization) and lipolytic action on pathogenic microbes (biocontrol). The confrontation test confirmed the ability of both bacterial isolates to inhibit the mycelium growth of the selected phytopathogens, highlighting their potential as effective biocontrol agents. Considering the multiple PGP traits exhibited by *B. siamensis* 32 DfEM and *B. amyloliquefaciens* 4I, it can be concluded that both strains qualify as functional PGPR. However, owing to their robust siderophore production and notable antifungal activity, they appear to be more suitable for biocontrol (biofungicide) applications rather than biofertilization. Further studies are recommended to optimize assay methods and investigate additional mechanisms to confirm the potential of these strains as effective bioinoculants for the sustainable and diversified promotion of plant growth.

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