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Quorum quenching activity of endophytic *Bacillus* sp. EBS9 from *Tecomella undulata* and its biocontrol applications

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ABSTRACT

This study investigates the quorum quenching (QQ) activity of an endophytic bacterium, *Bacillus* sp. EBS9, isolated from the native medicinal plant *Tecomella undulata* of Rajasthan, and its biocontrol potential against the soft rot pathogen *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*). QQ activity was confirmed by the loss of violacein pigment in *Chromobacterium violaceum* (MCC 2290). Quorum quenching metabolites were extracted using ethyl acetate, and the Quorum Quenching Extract (QQE) demonstrated positive activity in assays with *C. violaceum* CV026 (MCC 2216). HRLC-MS analysis identified diketopiperazines, L,L-Cyclo (leucylprolyl) and Cyclo (L-Phe-L-Pro), which are N-acyl homoserine lactones (AHLs) antagonists competing for LuxR receptor binding sites. In vitro and *in planta* assays evaluated QQB's biocontrol potential using treatment I (*Pcc*), treatment II (*Pcc* + QQB), and a control (sterile water). In the in vitro soft rot attenuation assay showed that treatment I caused severe maceration in vegetable slices, particularly in radish, exhibiting the highest maceration diameter (25.33 ± 3.52 mm) and percentage (46.14 ± 5.70 %). However, co-inoculation with QQB significantly reduced maceration across all tested vegetables. In the plate assay, germination rates decreased to approximately 50 % in both *Vigna radiata* and *Raphanus sativus* for treatment I, but improved to 86.67 % in treatment II. The seed vigour and germination indices also improved with QQB treatment in both plant species. In the pot assay after 30 days, in contrast to a 50 % decrease in root and shoot lengths in treatment I, treatment II led to a substantial recovery, with root lengths increase by 112.07 % and 138.76 %, while shoot length by 315.65 % and 163.63 % in *V. radiata* and *R. sativus*, respectively. This study highlights the QQ and biocontrol potential of *Bacillus* sp. EBS9 against *P. carotovorum* (*Pcc*), suggesting its promise in effective management of phytopathogens, which is crucial for agricultural productivity while minimizing environmental impact.

1. Introduction

Quorum quenching (QQ) is an important strategy for managing plant diseases as it disrupts quorum sensing (QS) mechanisms that many pathogenic bacteria rely on to establish their virulence. During QS, bacteria communicate through small signaling molecules called auto-inducers. These molecules bind to their respective receptors to coordinate various physiological processes (LaSarre and Federle, 2013). In many phytopathogens, QS mediated by N-acyl homoserine lactones (AHLs) regulates the production of virulence factors, such as plant cell wall-degrading enzymes, necrosis-inducing factors, and type III secretion systems (Ansari and Ahmad, 2018). Notable pathogens like *Agrobacterium tumefaciens*, which causes crown gall (Zheng et al., 2003), and *Pectobacterium* and *Dickeya* species, responsible for soft rot and spoilage in fruits and vegetables (Sibanda *et al.*, 2018; Baltenneck *et al.*, 2021; Saranya *et al.*, 2024), rely on AHL-based QS mechanisms. Disrupting QS through QQ can significantly reduce bacterial pathogenicity by preventing these coordinated activities. QQ can be achieved through various mechanisms that includes enzymatic degradation of AHL molecules, inhibition of their synthesis, or blocking their receptors (Turan and Engin, 2018). Implementing QQ strategies in agriculture provides a sustainable alternative to chemical pesticides, reducing harm to non-target organisms, minimizing environmental impact, and slowing the development of resistant pathogen strains.

Endophytes, microorganisms residing within plant tissues without harming their host, show significant potential as QQ agents (Paul *et al.*, 2023). These bacteria can produce QQ compounds that disrupt QS signals in pathogens, thereby preventing infection. Previous studies have documented QQ activity in various endophytes, such as *Streptomyces* LPC029 from *Gmelina arborea* (Chankhamhaengdecha *et al.*, 2013),

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Table 1
Treatment groups to evaluate the role of QQB in attenuation of QS regulated virulence of *Pcc*.

Treatment groups	*Pcc* (10^6 CFU/ml)	QQB (10^8 CFU/ml)	Sterile dH_2O
I	+	–	–
II	+	+	–
Negative Control	–	–	+

Enterobacter asburiae PT39 and *Bacillus firmus* PT18 from *Pterocarpus santalinus* (Rajesh and Rai, 2014), *Bacillus thuringiensis* KMCL07 from *Madhuca insignis* (Anandan and Vittal, 2019), *Bacillus cereus* Si-Ps1 from citrus plants (Akbari Kiarood *et al.*, 2020), and *Bacillus toyonensis* from *Arthrocaulon* sp. (Roca et al., 2024). Additionally, endophytes enhance plant growth by producing growth hormones, enhancing nutrient uptake, and mitigating biotic and abiotic stress (Sharma *et al.*, 2020; Mathur *et al.*, 2024), offering dual benefits of plant health promotion and pathogen protection.

Medicinal plants serve as promising sources of QQ endophytes due to their rich diversity of bioactive compounds. These plants have evolved to produce a wide range of antimicrobial secondary metabolites, which can be mirrored in their endophytic communities. As discussed by Sharma *et al.* (2020; 2023), endophytes from medicinal plants not only produce therapeutic compounds but may also influence the host plant's gene expression to enhance the synthesis of these compounds.

The exploration of quorum-quenching (QQ) endophytes from medicinal plants presents a promising avenue for developing novel biocontrol agents. In this study, we focused on isolating bacterial endophytes from *Tecomella undulata*, a native plant of Rajasthan known locally as Rohida. *Tecomella* is recognized for its numerous bioactive compounds and has been documented for its analgesic, antibacterial, antifungal, anti-inflammatory, anti-cancer, and anti-HIV properties (Jain *et al.*, 2012). Despite its medicinal significance, limited research has been conducted on the bacterial endophytes of *Tecomella*, particularly regarding their QQ activity. Therefore, the objective of this study was to isolate QQ bacterial endophytes from *T. undulata* and evaluate their potential as biocontrol agents against a phytopathogen. The findings of this study provide an alternative, eco-friendly approach to managing plant infections and diseases. This biocontrol strategy holds broad applicability in agriculture, providing enhanced protection for crops against pathogens and potentially increasing yield.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Chromobacterium violaceum (MCC 2290) and *C. violaceum* CV026 (MCC 2216) were used as biosensor strains. *C. violaceum* is a Gram-negative bacterium known for expressing the C10-HSL signal molecule which binds to its receptor and induces the production of a purple-coloured violacein pigment, a trait associated with QS (August *et al.*, 2000). The strain CV026 is a Mini-Tn5 mutant of wild-type *C. violaceum* that produces violacein pigment only when supplemented with short-chain AHLs (C4-C8) (McClean et al., 1997). *P. carotovorum* subsp. *carotovorum* (*Pcc*) (MCC 2112) was utilized to assess the biocontrol activity of quorum quenching bacteria. *Pcc* is a phytopathogen known to produce AHL molecules. *Bacillus cereus* (MTCC 1272) served as a positive control in screening bacterial isolates for QQ activity. This bacterium produces an AHL lactonase enzyme encoded by *aiiA* gene homologues, which degrades AHL by targeting the lactone bond and amide linkage (Chan *et al.*, 2010).

C. violaceum and CV026 was procured from the National Centre for Cell Science (NCCS), Pune, India. It was cultured in Luria-Bertani (LB) broth ((g/l) tryptone10.0, yeast extract 5.0, NaCl 10.0, pH 7.5 ± 0.2; Hi-Media) at 30 °C for 24 h and maintained on LB agar (15 g/l agar added to LB broth; Hi-Media) at 4 °C (McClean et al., 1997). *Pcc* was procured from NCCS, Pune and *B. cereus* from Microbial Type Culture Collection and Gene Bank (MTCC) Chandigarh, India. They were cultured in Nutrient Broth (NB) ((g/l) peptone 5.0, HM peptone B 1.5, yeast extract 1.5, NaCl 5.0, pH 7.5 ± 0.2; Hi-Media) at 28 °C for 24 h and maintained on Nutrient Agar (NA) (15 g/l agar added to NB; Hi-Media) at 4 °C.

2.2. Isolation of endophytes

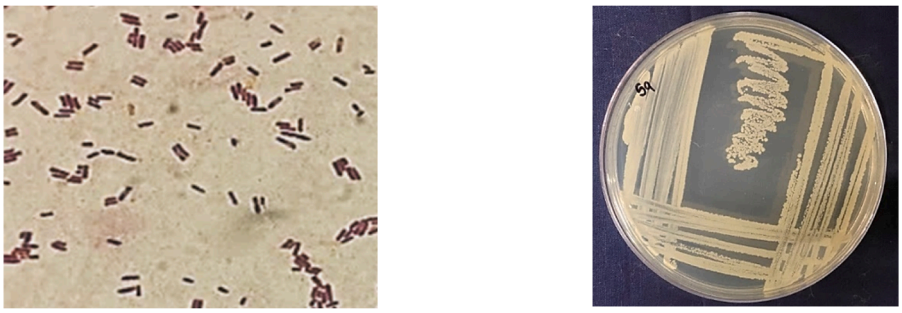
Fresh and healthy plant parts (leaves, stems, bark, and flowers) of *Tecomella undulata* were collected from the University of Rajasthan, Jaipur. These parts were surface sterilised using a stepwise procedure involving immersion in 70 % ethanol for 1 min, followed by 2 % sodium hypochlorite with Tween 20 (2 drops) for 4 min, another 30-seconds rinse in 70 % ethanol, and three consecutive washes in distilled water for 1 min each (Anjum and Chandra, 2015; Kumar *et al.*, 2016).

The sterilised plant parts were then aseptically cut and placed on NA media supplemented with nystatin (100 µg/ml) to suppress fungal growth, aiming to isolate endophytic bacteria. To verify the effectiveness of the sterilisation process, aliquots of the final rinse water were also plated on NA media. All plates were incubated at 37 °C for 24–72 h and monitored regularly for microbial growth. Morphologically distinct bacterial colonies were isolated and subsequently maintained on NA at 4 °C for further studies.

2.3. Plate screening assay for QQ activity

The isolated endophytes were screened for QQ activity using *C. violaceum* as a biosensor strain, following the agar overlay method. In this method, the isolates grown in NB for 24 h were streaked in a straight line on NA plates, and incubated for 24 h at 30 °C. C. violaceum was grown separately in LB broth for 24 h and after incubation, 50 µl of culture (~10^6 CFU/ml) was prepared in 5 ml semisolid LB agar (0.75 %) and overlaid on the streaked endophytes. The plates were incubated at 30 °C for 24 h and observed for the presence of colourless zones around the streaked isolates, indicating QQ activity (McLean *et al.*, 2004). *B. cereus* was used as a positive control in the assay. The selected isolate showing the most significant QQ activity was named Quorum

Fig. 1. Plate screening assay using *C. violaceum* showing QQ activity.



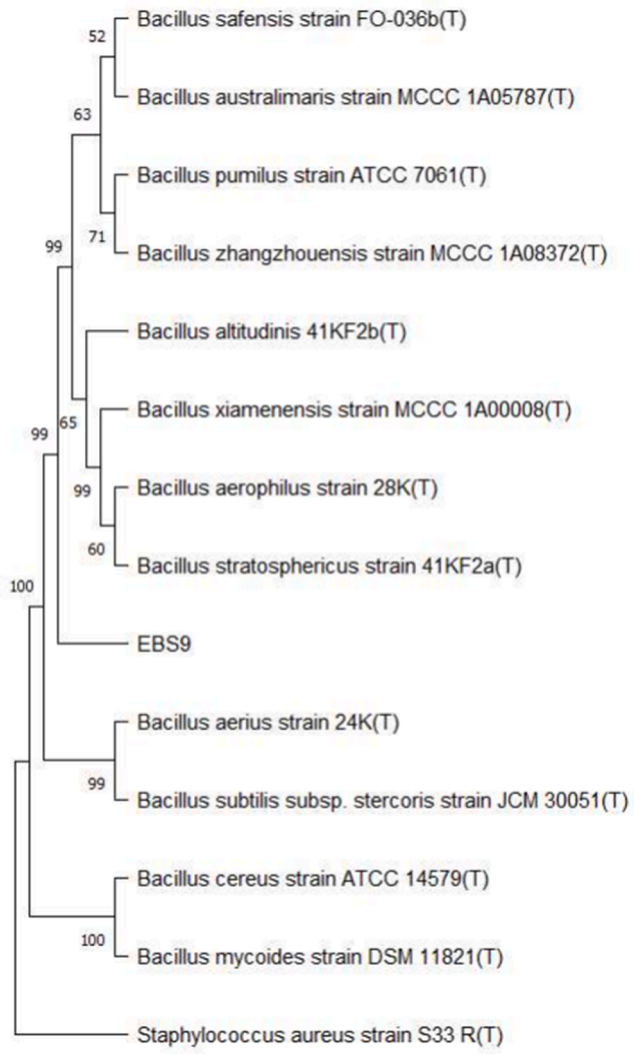


Fig. 2. Identification of QQB by (a) Gram's staining (1000X), (b) colony morphology, and (c) Phylogenetic tree based on 16S rRNA sequence from endophytic bacteria EBS9 and closely related species corresponding to Type strains (T) of the genera. Numbers at the nodes indicate bootstrap values from the Neighbor-joining analysis of 1000 resampled datasets. The bar represents the sequence divergence.

Quenching Bacteria (QQB) and used for further studies.

2.4. Identification of QQB

The potent QQB isolate was studied for its morphological colony characteristics, cell shape, and Gram staining (Cappuccino and Sherman, 2014). For molecular identification, the bacterial genomic DNA was isolated using the standard phenol/chloroform protocol (Sambrook *et al.*, 1989). PCR amplification of the 16S rRNA gene was performed using universal primers 27F [5′-CCA GAG TTT GAT CMT GGC TCA G-3′] and 1492R [5′-TAC GGY TAC CTT GTT ACG ACT T-3′]. The amplified DNA was purified and sequenced using an ABI® 3730XL automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA). Sequencing was conducted from both ends, utilizing additional internal primers to ensure each position was read at least twice. The sequence assembly was carried out using the Lasergene package. Identification of the isolate was

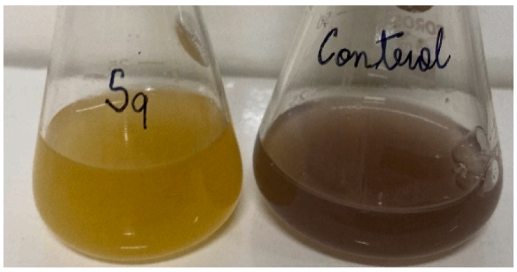


Fig. 3. Reduction in purple coloured pigment (violacein) in QQ activity assay.

performed using the EzBioCloud database (Yoon *et al.*, 2017). The FASTA sequence was submitted to NCBI to obtain an accession number. BLAST analysis was conducted, and a phylogenetic tree was constructed using the Neighbor-joining technique in the MEGA software version 11.

2.5. Extraction of QQ compounds and detection of QQ activity

The extraction of QQ compounds from the potent QQB was carried out using ethyl acetate. The QQB was cultured in NB at 30 °C in an orbital shaker for 2 days. Following incubation, the culture was centrifuged at 3800 rpm for 30 min at 4 °C. The supernatant was then filtered through a sterile membrane filter (0.45 µm pore size). The filtered supernatant was mixed with an equal volume of ethyl acetate (1:1 ratio) in a separatory funnel to extract the organic phase. The organic phase was subsequently evaporated in a desiccator at room temperature. The dried residue containing the QQ compounds was reconstituted in dimethyl sulfoxide (DMSO) to achieve a final concentration of 0.1 g/ml (Kachhadia *et al.*, 2022). This preparation was referred to as the Quorum Quenching Extract (QQE).

QQE (10 µl) was tested for QQ activity using *C. violaceum* CV026 ($OD_{600} = 0.1$) in LB broth (18 ml) containing C6-HSL (0.02 g/ml) by the flask incubation method. A negative control flask containing all components except the QQE was also prepared. The flasks were checked for inhibition of violacein pigment after incubation 24 h at 28 °C under shaking conditions (Choo et al., 2006).

2.6. HRLC-MS analysis of QQE for identification of QQ compounds

The QQE of endophytic bacterial isolate (QQB) was further analysed by HRLC-MS using Hypersil GOLD C18 column. The injection volume and flow rate were 5 µl and 0.3 ml/min at 1200 bar, respectively. Gradient mobile phases used were: Phase A (0.1 % formic acid), and phase B (90 % acetonitrile, 10 % water, 0.1 % formic acid). The linear gradient elution process was: 95 % A with 5 % B for 1 min, 100 % B for 20 min, 100 % B for 30 min, then again 95 % A with 5 % B for 31 min and last 100 % A for 35 min. Electrospray ionisation-mass spectrometry (ESI-MS) experiments were carried out using a Q-TOF G6550A mass spectrometer. Both positive and negative mode ESI scans were carried out to identify acidic and basic compounds and polar and non-polar molecules in the extract. The acquisition method was set as: minimum range 120 (m/z), maximum range 1200 (m/z), scanning rate 1 spectra/s. Settings for the iFunnel MS Q-TOF segment of instrument was maintained as: gas flow rate 13 l/min at 250 °C temperature, sheath gas flow rate 11 l/min at 300 °C temperature, and 35 psi nebulizer gas flow pressure (Singh et al., 2022).

2.7. Role of QQB in attenuation of QS-regulated virulence of *PCC*

Pcc is a phytopathogen that induces QS-regulated virulence in various host plants. The ability of the QQB to mitigate the virulence of *Pcc* was tested across various vegetables and plant samples using three distinct experiments (I, II, and III). For each experiment, the vegetable and plant samples were divided into two treatment groups as outlined in Table 1. In treatment I, samples were inoculated with *Pcc*, and the resultant virulence was evaluated using predefined parameters. In treatment II, the samples were treated with a combination of *Pcc* and QQB. The reduction in virulence caused by QQB was then measured to evaluate its effectiveness in attenuating the QS-regulated virulence of *Pcc*. This comparative approach allowed for a clear assessment of QQB's potential as a biocontrol agent in reducing *Pcc*-induced plant diseases.

2.7.1. Soft rot attenuation assay (Experiment I)

The QQ-based biocontrol activity was evaluated on various host vegetables against soft rot caused by *Pcc* using a soft rot attenuation assay, according to a modified method of Garge and Nerurkar (2016). Healthy potato, radish, cucumber, carrot, and cabbage samples were rinsed with tap water and surface sterilised using 0.01 % mercury chloride, 70 % ethanol, followed by sterile distilled water. For the assay, vegetable slices (5 mm thick) and cabbage pieces (5 cm x 5 cm) were prepared, and their initial weights were recorded. Each surface-sterilised vegetable slice placed in Petri plates received 5 µl of either *Pcc* (treatment I) or a combination of *Pcc* and QQB (treatment II). Sterile distilled water (5 µl) served as the control. All plates were incubated at 30 °C for 24 h After incubation, the maceration diameter (in mm) of the affected regions on the slices was measured. The macerated tissue was carefully removed, and its weight was recorded. The maceration percentage was calculated using the following formula:

$$\text{Maceration}(\%) = \frac{\text{Weight of macerated tissue}}{\text{Initial weight of slice}} \text{x } 100$$

The comparison of maceration diameter and maceration percentage between treatment I and II was made to evaluate the attenuation potential of QQB.

2.7.2. Biocontrol activity in plants using plate assay (Experiment II)

The experiment was carried out on seeds of *Raphanus sativus* (radish) and *Vigna radiata* (mung bean) using a modified method of Garge and Nerurkar (2017). The mung beans and radish seeds were surface sterilised as previously described. Five surface-sterilised seeds were placed in sterile Petri plates containing moistened tissue paper, and 500 µl of either treatment I (*Pcc*) or treatment II (*Pcc* + QQB) was applied. Sterile distilled water (500 µl) served as the control. All plates were incubated at 30 °C for 7 days.

On the seventh day, seed germination parameters, including seed germination percentage, seed vigour index, and germination index, were measured. Additionally, plant growth parameters such as root number, root length, and shoot length were assessed in both treatment groups (Mathur *et al.*, 2022).

Germination Percentage: The seed germination percentage was calculated as the average number of seeds germinating over seven days using the following formula (Al-Ansari and Ksiksi, 2016):

$$\text{Germination } \% = \frac{\textit{No. of seeds germinated}}{\textit{Total no. of seeds plated}} \text{x } 100$$

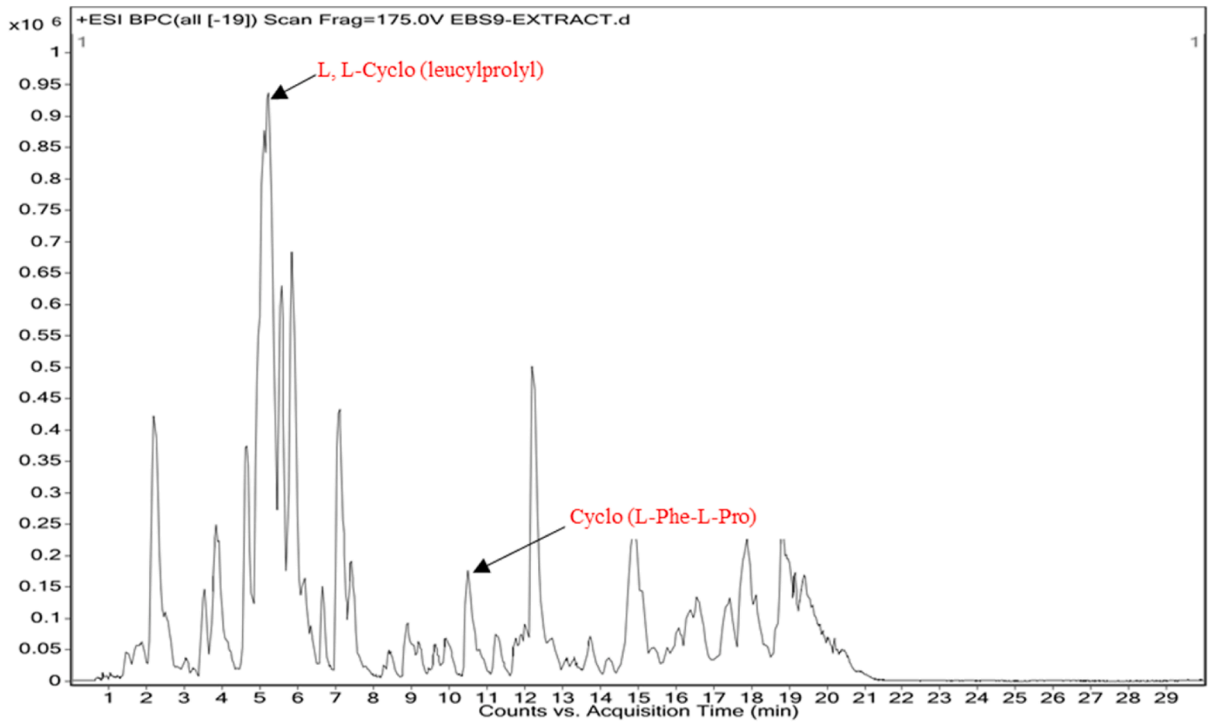
Seed Vigour Index: Seed Vigour Index (SVI) was computed using the formula (Anupama *et al.*, 2014):

$$\textit{Seed Vigour} = \textit{Seedling length } (mm) \textit{ x Germination}\%$$

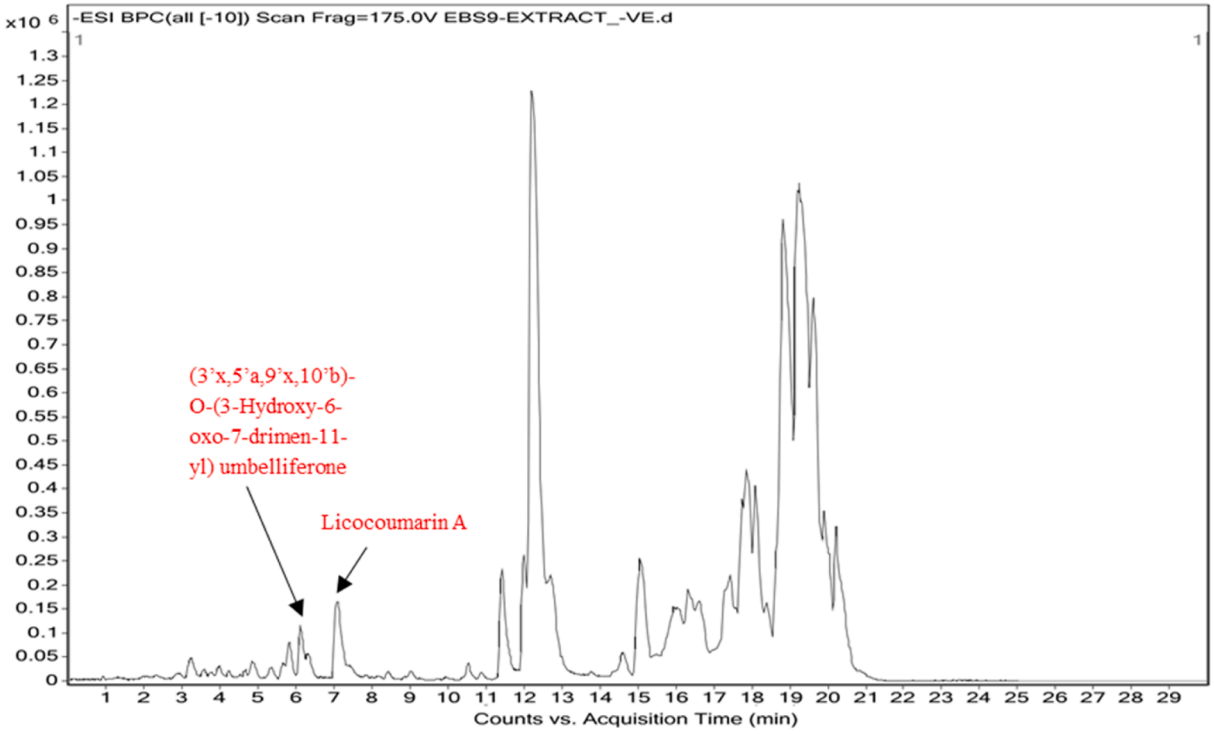
Germination Index: The numbers of seedlings budding daily was counted from treatment days 0–7 and Germination Index (GI) was evaluated using the formula (Fetouh and Hassan, 2014):

$$\text{Germination Index} = \sum \frac{n}{x}$$

where, n is the number of seedlings germinated on day x and x is the day after planting.



(a)



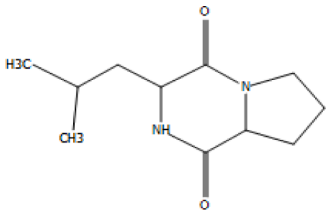
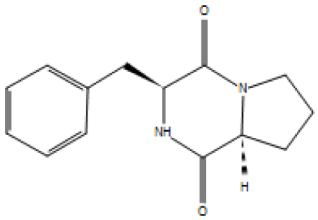
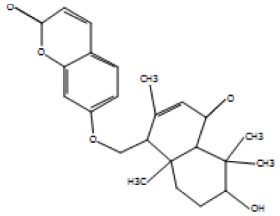
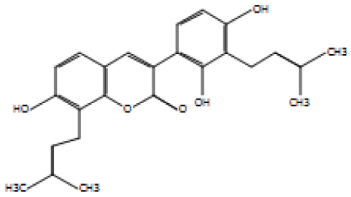
(b)

Fig. 4. HRLC-MS chromatogram of QQE showing (a) Positive and (b) Negative ESI scans.

2.7.3. Biocontrol activity in plants using pot assay (Experiment III)

The biocontrol activity of QQB against Pcc was also evaluated using the modified method of Vega et al. (2020) in a pot assay. Ten surface-sterilised seeds of mung beans and radish (described in section 3.7.2) were transplanted into plastic pots (25 cm x 20 cm) containing 30 kg of autoclaved soil. Each pot received 30 ml of treatment I (*Pcc*) and treatment II (*Pcc* + QQB). Pots treated with 30 ml of sterile distilled water served as the control. The pots were maintained in a greenhouse at 30 °C with an 8 h photoperiod and were watered daily with 20 ml of water. After 30 days, the root number and the root and shoot lengths

Table 2
List of QQ compounds identified in QQE.

Compound Name	Molecular formula	Compound structure	References
L, L-Cyclo (leucylprolyl)	$C_{11}H_{18}N_2O_2$		Kachhadia *et al.*, 2022
Cyclo (L-Phe-L-Pro)	$C_{14}H_{16}N_2O_2$		Kachhadia *et al.*, 2022
(3′x,5′a,9′x,10′b)-O-(3-Hydroxy-6-oxo-7-drimen-11-yl) umbelliferone	$C_{24}H_{28}O_5$		Amin *et al.*, 2020; D'Almeida *et al.*, 2017
Licocoumarin A	$C_{25}H_{26}O_5$		Manner and Fallarero, 2018; Kumar, 2022

were measured and compared between the treatment groups.

2.8. Statistical analysis

All experiments were performed in triplicate, and the data were represented as mean ± standard deviation. The statistical analysis was conducted using SPSS software (Version 22). An independent sample two-tailed *t*-test was used to assess the significance of differences between treatments I and II on maceration diameter and maceration percentage in the soft rot attenuation assay (Experiment I). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test at a 0.05 significance level was used to evaluate the effects of treatments I and II on seed germination parameters (germination percentage, germination index, and seed vigour index) and plant growth parameters (root and shoot length, and root number) in Experiments II and III. Correlation analysis was also conducted, and the Karl Pearson correlation coefficient was calculated for plant growth parameters in Experiment III.

3. Results and discussion

3.1. Isolation of endophytes and screening for QQ activity

A total of twenty-four bacterial isolates were obtained from the surface sterilised plant parts. The plate screening assay using C. violaceum revealed two endophytic bacterial isolates, EBF3 and EBS9, which exhibited a decrease in violacein around their growth in agar overlay method, indicating positive QQ activity. These results were comparable to the positive control (Fig. 1). The isolate EBS9 was selected as a potent Quorum Quenching Bacterium (QQB) due to its larger colourless zone. Kachhadia et al. (2022) used a similar strategy for selecting a potent quorum quencher.

Previous studies have reported that QS-regulated traits in C. violaceum and other bacteria are unrelated to bacterial growth and their inhibition will not have a bactericidal effect. Any modification in these traits is believed to result from QS inhibition (Tiwary et al., 2017; Dimitrova et al., 2023). Violacein produced by C. violaceum is regulated by the AHL-mediated cviI-cviR QS system. The gene cviI synthesises the AHL signal C6-homoserine lactone (HSL), while cviR codes for a DNA-binding cytoplasmic transcription factor that acts as the receptor. During high cell density, AHL binds with CviR, forming a complex that activates the transcription of genes from the vio operon, resulting in violacein production (Kothari et al., 2017). Therefore, the absence of violacein pigment around QQB growth indicates disruption of the QS process and confirms its QQ activity.

3.2. Identification of QQB

The potent QQB (EBS9) was identified as Gram-positive bacillus, forming moderately sized, pale white circular colonies (Fig. 2a and b). The 16S rRNA analysis was carried out and an accession number OR253786 for QQB was obtained from GenBank after the submission of the FASTA sequence. EBS9 was identified as belonging to the genus *Bacillus* based on both BLAST analysis and phylogenetic assessment (Fig. 2c). The morphological and microscopic characteristics of QQB were consistent with those of the genus *Bacillus*. Consequently, the endophytic QQB was confirmed to be *Bacillus* sp. EBS9.

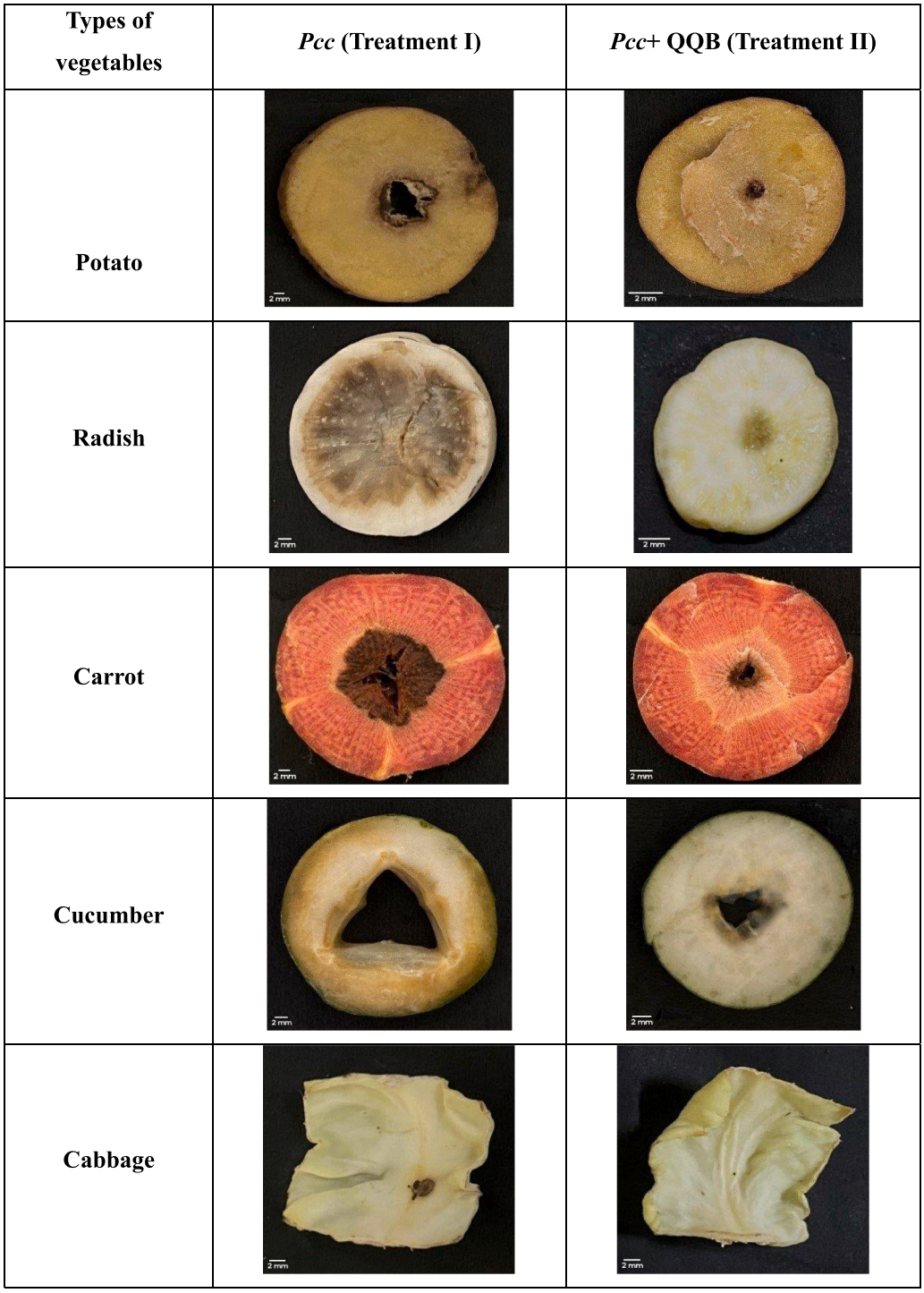


Fig. 5. Soft rot attenuation assay on potato, radish, carrot, cucumber, and cabbage.

This finding aligns with previous reports that species of the genus *Bacillus* from the phylum Firmicutes are prevalent endophytic bacteria associated with various plants, as determined by culture-dependent methods (Xia et al., 2015). Endophytic *Bacillus* species with QQ activity have been reported from tobacco (*Nicotiana tabacum*) (Ma et al., 2013), *Cannabis sativa* L. (Kusari et al., 2014), and *Madhuca insignis* (Anandan and Vittal, 2019). Lopes et al. (2018) reviewed various endophytic *Bacillus* species and their bioactive compounds, noting QQ activity as one of their properties.

3.3. Extraction of QQ compounds and detection of QQ activity

The QQ compounds were extracted from the two-day-old QQB culture using ethyl acetate. A dried ethyl acetate extract weighing 112.8 mg was obtained, reconstituted to 0.1 g/ml with DMSO and referred to as the Quorum Quenching Extract (QQE).

When subjected to the QQ activity assay, a significant reduction in violacein content in the flask incubation assay using CV026 was observed (Fig. 3), confirming the presence of QQ activity in the QQE. CV026 is a Mini-Tn5 mutant derived from *C. violaceum* and produces pigment only upon the exogenous supply of AHLs (McClean *et al.*, 1997).

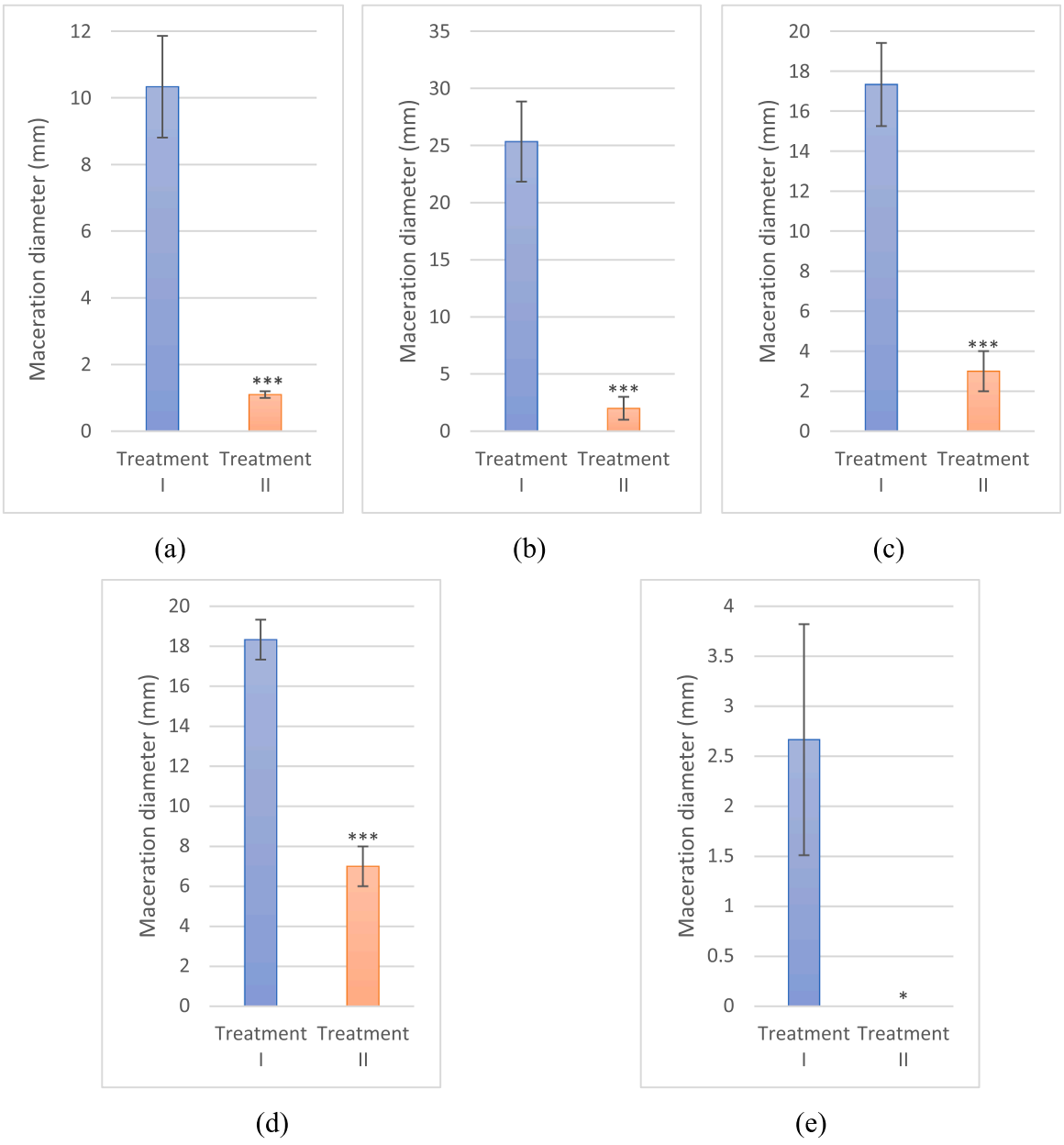


Fig. 6. Effect of soft rot attenuation assay in terms of maceration diameter (in mm) on slices of (a) potato, (b) radish, (c) carrot, (d) cucumber, and (e) cabbage.

The inhibition of violacein production in CV026, in the presence of QQE and C4-HSL, suggests interference with the CV026 CviIR-dependent QS system by the compounds present in the extract.

Ethyl acetate extracts from other bacteria have also shown quorum sensing inhibition, supporting these findings. For instance, ethyl acetate extracts from *Halobacillus salinus* inhibited QS in *Vibrio harveyi* (Teasdale et al., 2009), *Streptomyces coelicoflavus* inhibited QS in *Pseudomonas aeruginosa* PAO1 (Hassan et al., 2016), and *Bacillus cereus* RC1 inhibited violacein production in *C. violaceum* (Kachhadia et al., 2022).

3.4. HRLC-MS analysis of QQE for identification of QQ compounds

Untargeted metabolomic analysis of the QQE using HRLC-MS resulted in the separation of a complex mixture of secondary metabolites. Positive and negative ESI scans revealed the presence of 80 and 49 compounds, respectively, which were subsequently searched in the literature for their QQ activity. In the positive mode ESI scan, two cyclic dipeptides, L,L-Cyclo (leucylprolyl) and Cyclo (L-Phe-L-Pro) were identified (Fig. 4a). These cyclic dipeptides, known as diketopiperazines (DKPs), are reported to have a wide range of biological activities, including modulation of QS in bacteria (De Carvalho and Abraham, 2012). Their QQ activity has been documented (Kachhadia et al., 2022), and their presence in the extract suggests a possible role in QQ. In the negative mode ESI scan, secondary metabolites such as (3′x,5′a,9′x, 10′b)-O-(3-Hydroxy-6-oxo-7-drimen-11-yl) Umbelliferone and Licocoumarin A were found (Fig. 4b), which also indicate contributions to the QQ activity of QQE according to the literature (D'Almeida et al., 2017; Kumar, 2022). The molecular formulas and structures of these compounds are presented in Table 2.

Some DKPs produced by *Bacillus* spp. as secondary metabolites with varying bioactivities have already been documented in the literature (Bofinger et al., 2017; Nishanth Kumar et al., 2013; 2014). Earlier studies demonstrated that DKPs inhibited bioluminescence production in *E. coli* (pSB401) (Holden et al., 1999) and *Vibrio fisheri* (Campbell and Blackwell, 2009). Many studies have shown that DKPs can disrupt the QS system in pathogens, leading to reduced expression of virulence

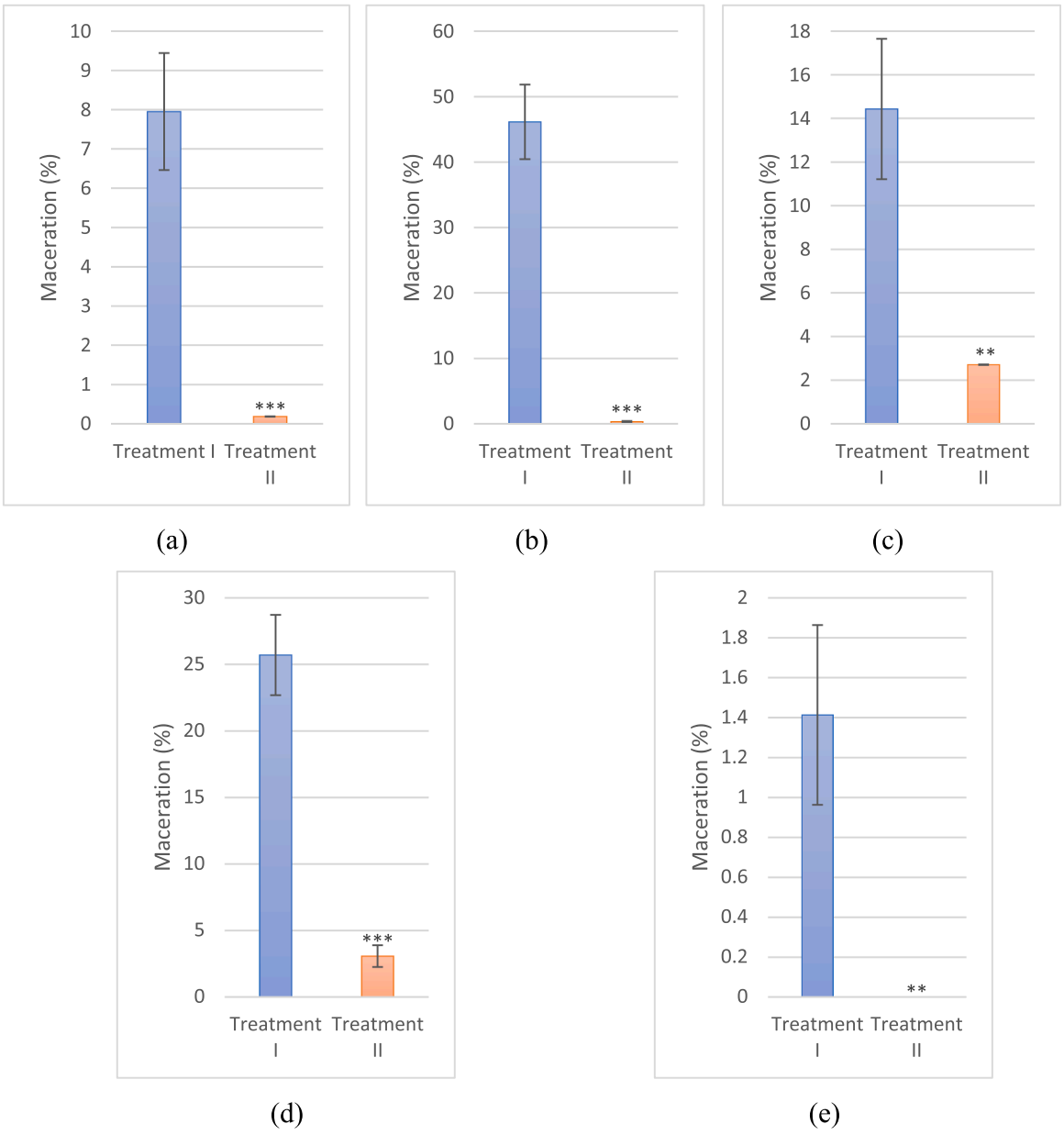


Fig. 7. Effect of soft rot attenuation assay in terms of maceration percentage on slices of (a) potato, (b) radish, (c) carrot, (d) cucumber, and (e) cabbage.

factors. For example, Cyclo (L-Pro-L-Tyr) and Cyclo (D-Ala-L-Val) produced by *Alternaria alternata* inhibited the AHL-based QS system of *Serratia liquefaciens* MG1 (Ryan and Dow, 2008). Cyclo (L-Phe-L-Pro) and Cyclo (L-Pro-L-Tyr) from *LactobaciLus reuteri* inhibited the AIP-based QS of *Staphylococcus aureus* (Li et al., 2011). Abed *et al.* (2013) also observed that DKPs isolated from dichloromethane extracts of a halophilic Marinobacter sp. SK-3 inhibited the QS process in biosensor strains. Kachhadia et al. (2022) observed that DKPs such as Cyclo (D-phenylalanyl-L-prolyl), Cyclo (Phe-Val), Cyclo (Pro-Ala), Cyclo (L-prolyl-L-valine), Cyclo (Leu-Leu), and Cyclo (Leu-Pro) present in the ethyl acetate extract of QQ *Bacillus cereus* RC1 inhibited QS in the biosensor strain and also attenuated soft rot symptoms caused by *Lelliottia amnigena*. These previous studies support the results obtained and suggest that the QQ compounds present in QQE were the cyclic dipeptides L,L-Cyclo (leucylprolyl) and Cyclo (L-Phe-L-Pro), which inhibited QS-regulated violacein production in CV026.

Literature suggests that these DKPs can act as AHL antagonists and compete for the same binding site on the LuxR receptor protein (Holden et al., 1999). For example, the DKP 2,5-piperazinedione reduced the expression of QS-regulated traits in *Pseudomonas aeruginosa* PAO1 by interfering with the binding of the AHL signal 3-oxo-C12-HSL to its receptor protein (Musthafa et al., 2012). Cyclo (Trp-Ser), found in the ethyl acetate extract of *Rheinheimera aquimaris* QSI02, isolated from the Yellow Sea in Qingdao, China, inhibited violacein production in CV026, biofilm formation, and other QS-regulated traits in *P. aeruginosa* PAO1. Molecular docking and molecular dynamics simulation studies suggested that Cyclo (Trp-Ser) works as a competitive inhibitor for the binding of AHL to the CviR receptor protein in CV026. In *P. aeruginosa* PAO1, it appears to affect the stability of the LasR receptor protein, leading to the inhibition of the QS-regulated phenotype (Sun et al., 2016). Wang et al. (2022) also reported that chemically synthesized cyclic dipeptides inhibited QS in CV026 and PAO1. In silico analyses suggest that they bind the QS receptor's active site, inducing conformational changes that downregulate QS-regulated virulence factor expression.

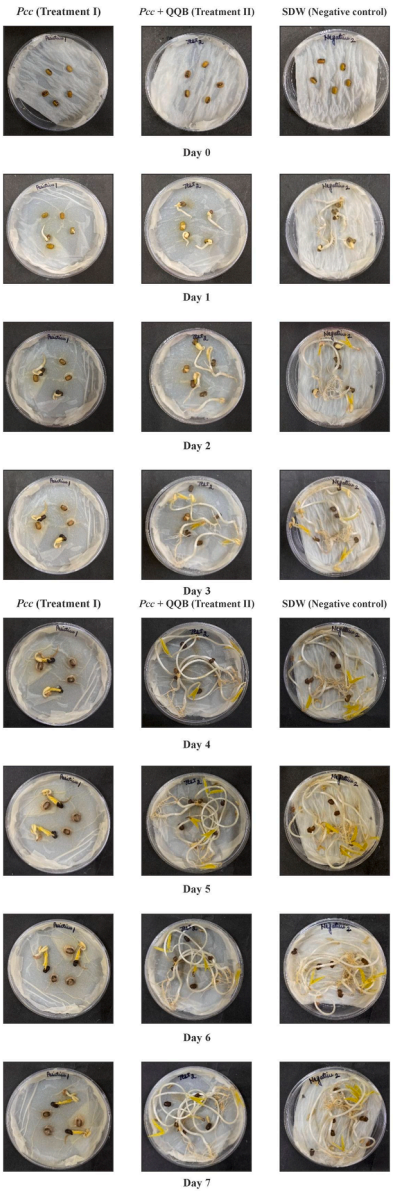


Fig. 8. Biocontrol activity in plants using plate assay in *Vigna radiata* at germination level.

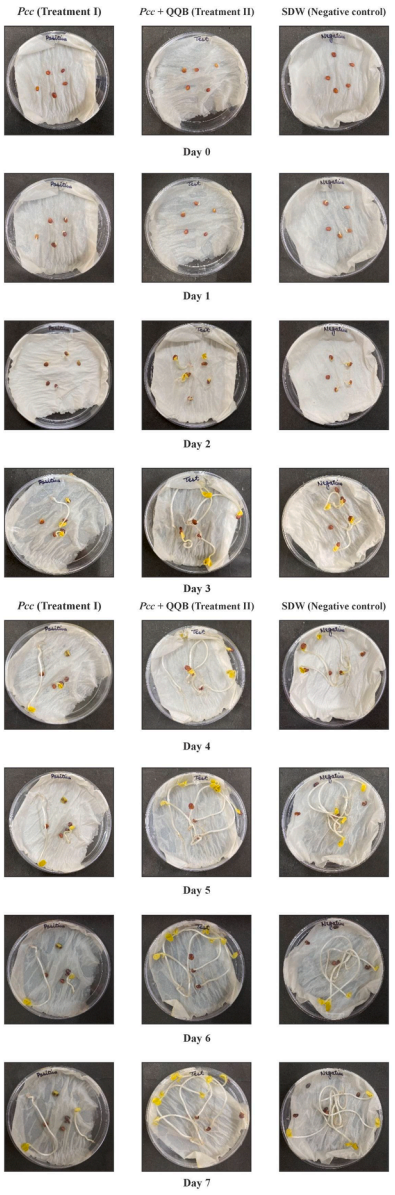


Fig. 9. Biocontrol activity in plants using plate assay in *Raphanus sativus* at germination level.

3.5. Role of QQB in attenuation of QS-regulated virulence of PCC

3.5.1. Soft rot attenuation assay (Experiment I)

The biocontrol activity of QQB against soft rot caused by *Pcc* was assessed in various vegetables: potato, radish, carrot, cucumber, and cabbage. When inoculated with *Pcc* alone (treatment I), significant maceration was observed, indicating *Pcc's* pathogenic ability (Fig. 5). This maceration is due to enzymes produced by *Pcc* that decompose plant cell walls (Lee et al., 2013). However, co-inoculation with *Pcc* and QQB (treatment II) significantly reduced maceration diameter (Fig. 6) and percentage (Fig. 7). The highest maceration was observed in radish slices for treatment I, with a diameter of 25.33 ± 3.52 mm and a percentage of 46.14 ± 5.70 %. With treatment II, the maceration was significantly reduced to 2.0 ± 1.0 mm and 0.31 ± 0.11 %, respectively. Similar reductions were observed in cucumber (from 25.71 ± 3.02 % to 3.07 ± 0.82 %) and carrot slices (from 14.43 ± 3.22 % to 2.71 ± 0.01 %). For potato slices, maceration diameter (10.33 ± 1.53 mm) and percentage (7.95 ± 1.49 %) were lesser, but it was significantly reduced when subjected to treatment II. For cabbage, treatment I exhibited the least maceration (2.67 ± 1.15 mm), which was further reduced with treatment II.

Independent *t*-test comparisons revealed that the differences observed in the soft rot attenuation assay in terms of maceration diameter and maceration percentage between treatment I and treatment II were highly significant in potato, radish, and cucumber slices ($p \leq 0.001$). For cabbage, the differences in maceration diameter and percentage were significant ($p = 0.016$ and $p = 0.006$, respectively). In the case of carrot slices, the reduction in maceration diameter was highly significant ($p = 0.000$), and the reduction in maceration percentage was also significant ($p = 0.003$).

The results suggest that the QQ activity of the endophytic QQB is responsible for the observed reduction in quorum sensing (QS)-regulated traits of *Pcc*. The ExpI/ExpR QS system in *Pcc*, homologous to the LuxI/LuxR system, produces two AHL signals, 3-oxo-C8-HSL and 3-oxo-C6-HSL. These signals regulate the production of virulence factors such as cellulases, pectinases, phospholipases, xylanases, and proteases, which contribute to the maceration observed in vegetable slices inoculated with *Pcc* (Lee et al., 2013). The QQB isolated in this study interferes

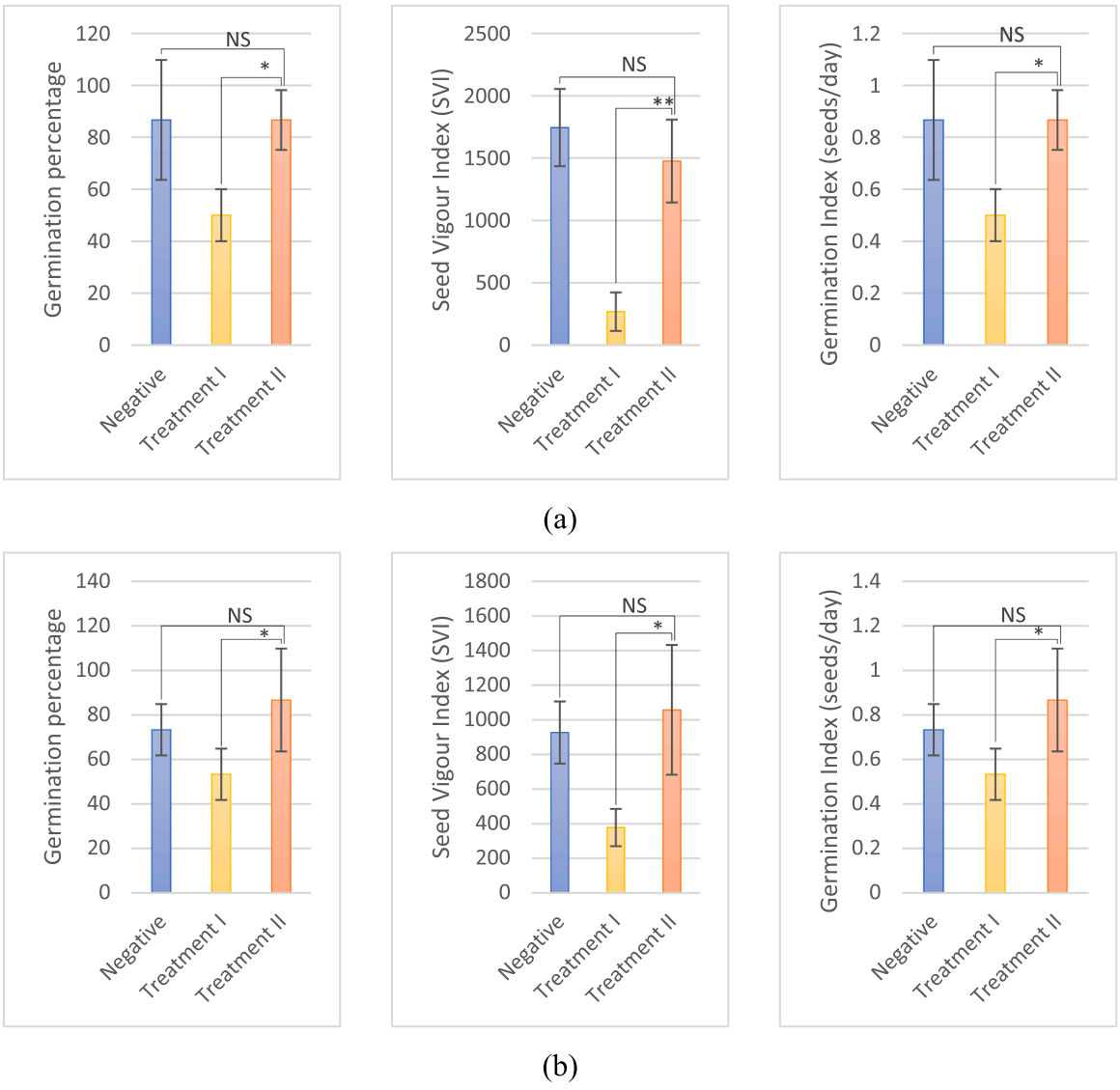


Fig. 10. Effect of biocontrol activity in plants using plate assay on seed germination parameters (a) *Vigna radiata* and (b) *Raphanus sativus*.

with this QS mechanism, thereby achieving biocontrol against *Pcc*.

Supporting this, Garge and Nerurkar (2017) reported that inoculating *Pcc*BR1 alone caused severe host tissue maceration, that was significantly reduced when *Pcc*BR1 was co-inoculated with *Bacillus* isolates on vegetable slices. Similarly, a combined application of *Acinetobacter* sp. strain XN-10 and *Pcc* Z3–3 reduced maceration in potato (from 35.3 % to 6.3 %), carrot (from 31.3 % to 9.0 %), and Chinese cabbage (from 52.0 % to 1.7 %), with AHL hydrolysis and dihydroxylation contributing to the QQ activity (Zhang et al., 2020). Another study revealed 100 % inhibition of *Pcc* Z3–3 in radish slices and a substantial reduction in maceration in potato slices in the presence of QQ *Ochrobactrum intermedium* D-2 (Fan et al., 2020). Furthermore, *Pseudomonas segetis* strain P6, isolated from the rhizosphere of *Salicornia europaea*, reduced soft rot in potatoes and carrots caused by *Dickeya solani, Pectobacterium atrosepticum*, and *Pcc*. Additionally, P6 increased the height and weight of tomato plants, indicating its plant growth-promoting activities (Rodríguez et al., 2020). Previous studies (Holden *et al.*, 1999; Sun *et al.*, 2016; Wang *et al.*, 2022) suggest that DKPs bind to receptors, competitively inhibiting the binding of AHL molecules and subsequently reducing the expression of QS genes. This leads to the speculation that in treatment II, the AHL signals produced by *Pcc* were unable to bind to their receptors due to competitive inhibition by DKPs produced by QQB, resulting in decreased virulence of *Pcc*, as evidenced by reduced maceration. The results are also supported by other studies that reported that DKPs such as Cyclo (L-prolyl-L-valine), Cyclo (Pro-Leu), and Cyclo (D-phenylalanyl-L-prolyl) from *Pseudomonas aeruginosa* RKC1 have been demonstrated to reduce biofilm formation in the soft rot pathogen *Lelliottia amnigena* RCE, and inhibit maceration in carrot, potato, and cucumber slices (Kapadia *et al.*, 2022).

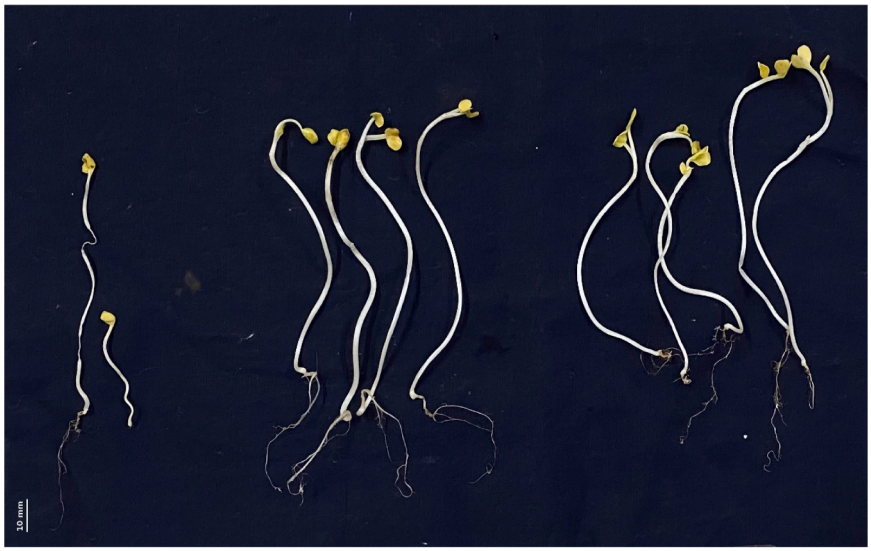
3.5.2. *Biocontrol activity in plants using plate assay (Experiment II)*

The biocontrol activity of QQB against *Pcc* on seed germination and plant growth parameters was evaluated using surface-sterilised seeds of *Vigna radiata* (Fig. 8) and *Raphanus sativus* (Fig. 9). Germination percentage significantly decreased when seeds were inoculated with *Pcc* (treatment I), dropping to 50.0 ± 10.0 % for *V. radiata* and 53.33 ± 11.55 % for *R. sativus*. However, with the combined inoculation of QQB and *Pcc* (treatment II), germination percentages increased to 86.67 ± 11.55 % for *V. radiata* and 86.67 ± 23.09 % for *R. sativus*, both statistically significant improvements ($p \leq 0.05$). Similar germination rates were observed when seeds were treated with sterile distilled water (negative control) (Fig. 10).

A significant improvement was also noted in the seed vigour index. For *V. radiata*, the index increased from 268.0 ± 154.0 (treatment I) to

Pcc (Treatment I) | SDW (Negative control) | *Pcc* + QQB (Treatment II)

(a)



Pcc (Treatment I) | SDW (Negative control) | *Pcc* + QQB (Treatment II)

(b)

Fig. 11. Growth of plant in biocontrol plate assay (a) *Vigna radiata* (b) *Raphanus sativus*.

1476.0 ± 331.95 (treatment II) ($p = 0.002$). In *R. sativus*, it rose from 378.0 ± 108.0 to 1057.33 ± 374.47 ($p = 0.015$). The germination index improved from 0.50 ± 0.10 (treatment I) to 0.87 ± 0.12 seeds/day (treatment II) for *V. radiata* and from 0.53 ± 0.12 to 0.87 ± 0.23 seeds/day for *R. sativus*. These enhancements in all three parameters were comparable to or exceeded those of the control (Fig. 10).

In addition to germination parameters, plant growth parameters were measured seven days post-treatment (Fig. 11). For *V. radiata*, root length, shoot length, and root number decreased with treatment I to 5.75 ± 0.75 mm, 16.0 ± 6.0 mm, and 1.5 ± 0.50, respectively. However, these parameters significantly increased with treatment II to 48.97 ± 8.99 mm (root length), 144.70 ± 3.10 mm (shoot length), and 9.07 ± 0.12 (root number), all highly significant ($p \leq 0.001$). Similarly, for *R. sativus*, treatment I reduced root length to 5.37 ± 1.01 mm, shoot length to 65.07 ± 8.43 mm, and root number to 1.0 ± 0.0. In contrast, treatment II significantly increased these values to 25.0 ± 5.01 mm (root length), 94.93 ± 12.19 mm (shoot length), and 3.02 ± 0.51 (root number), with increases in root length and root number being highly significant ($p = 0.001$) and the increase in shoot length being significant ($p = 0.007$) (Fig. 12).

These results suggest that QQB significantly improves both germination and plant growth parameters, demonstrating biocontrol activity against *Pcc*. Similar findings were reported by Garge and Nerurkar (2017), who observed increased germination percentage and enhanced plant growth parameters in *V. radiata* seeds when treated with a combination of biocontrol *Bacillus* species (As30, Gs42, and Gs52) and

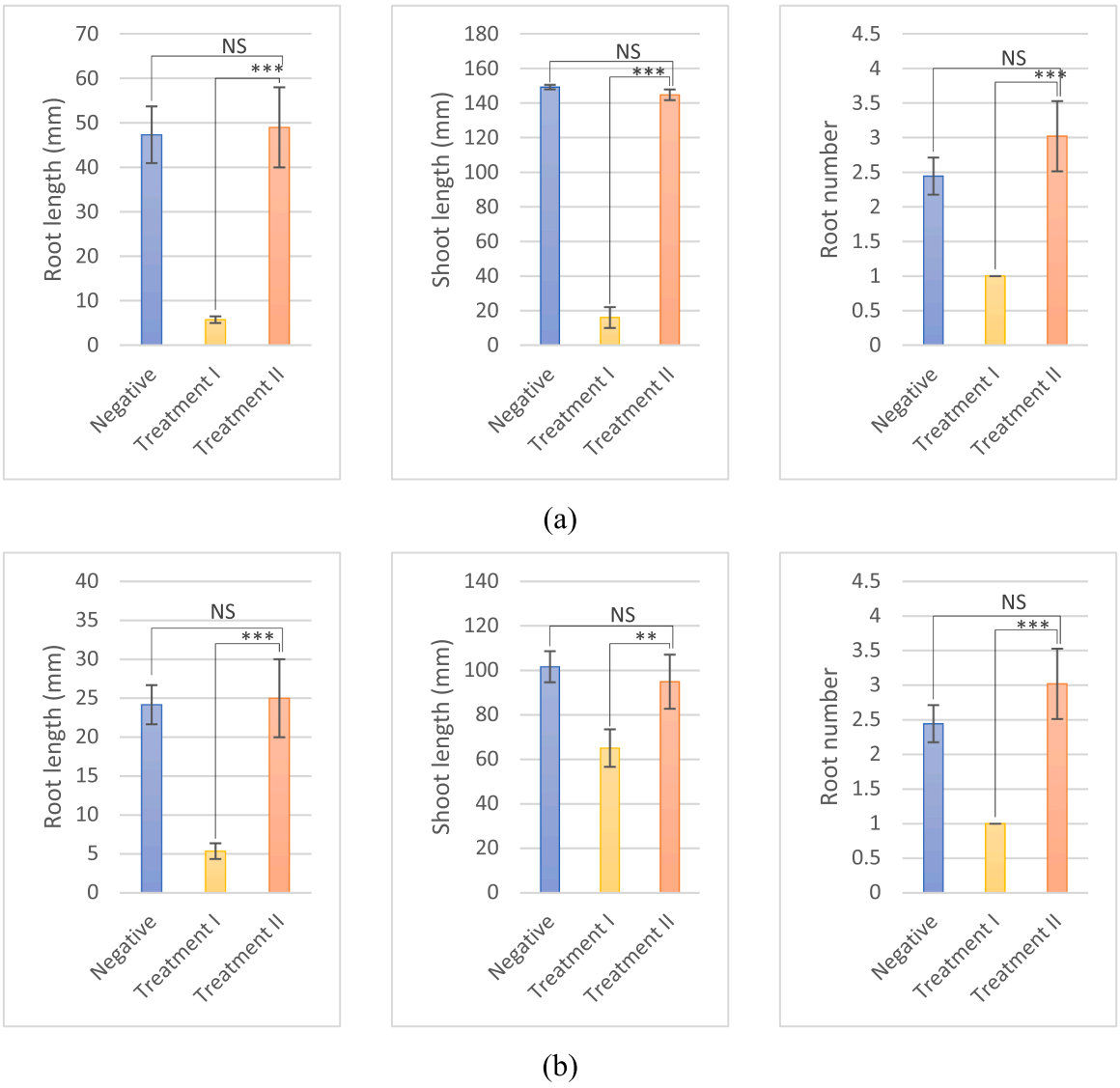


Fig. 12. Effect of biocontrol activity in plants using plate assay on plant growth parameters of (a) *Vigna radiata* and (b) *Raphanus sativus*.

PccBR1. This combination resulted in increased mean root and shoot length and weight.

3.5.3. Biocontrol activity in plants using pot assay (Experiment III)

In Experiment III, the biocontrol efficacy of QQB against *Pcc* was evaluated in greenhouse conditions using *V. radiata* and *R. sativus*. Plants were subjected to two treatments for 30 days, after which growth parameters were measured. Seeds treated solely with *Pcc* (treatment I) exhibited significant reductions in all measured growth parameters, demonstrating the detrimental effects of the pathogen (Fig. 13). Specifically, in *V. radiata*, root length, shoot length, and root number decreased by 64.67 %, 75.42 %, and 74.67 %, respectively, while in *R. sativus*, these reductions were 59.88 %, 65.42 %, and 51.92 %.

In contrast, when a combination of *Pcc* + QQB (treatment II) was applied, negative impact of the pathogen was significantly mitigated. In *V. radiata*, root length increased from 13.33 ± 4.16 mm in treatment I to 28.27 ± 9.47 mm in treatment II, representing a 112.07 % recovery. Similarly, *R. sativus* showed an increase in root length from 10.5 ± 0.87 to 25.07 ± 0.51 mm, a 138.76 % improvement. Shoot length also improved markedly, with increases of 315.65 % in *V. radiata* and 163.63 % in *R. sativus*. Specifically, shoot length in *V. radiata* increased from 38.27 ± 1.92 mm (treatment I) to 159.07 ± 1.62 mm (treatment II), while in *R. sativus*, it increased from 53.17 ± 4.19 mm (treatment I) to 140.17 ± 2.20 mm (treatment II). Root number, which had been reduced by treatment I, also significantly increased in both *V. radiata* (4.39 ± 0.34) and *R. sativus* (2.19 ± 0.36) following treatment II, corresponding to increases of 230.07 % and 119 %, respectively. These findings suggest that QQB attenuates *Pcc* quorum sensing signals, thereby promoting plant growth (Fig. 14).

Correlation analysis revealed a strong positive relationship between growth parameters in the control, indicating balanced, healthy plant growth under normal conditions. In *V. radiata*, treatment I disrupted this balance, resulting in a weak positive correlation between shoot and root length ($r = 0.383$) and between shoot length and root number ($r = 0.150$). However, the strong positive correlation between root length and root number ($r = 0.971$) suggested that root growth parameters were more resilient to *Pcc*-induced stress. In treatment II, these correlations improved: shoot vs. root length ($r = 0.518$), root length vs. root number ($r = 0.703$), and shoot length vs. root number ($r = 0.973$), suggesting that QQB alleviated the negative effects of *Pcc* and restored the relationship between growth parameters nearly to control levels. In *R. sativus, Pcc* treatment (treatment I) resulted in a moderately negative correlation between shoot length and root length ($r = -0.447$), indicating disruption in balanced growth. *Pcc* + QQB treatment (treatment

SDW (Negative control) | *Pcc* + QQB (Treatment II) | *Pcc* (Treatment I)

(a)

Pcc (Treatment I) | *Pcc* + QQB (Treatment II) | SDW (Negative control)

(b)

Fig. 13. Growth of plant in biocontrol pot assay (a) *Vigna radiata* (b) *Raphanus sativus*.

II) restored strong positive correlations: shoot vs. root length (r = 0.762), shoot length vs. root number (r = 0.772), and root length vs. root number (r = 1.00). This indicates that QQB restored normal growth relationships that had been disrupted by *Pcc*.

Post hoc comparisons using Tukey's B test revealed that the difference in shoot length between treatments I and II was highly significant (p = 0.000) in both plants. The difference in root length was significant in *V. radiata* (p = 0.024) and highly significant in *R. sativus* (p = 0.000). Root number differences were highly significant in *V. radiata* (p = 0.000) and significant in *R. sativus* (p = 0.002). ANOVA results from both Experiments II and III indicated that differences between treatments I and II were statistically significant for all measured parameters.

A similar *in planta* study against blackleg disease caused by PccBR1 in cucumber showed increased root and shoot lengths in germinated seedlings coated with a combination of the pathogen and actinobacteria before being planted in pots (Vesuna and Nerurkar, 2020).

These findings demonstrate that *Pcc* infection severely reduced plant growth, particularly root and shoot lengths, with decreases exceeding 50 %. However, the combination of *Pcc* and QQB significantly mitigated these effects, with *V. radiata* shoot length surpassing control levels. This highlights the role of QQB in protecting plants from *Pcc*-induced damage, positioning it as a promising biocontrol agent for agricultural use. The potential of QQB to produce diketopiperazines (DKPs) may contribute to these protective effects by disrupting the pathogenic effects of *Pcc*, reinforcing the overall efficacy of QQB in enhancing plant health.

4. Conclusion

This study presents evidence for the potential of the endophytic bacterium *Bacillus* sp. EBS9, isolated from *Tecomella undulata*, as an effective biocontrol agent against *Pcc*. The bacterium exhibits significant QQ activity, which disrupts QS signals within *Pcc*, thereby attenuating its virulence. This attenuation was demonstrated across both in vitro assays (including soft rot reduction and plate assays) and *in planta* experiments, where the bacterium notably interfered with the pathogen's QS-dependent mechanisms. Furthermore, the QQ activity exhibited by *Bacillus* sp. EBS9 is likely attributed to the production of diketopiperazines (DKPs). These results underscore the role of EBS9 in disrupting pathogenic communication and coordination, offering a holistic approach to disease management. By disrupting QS in target phytopathogens, these bacteria also provide an eco-friendly alternative to traditional chemical controls. However, the research highlights the necessity of further large-scale trials to assess the bacterium's impact on non-target microbial communities. Additionally, it is essential to confirm its safety and efficacy as a biocontrol agent across diverse agricultural settings.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT4.0 in order to improve language and readability. After using this tool/

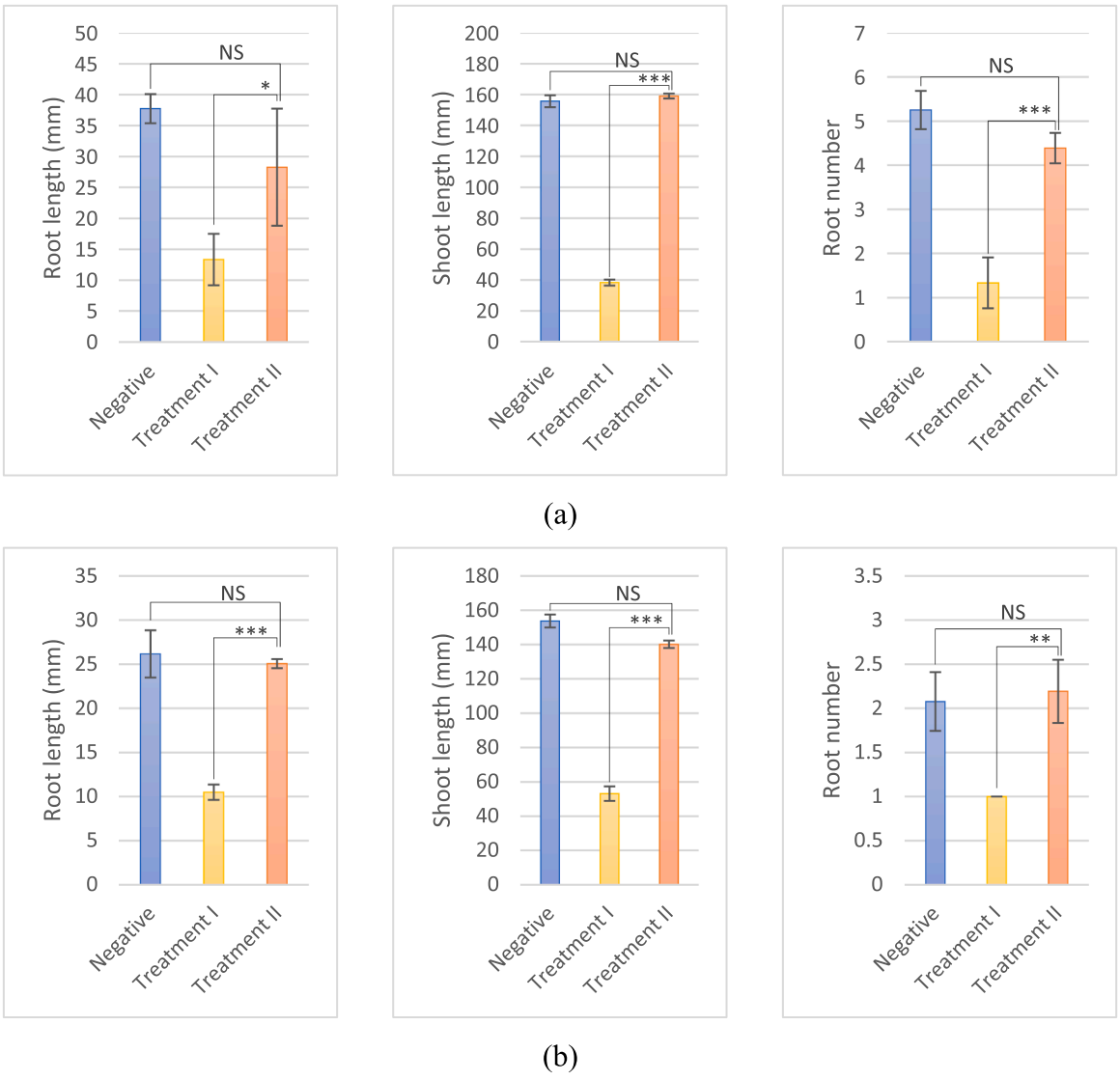


Fig. 14. Effect of biocontrol activity in plants using pot assay on plant growth parameters of (a) *Vigna radiata* and (b) *Raphanus sativus*.

service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

CRediT authorship contribution statement

Etisha Paul: Investigation, Writing – original draft. **Charu Sharma:** Conceptualization, Methodology, Data curation, Formal analysis, Supervision, Writing – original draft, Writing – review & editing. **Payal Chaturvedi:** Methodology, Data curation, Formal analysis, Supervision, Validation, Writing – review & editing. **Pradeep Bhatnagar:** Conceptualization, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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