PLANT SCIENCE TODAY
ISSN 2348-1900 (online)
Vol 11(sp3): 124-136
https://doi.org/10.14719/pst.4288

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RESEARCH ARTICLE

Biological management of rhizome rot in ginger (*Zingiber officinale*) plants and stored ginger seeds

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OPEN ACCESS

ARTICLE HISTORY

Received: 09 August 2024
Accepted: 15 November 2024

Available online
Version 1.0 : 25 December 2024
Version 2.0 : 15 January 2025

Check for updates

Additional information

Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

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Indexing: Plant Science Today, published by Horizon e-Publishing Group, is covered by Scopus, Web of Science, BIOSIS Previews, Clarivate Analytics, NAAS, UGC Care, etc
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Nandana M S, Anith K N. Biological management of rhizome rot in ginger (*Zingiber officinale*) plants and stored ginger seeds . Plant Science Today. 2024; 11(sp3): 124-136. https://doi.org/10.14719/pst.4288

Abstract

Two endospore-forming endophytic bacteria (EEB1 A8 and EEB2 B13) were isolated from ginger rhizome collected from the fields of College of Agriculture, Vellayani and identified as *Bacillus* spp. Their antagonistic potential against *Pythium myriotylum*, the soft rot pathogen of ginger, was analyzed *in vitro* by dual culture plate assay and agar well diffusion method. They tested positive for biocontrol traits such as the production of hydrogen cyanide, siderophore and volatile organic compounds. The bacterial isolates were applied to ginger plants to test biocontrol efficiency against *P. myriotylum*, individually and in combination with *Piriformospora indica*, a fungal root endophyte capable of promoting plant growth and enhancing plant defense. A lesser percent disease index (PDI) was observed in plants where the combination of EEB1 A8 and *P. indica* was applied. The activity of enzymes pertinent to protective mechanisms against pathogens, like peroxidase, phenylalanine ammonia-lyase, super oxide dismutase (SOD) and polyphenol oxidase (PPO) in the plants were analyzed before challenging, 1st, 3rd and 5th day after challenging with pathogen. The values followed the same trend in all treatments as it increased after inoculation up to 3rd day and then decreased for peroxidase (PO) and SOD. The plants treated with EEB1 A8 and combination of EEB1 A8 and *P. indica* showed the highest values for the level of defense enzyme production. In a storage study, when ginger seed rhizomes were treated with individual bacterial isolates and as a consortium of both, followed by inoculation with the pathogen *P. myriotylum*, rhizomes treated with consortium were more tolerant to rhizome rot. Applying endospore-forming bacteria with biocontrol properties is a propitious method for controlling rhizome rot in ginger, in the field and during seed storage.

Keywords

biological control; endospore-forming endophytes; rhizome bacterization; soft rot

Introduction

Ginger (*Zingiber officinale*), under Zingiberaceae family, is a widely used rhizomatous spice in alternative medicine systems worldwide. Ginger exhibits its remarkable versatility in culinary applications. Whether incorporated into soups, salads, dressings, or baked goods, ginger imparts a warm and pungent flavour. The crop is susceptible to several diseases, including leaf spot, bacterial wilt, rhizome rot and storage rot (1). Among these, rhizome rot is the most devastating one, resulting in an estimated yield loss of more than 20%. This disease is brought on by oomycete, *Pythium* spp., which can exist in the soil for longer periods, leading to significant crop losses if

infection occurs during the incipient phase of plant growth and seed storage. *P. aphanidermatum* is the most pervasive species responsible for rhizome rot, while *P. myriotylum* is a fast-spreading species in the field (2).

Interest on endophytic microorganisms as biological antagonist has been extensively increasing due to their ability to produce novel bioactive molecules, that play crucial roles in managing phytopathogens. In sustainable agriculture, bacterial and fungal endophytes are commonly used as inoculants to improve crop yield and effect biocontrol (3). To unlock the promising potential of endophytes, diverse endophyte communities from various taxonomically distinct and metabolically diverse plants are to be isolated. A group of endophytic bacteria that have attracted attention is the endospore-forming ones. Under specific starvation conditions, bacteria from certain genera can produce endospores which have resistance to multiple stress factors. Previous reports have shown that endospore-forming endophytes play an effective role in alleviating both abiotic and biotic stress conditions in plants (4, 5).

Piriformospora indica is a widely recognized fungal root endophyte, for its benefits such as growth promotion and the defensive mechanisms against pathogens in vast spectrum of host plants (6). It enhances the growth of various crops, including agricultural, plantation and medicinal species by establishing a symbiotic relationship. The positive impacts of *P. indica* observed in plants encompass enhanced plant growth, elevated biomass production, boosted secondary metabolite synthesis, and upgraded nutritional quality (6-9). It plays a protective role against bacterial, viral and fungal pathogens, contributing to the appropriate vegetative and reproductive development of tomato plants (10, 11). *P. indica* colonization in black pepper root system improved growth in tissue cultured plantlets on inoculation with *P. indica* (12). Earliness in flowering, yield enhancement and improved piperine composition in the berries of black pepper plants on inoculation with *P. indica* have been reported (13).

Various studies have reported that co-culturing of microorganisms resulted in advantages like production of metabolites that can be used as drugs. *Bacillus pumilis* and fungal root endophyte *P. indica*, when grown together in a co-culturing system, in coconut water had enhanced development of tomato seedling compared to application of individual organism and mixed inoculation of separately grown bacteria and fungi. It was also noticed that the root colonization arrangement of *P. indica* in plants treated with co-cultured inoculum was different from other treatments (14, 15). This paves a way to combine beneficial bacteria and fungi as co-inoculants.

Microbial communities within rhizomes of diverse plant species, including those of medicinal significance, remain relatively unexamined. This study reports the profiling of the endospore forming endophytic bacteria associated with ginger and their biocontrol potential against the phytopathogen *P. myriotylum*. Evaluation of their antagonistic activities against *P. myriotylum* were done by *in vitro* experiments, *in vivo,* by a pot culture experiment and in stored ginger seeds. Efficiency as biocontrol agents along with *P. indica* in soft rot disease suppression also were studied by co-inoculation of the bioagents.

Materials and Methods

Isolation of endophytic bacteria producing endospore from ginger rhizomes

Bacterial isolation was performed from the endorhizosphere of local varieties and wild relatives of ginger by following the two-step enrichment process described earlier (4). The surface of ginger rhizomes (5 g) was sterilized with 4% sodium hypochlorite and then washed in sterile water and treated with ethanol (70% for 2 min.). The surface sanitized rhizomes were again washed in sterile water thrice and the last wash was collected in sterilized vials and 0.1 mL of it was taken and directly spread plated in nutrient agar (NA) plates and held for 2 days for sterility check. The absence of bacterial colonies on plates and turbidity in vials suggest adequate surface sterilization of the rhizomes. The samples were then exposed to heated air for drying in an oven at 35 °C for 2 days (the first enrichment step). Subsequently, the rhizomes were crushed in a sterile mortar in phosphate saline buffer (PSB), and the suspension collected in sterile vials. The sap was then subjected to a second enrichment step by incubating it in a water bath at 75 °C for 10 min. The content was serially diluted followed by spread-plating on nutrient agar plates. Colonies from the plates were picked and pure cultured after 2 days. Colony characters (form, colour, elevation and margin) of bacteria were observed and same were classified together. After sub-culturing, the isolated organisms were stored on nutrient agar slants in refrigerator and in 25% glycerol at -80 °C.

Characterization of endospore-forming endophytic bacterial isolates

After a preliminary screening for antagonistic properties against *P. myriotylum,* two endospore-forming bacterial isolates from ginger rhizomes were biochemically characterized by various tests, using ready-made HiMedia© Biochemical Test Kit. Carbohydrate utilization patterns were also assessed for various sugars. The isolates were preliminarily identified at the genus level based on the results of the biochemical tests, Bergey's Manual of Determinative Bacteriology. Isolates obtained were subjected to Schaeffer-Fulton staining and observed under a light microscope for the presence of endospores (4).

The 16S rRNA based molecular identification of isolates was performed using universal primers: 16S-RS-F (forward: 5'-CAGGCCTAACACATGCAAGTC-3') and 16S-RS-R (reverse: 5'-GGGCGGWGTGTACAAGGC-3') available at Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram, Kerala. BioEdit program was used for aligning contig sequence and the organism was identified from the BLAST search output (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Phytopathogen and culture conditions

Ginger rhizomes were sampled based on the symptoms like yellow colour of leaves from tip downward through margins and water-soaked symptoms in the collar region of the pseudostem, from farm of College of Agriculture, Vellayani. Isolates were acquired from rhizomes by following the method described earlier (16). Rhizomes with rot symptom were surface sterilized with sodium hypochlorite (4%), cut into sections of approximately 5 mm^2, washed twice with sterile water and blot dried. The pieces were transferred on to potato dextrose agar (PDA) plates added with 50 µg/mL Penicillin and incubated overnight at 28 °C in dark. Hyphal tips from the growing edge were transferred to fresh medium for pure culture. Morphological and microscopic characteristics of the pathogen were studied by staining it with lactophenol cotton blue and observing under a compound microscope. To verify its pathogenicity, ginger rhizomes were given pinprick injuries that serve as infection ports and inoculated with spore suspension and once an infection had been established, the pathogen was re-isolated on PDA plates. A suspension containing spores was prepared from one-week-old PDA cultures maintained at 28 °C in dark. The number of spores in the solution was quantified with a haemocytometer and the concentration was revised with sterile water to 1×10^6 spores/mL before use. Molecular identification was done by sequence similarity obtained using internal transcribed spacer region (ITS) sequence-based technique available at RGCB, Thiruvananthapuram. The identity of fungal pathogen was determined from the BLAST search output.

In vitro antagonism of bacterial isolates against *Pythium myriotylum*

The selected isolates were evaluated for their ability in restraining the growth of *P. myriotylum* on by dual culture plate assay in PDA medium (17). To initiate the assay, the fungal pathogen was cultivated on PDA plates for 3 days, and mycelial discs were cut out from the advancing growing region of the fungal colony. These discs were then positioned at the centre of PDA plates. On both sides of the mycelial disc, bacterial streaks of 1.5 cm were made at an extent of 3 cm from the centre. The plates were maintained at a temperature of 28 ± 2 °C. The inhibition zone (mm) was measured from the test plates containing *P. myriotylum* after 72 h.

The inhibitory effect of the bacterial bioagent's culture filtrate against *P. myriotylum* was evaluated by agar well diffusion method (11, 18). Bacterial cells were picked from pure culture of the isolates and dispensed to 100 mL nutrient broth and kept 24 h in an incubator with shaker at 28 °C with a steady shaking at 100 rpm, and 10 mL from it was centrifuged at 4500 rpm for 15 min, resulting in the collection of aseptically obtained supernatant containing secreted compounds and substances produced by the bacterial bioagents. To ensure further sterility, the supernatant underwent filter sterilization using a nitrocellulose bacteriological filter of 0.2 µm. The resulting filtrate, devoid of bacterial cells, was retrieved aseptically and preserved at 4 °C for future use.

PDA plates with mycelial plug of *P. myriotylum* positioned at the centre were incubated at 28 °C for one day. Wells of 8 mm diameter were carved out at the opposing sides of the plate after incubation. 100 µL of 1% molten agar was poured in the well up to halfway for preventing the leakage of culture filtrate. After the solidification of agar, 100 µL of the culture filtrate of each isolate was infused to the wells in respective plates with four replications. Plates were incubated for 48 h at 28 °C. The inhibition zone of the pathogen's mycelial growth surrounding the well was recorded.

Evaluation of biocontrol traits of endophytic bacterial isolates

Detection of hydrogen cyanide production

Hydrogen cyanide (HCN) generation by the endospore-forming bacterial isolates were assessed using the procedure described earlier (19). Each isolate was inoculated by streaking on nutrient agar medium and nutrient broth, modified by adding glycine (4.4 g/L). Sterilized filter paper disc dipped in picric acid solution (in 2% sodium carbonate aqueous solution) was positioned at the inner part of the lid of the plate and maintained at 28 °C for 4 days. Brown colour production in filter paper denoted HCN generation. A positive control inoculated with *P. fluorescens* strain PN026 was also kept.

Siderophore production

For the estimation of siderophore production modified method of Schwyn and Neilands was used (20). The required dye was prepared by integrating three reagents. The first solution was 0.12% chrome azurol sulfonate, the second formulated by dispensing $FeCl_3$-$6H_2O$ (0.0027 g) in 10 mM HCl (10 mL) and the third with 0.18% HDTMA solution. For the preparation of the blue dye, 1 mL of CAS solution and 9 mL of $FeCl_3$-$6H_2O$ solution were combined. The CAS-$FeCl_3$ solution was mixed with 40 mL HDTMA solution.

For CAS agar preparation, 100 mL of MM9 salt solution (25 g NaCl, 50 g NH_4Cl and 15 g KH_2PO_4, in 500 mL of ddH_2O) was poured to 750 mL of ddH_20 and 32.24 g PIPES buffer was dissolved in it. This turned the solution green. The solution was autoclaved after adding 15 g agar. At the time of plating, 10 mL of 20% glucose (sterilized) solution and sterilized Casamino acid (30 mL) were dispensed to the MM9/PIPES formulation. Finally, Blue Dye (100 mL) was steadily poured with ample stirring to blend completely and after solidification of the medium, each isolate (24 h old) was spot inoculated and incubated at 30 °C for 24 to 72 h. The development of a zone with orange halo surrounding the colonies indicates positive reaction. A positive control inoculated with *P. fluorescens* strain PN026 and 3 bacterial strains that do not produce siderophore, as negative controls, were also kept.

Volatile organic compounds (VOC)

Lower part of two Petri plates were taken and 1 plate was poured with nutrient agar and the other one with PDA. The bacterial isolates were cross-streaked heavily on the nutrient agar plates and a mycelial disc of *P. myriotylum* was placed on PDA plate. The fungal plates were placed in an

inverted position above the bacterial plates. The two plates were tightly covered with cling film to prevent the escape of VOC generated by the bacteria. The VOC produced was assessed by the inhibition of radial growth of the fungal pathogen.

In vivo disease suppression

An experiment was designed in a completely randomized design (CRD) by growing ginger plants in pots to assess the biocontrol activity of endospore-forming endophytic bacteria and *P. indica* against *P. myriotylum* in ginger. Ginger rhizomes of Athira variety from Kerala Agricultural University were used in the experiment. The bacterial inoculum was prepared as described elsewhere (21). A bacterial suspension was obtained by drenching the culture plates with 10 mL sterilized distilled water under aseptic conditions. Cell numbers in the suspension of bacteria used for seed rhizome treatment were fixed to 10^8 cells per mL. Rhizomes of ginger were sterilized peripherally with 1% NaOCl solution for 4 min and after washing in sterile water, placed in the bacterial cell suspension for 4 h and then air-dried overnight in sterile airflow to ensure uniform coating to the seed rhizomes (22). Potting mixture (coir pith and vermiculate in the ratio of 1:2) was autoclaved at 121 °C for 1 h for each 3 days. Treated rhizomes were raised in the above potting mixture in protrays for 30 days and then transplanted to pots (with cow dung, soil and sand in 1:1:1 ratio). In treatments with *P. indica* application, the mycelium of the fungal endophyte, which had been cultivated for 15 days in 100 mL of PDB medium, retrieved by straining via muslin mesh cloth, was weighed and uniformly blended with the potting mixture (1% (w/v)). A second application of 10 mL endophytic bacterial suspension (fixed to 10^8 cells per mL) was done by drenching the pots 15 days after transplanting.

A blend of ground oat hulls (45 g of Quaker Oats) and sand (500 cm^3) was prepared. 300 mL of it was transferred into 500 mL flasks and autoclaved at 121 °C for 1 h, twice in 1 day interval. The medium was transferred into sterile Petri plates (90 mm) in aseptic condition to increase the surface area and inoculated with 5 mm^2 mycelial disc of *P. myriotylum* grown in PDA medium and maintained at 28 °C for 3 weeks for mycelial growth. The pathogen along with medium (5 g) was inoculated at 3-4 cm depth from the collar region of healthy ginger plants for challenge inoculation, 60 days after transplanting.

Single inoculation with the bacterial strains, *P. indica* and combination of the *P. indica* and individual endophytic bacteria were included as treatments along with an absolute control and a chemical control with copper oxychloride (0.2%) drenching prior to pathogen inoculation. The ability to suppress rhizome rot in ginger by the treatments were evaluated in terms of disease incidence (DI%) and Percentage disease index (PDI). The disease incidence was calculated in the first, second and third week after inoculation of the pathogen, using the formula (23).

$$\text{Disease incidence (DI\%)} \quad \frac{\text{Number of infected plants}}{\text{Total No. of plants}} \text{ X100}$$

The disease severity was assessed by determining the percentdisease index (% PDI) using standard score chart for rhizome rot of ginger (24). Score scale to assess the disease severity of rhizome rot of ginger is as follows:

0 indicates no symptoms; 1 represents up to 25%; 3 signifies 26-50%; 5 denotes 51-75% of tiller death; 7 indicates more than 75%, affected tiller after 25 days of inoculation and 9 signifies more than 75% tiller death within 25 days.

Based on the score chart, disease severity was calculated in the first, second and third week after inoculation with the pathogen using the formula:

$$\text{\% disease index (PDI)} = \frac{\sum \text{ of scores}}{\text{N X Maximum score}} \text{ X 100}$$

Where, N = number of plants per treatment.

Activity of defense related enzymes

Enzyme activity related to defense was were measured before and 1st, 3rd and 5th day after inoculating with the pathogen. Samples were obtained from the experiment above. Each treatment's leaf samples (500 mg) were macerated using pre-chilled pestle and mortar and homogenized in 10 mM NaH_2PO_4 buffer. After homogenization, it was centrifuged for 20 min at 12,000 rpm at 4 °C. The resulting supernatant was taken in 2 mL Eppendorf tubes and preserved at -20 °C. This supernatant served as the extract for estimating various enzymes.

Assay of peroxidase (PO)

The protocol by Rathmell and Sequeira (25) was followed. The extract (50 µL) was blended with 1 mL NaH_2PO_4 buffer (10 mM, pH 6.0), 1 mL of pyrogallol and 1 mL of 1% H_2O_2. The primary rate of increment of absorbance was recorded for 5 min at 1 min intervals at 436 nm in a spectrophotometer. PO activity was articulated as units of PO/min/g of fresh tissue.

Assay of polyphenol oxidase (PPO)

As per the protocol of Meyer and Abdallah (26), 200 µL of the extract was homogenized with 700 µL of NaH_2PO_4 buffer (pH 6.0). This mixture was incorporated with 100 µL of catechol (0.2 M). The initial change in absorbance was measured spectrophotometrically for 5 min at 420 nm. PPO activity was articulated as units of PPO/min/mg of fresh tissue.

Assay of phenylalanine ammonia lyase (PAL)

Activity of PAL was assessed as per the method of Brueske (27). After adding 500 µL of Tris hydrochloric acid buffer of pH 8.5 (0.5 M) and 500 µL of L-phenylalanine (0.15M) into 500 µL of enzyme-extract, the mixture was incubated at 37 °C for 60 min. The bio activity of enzymes was ceased by infusing 500 µL of 1 M trichloroacetic acid (TCA) and incubating it for 5 min at 40 °C for 5 min. After centrifugation the absorbance was read at 270 nm using a UV-visible spectrophotometer (SpectronicR Genesys 5). The reaction

rate was articulated as µg of trans-cinnamic acid produced per g of fresh tissue. Different concentrations of trans-cinnamic acid were used as standard.

Assay of Super oxide dismutase (SOD)

As per the method described by Beauchamp and Fridovich (28) 3 mL reaction mixture was prepared by mixing potassium phosphate buffer (50 mM), riboflavin (2 µM), methionine (13 mM), NBT (75 µM) and EDTA (0.1 mM) and it was mixed with 50 µL of enzyme extract. The spectrophotometer was calibrated with blank (without NBT and enzyme). A control containing NBT but without enzyme was considered as a reference. Double distilled water was added to blank and reference control to equalise the volume. All the samples were kept under 4, 100 W bulbs for 15 min and the absorbance was recorded at 560 nm. The unit was expressed as 1 unit of SOD activity, which means a 50% repression of the reaction between riboflavin and NBT.

Disease suppression during storage of ginger seed rhizomes

Cell suspension of individual bacterial isolates fixed to 10^8 cells per mL and a consortium with both the isolates were used for seed rhizome treatment. Rhizomes of equal weight were sprayed with the bacterial cell suspension and air dried overnight to ensure uniform coating on the seed rhizomes. Twenty-four h after drying, seed rhizomes were given pinprick injuries that serve as infection ports, and soaked in spore solution (10^6 spores/mL) of *P. myriotylum* for 4 h with a frequent shaking for maintaining the solution blended uniformly. Following air drying at 25 ºC, treated rhizomes were kept in Petri plates (150 mm diameter) with wet cotton within to uphold an elevated humidity (around 85% RH) and preserved at 28 ºC (29). Copper oxychloride (0.2%) treated rhizomes and untreated rhizome treated with pathogen were kept as chemical and pathogen control respectively. The disease severity was assessed by determining the PDI using standard score chart for rhizome rot of ginger. It was rated on a 0–4 scale for each 25% increase in affected area, where 0 indicate no symptom and 4 indicate 100% area affected.

$$\text{Percent disease index (PDI)} = \frac{\text{Sum of scores}}{\text{N X Maximum score}} \text{ X } 100$$

Where, N being the number of rhizomes per treatment.

% disease index based on the score chart was calculated considering the observation taken on 10^{th} day after spraying the spore solution of pathogen.

Results

Characterization of endospore-forming endorhizosphere bacterial isolates

Two endospore-forming endophytic bacteria were isolated from ginger rhizome on NA medium and were preliminarily identified as *Bacillus* spp. The isolates were Gram-positive and exhibited endospore production in Schaeffer-Fulton staining. Biochemical, Morphological and 16S rRNA gene sequence analysis confirmed the placement of the bacterial isolates in the genus *Bacillus* (Table 1-3, Fig. 1).

Table 1. Characteristics of endospore-forming endorhizospheric bacterial isolates from ginger.

Isolates	Cell shape and arrangement	Colony characters				Gram staining	Endospore formation
		Colour	Form	Elevation	Margin		
A8	Single rod	Cream	Curled	Raised	Undulate	G+	+
B13	Single rod	Creamy white	Circular	Umbonate	Undulate	G+	+

Table 2. Biochemical characters of endospore-forming endorhizospheric bacterial isolates from ginger.

Biochemical test	Isolates	
	A8	B13
Indole	-	-
Methyl red	+	+
Voges Proskauer''s	+	+
Citrate utilization	+	-
Glucose utilization	-	-
Adonitol utilization	-	-
Arabinose utilization	-	-
Lactose utilization	-	-
Sorbitol utilization	-	-
Mannitol utilization	-	-
Rhamnose utilization	-	-
Sucrose utilization	-	-

Phytopathogen

Molecular identification of the phytopathogen was accomplished using internal transcribed spacer region (ITS) sequence-based technique and showed relatedness of 95.82% with the species *Pythium myriotylum* (Table 4).

In vitro antagonism of bacterial isolates against P. myriotylum

Both the isolates exhibited significant zone of inhibition of 2.75 cm on the 5^{th} day after inoculation against the phytopathogen in dual culture plate assay, which indicated the direct inhibitory effect of the isolates against the phytopathogen. Same trend was observed in agar well diffusion assay in which the bacterial isolate's culture filtrate was used against a phytopathogen. A significant zone of inhibi

Table 3. BLAST search details of 16S rRNA gene sequences producing most significant alignment of sequences of endospore-forming endorhizospheric bacterial isolates from ginger.

Isolates	Max score	Total score	Query cover (%)	Percentage identity	Best match in Genbank data base	Accession no.
A8	1858	1858	81	99.83%	*Bacillus velezensis* strain FZB42	PP75851.1
B13	706	1248	87	99.07%	*Bacillus* sp. strain RGM_3184	PP75852.1

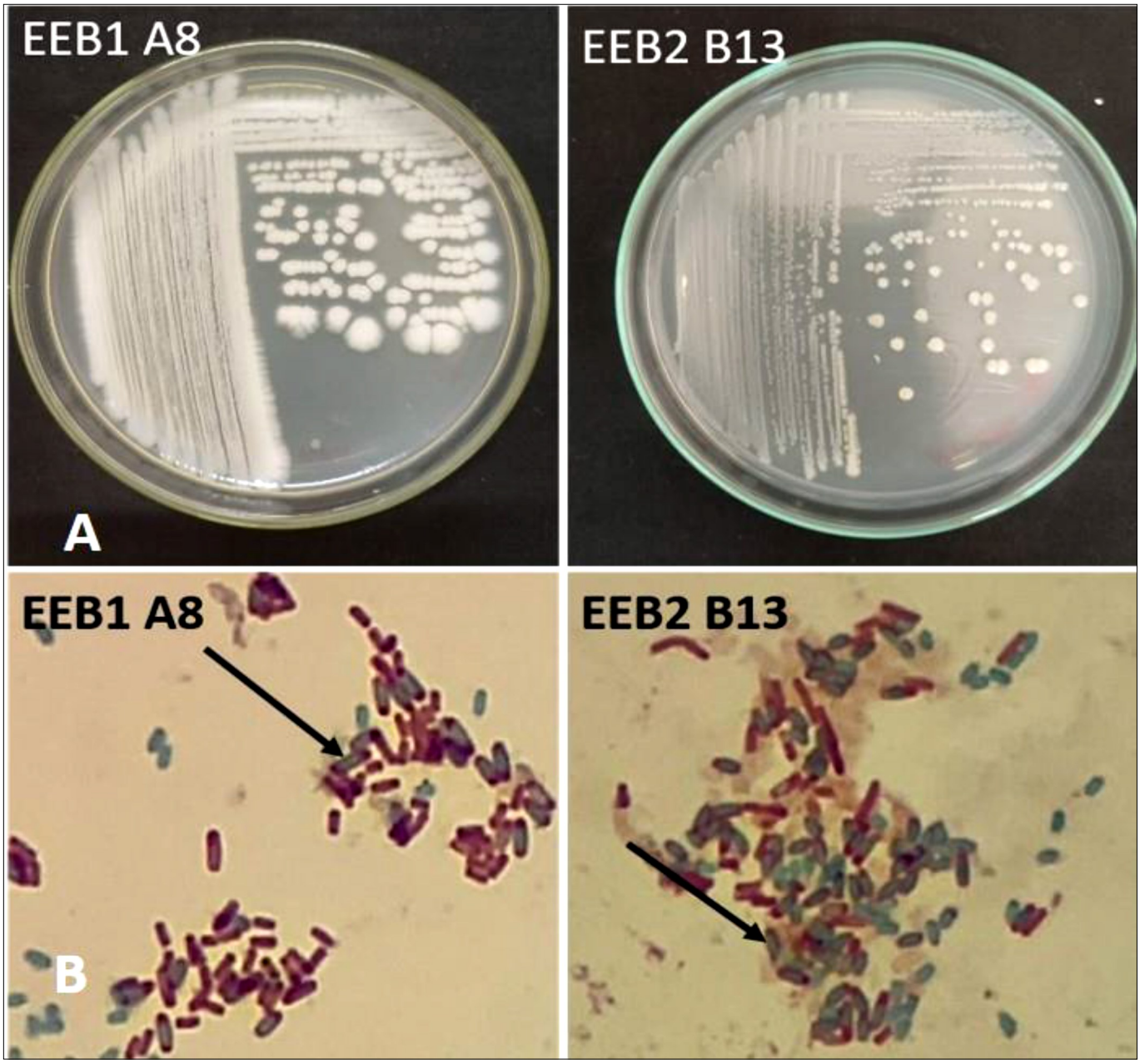

Fig. 1. Cultural and morphological characteristics of *Bacillus* isolates. **A**: (upper panel) Isolates grown on nutrient agar plate. **B**: (lower panel) Microscopic view (100X) of EEB isolates after Schaeffer-Fulton staining process. The arrows represent endospores.

Table 4. BLAST search details of internal transcribed spacer region (ITS) sequence of phytopathogen isolated from ginger.

Isolate	Max score	Total score	Query cover (%)	Percentage identity	Best match in Genbank data base	Accession no.
Pythium sp	1377	1377	100	95.82%	*Pythium myriotylum* A584	PP760336.1

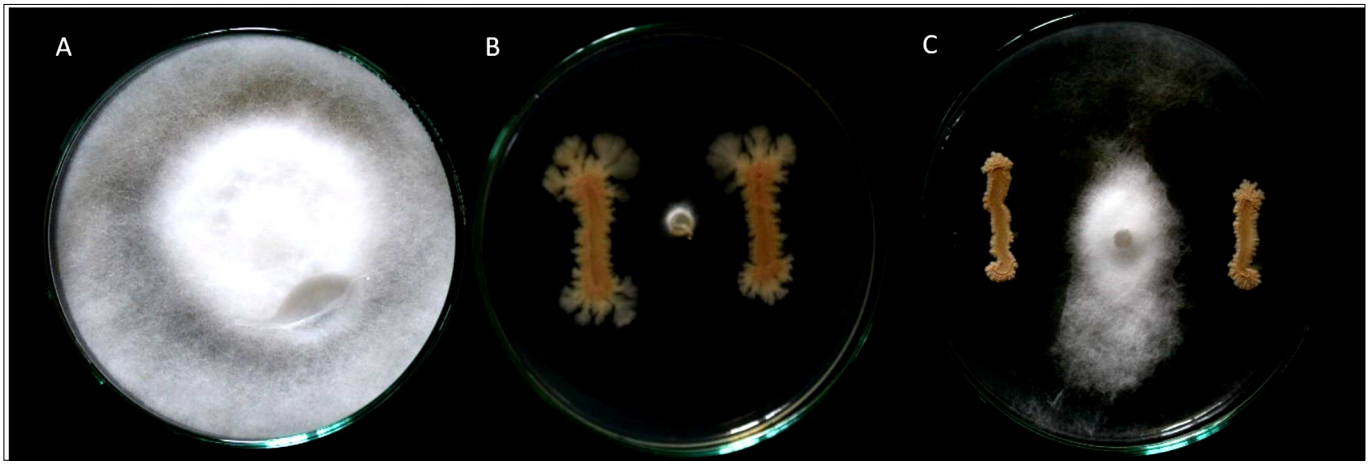

Fig. 2. Inhibition of *Bacillus* isolates against the phytopathogen *Pythium myriotylum* in dual culture plate assay. **A**: Control **B**: Isolate A8 **C**: Isolate B13.

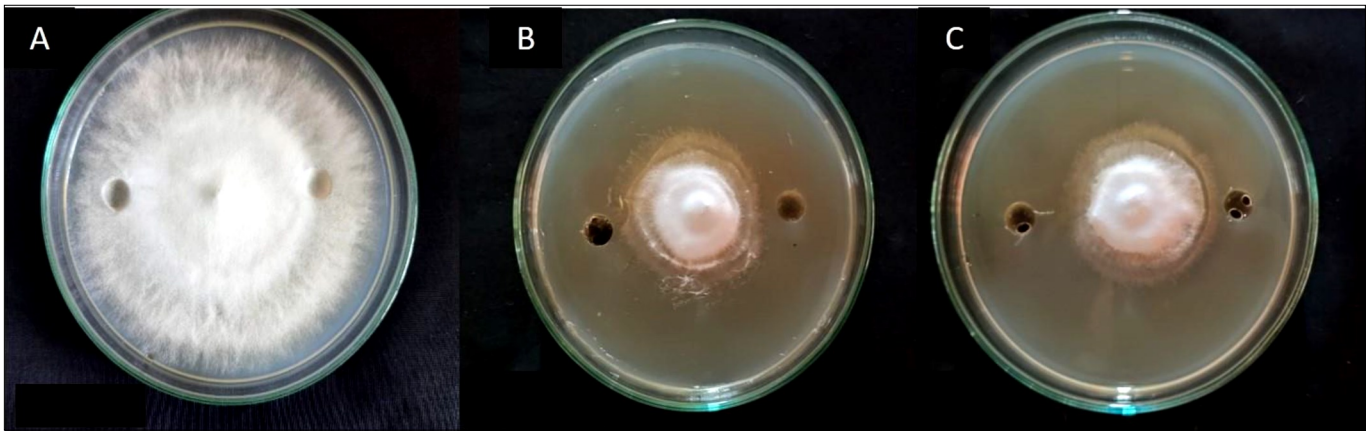

Fig. 3. Inhibition of cell free extracts of *Bacillus* isolates against the phytopathogen *Pythium myriotylum* in Agar well diffusion assay. **A**: Control **B**: Isolate A8 **C**: Isolate B13.

tion of 2.25 cm and 3.25 cm shown by EEB1 A8 and EEB2 B13 respectively, in the plates of agar well assay established the indirect antagonistic effect of the isolates against the pathogen *P. myriotylum* (Fig. 2, 3).

Biocontrol traits of endophytic bacterial isolates

The filter paper placed in NA plates and broth changed colour to brown, suggesting the production of hydrogen cyanide (HCN) by the isolates (Fig. 4). Siderophore production was positively tested for both isolates, as an orange halo formed surrounding the bacterial growth on CAS agar medium (Fig. 5). The inhibition percentage in the pathogen's radial growth was 39.58% and 43.75% for isolates EEB1A8 and EEB2 B13 respectively (Fig. 6) in the VOC production test. Both isolates exhibited positive results for all biocontrol traits tested *in vitro,* suggesting they are effective biocontrol agents (Table 5).

In vivo disease suppression

No plants exhibited soft rot symptoms during the first week after challenge inoculation. In the second week, the treatment with the isolate EEB1 A8 and combination of EEB1 A8 and *P. indica* showed the lowest disease incidence (3.33%) and the percentage disease index was lowest for the treatment with isolate EEB1 A8 alone (21.33%). During the 3rd week, the treatment with the isolate EEB1 A8 showed the lowest disease incidence and the same treatment along with treatment with combination of EEB1 A8 and *P. indica* showed lowest percentage disease index. Plants in pathogen inoculated control showed the highest DI and PDI and no plants treated with copper oxychloride were affected by the pathogen. The values clearly indicated that the plants treated with the bioagents individually (EEB1 A8, EEB2 B13 and *P. indica*) and in combination showed significant resistance to pathogen compared to control, in which the plants treated with EEB1 A8 and its combination with *P. indica* demonstrated highest re-

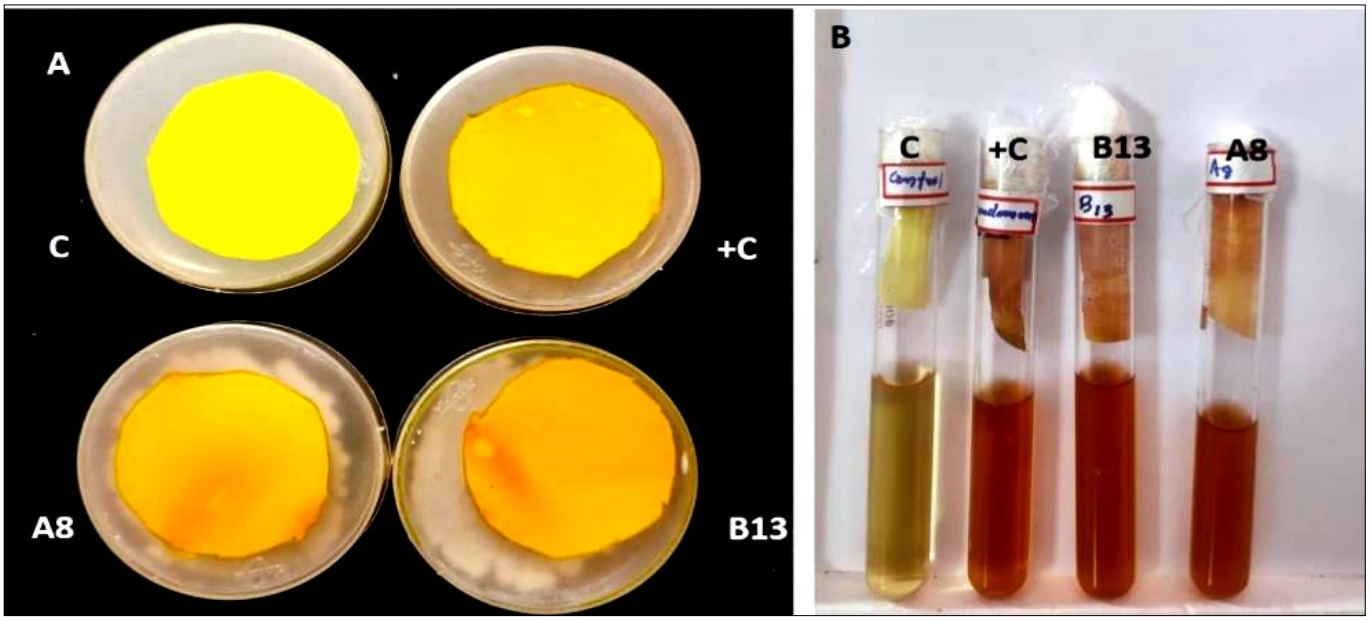

Fig. 4. Production of hydrogen cyanide (HCN) by the isolates A8 and B13. **C**: Control without any bacteria inoculated, **+C**: Positive control inoculated with *Pseudomonas fluorescens* PN026 in agar medium (**A**) and in broth (**B**).

Table 5. Biocontrol traits of endophytic bacterial isolates.

Isolates	Siderophore production	HCN production	Volatile organic compound (Percentage inhibition) *
A8	+	+	39.58 ± 8.83
B13	+	+	43.75 ± 2.94
LSD			3.14

Mean (± SD) of 4 replications (n = 6).

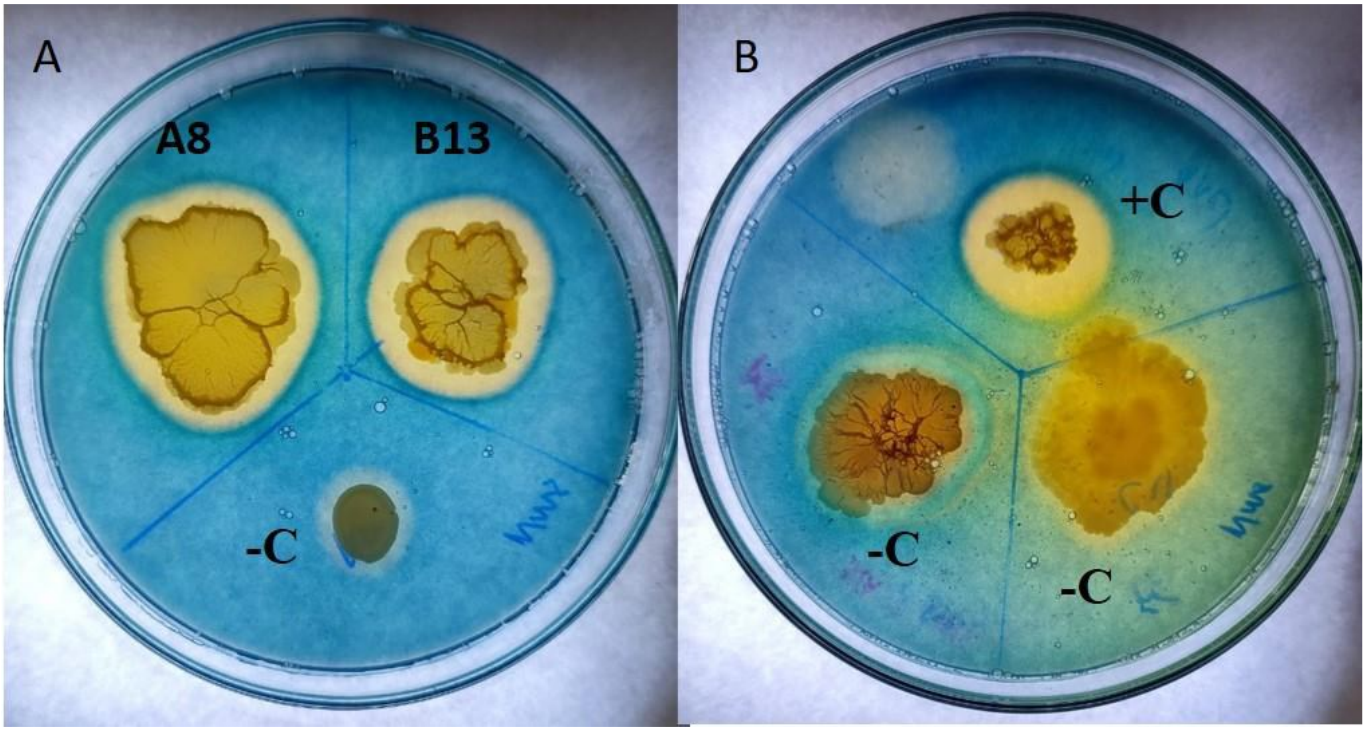

Fig. 5. A: CAS agar plates inoculated with isolates A8 and B13 **B**: **+C** - Positive control inoculated with *Pseudomonas fluorescens* PN026; **C** - Negative controls with bacteria which do not produce siderophore.

Fig. 6. Inhibition of *Pythium myriotylum* by volatile organic compounds produced by *Bacillus* isolates. **A**: Control **B**: Isolate A8 **C**: Isolate B13.

Table 6. Disease incidence and percentage disease index in ginger plants on challenge inoculation.

Treatments	1st week		2nd week		3rd week	
	DI%	PDI	DI%	PDI	DI%	PDI
A8	0	0	3.33 ± 0.77^{cd}	21.33 ± 1.53^{d}	6.67 ± 5.77^{c}	25.67 ± 2.08^{d}
B13	0	0	20.00 ± 0.20^{b}	28.00 ± 3.61^{bc}	26.67 ± 23.09^{cd}	32.67 ± 3.56^{c}
P. indica	0	0	16.67 ± 5.77^{bc}	28.00 ± 2.00^{bc}	40.00 ± 0.00^{bc}	35.33 ± 2.52^{c}
Combination of A8 and *P. indica*	0	0	3.33 ± 0.74^{cd}	24.00 ± 2.65cd	10.00 ± 0.00^{dc}	26.33 ± 2.08^{d}
Combination of B13 and *P. indica*	0	0	46.67 ± 11.55^{a}	29.33 ± 3.51^{b}	53.33 ± 11.55^{b}	38.00 ± 2.64^{b}
Pathogen control	0	0	60 ± 0.00^{a}	53.67 ± 1.53^{a}	73.33 ± 11.55^{a}	53.67 ± 3.21^{a}
Copper oxychloride (0.2%)	0	0	0	0	0	0
LSD			16.66	9.19	19.91	4.27

Mean (± SD) of 3 replications each containing 5 plants. **PDI**- Percentage Disease Index; **DI**- Disease Incidence, Values in a column followed by the same letters do

sistance. The DI and PDI values, which specify the disease spread and disease severity, of the plants treated with EEB1 A8 and its combination with *P. indica* were statistically comparable with the treatment with copper oxychloride, which clearly designated the efficiency of the biocontrol agents against the pathogen was comparable to chemical control (Table 6, Fig. 7).

Activity of defense related enzymes in ginger

The variation in activity of SOD among the treatments was not significantly different before inoculation of the pathogen. After inoculation, activity increased in the first day and followed the trend up to third day and then decreased in the fifth day without any substantial difference among the treatments. The highest activity was noticed in plants administrated with the isolate EEB1 A8, either alone or in

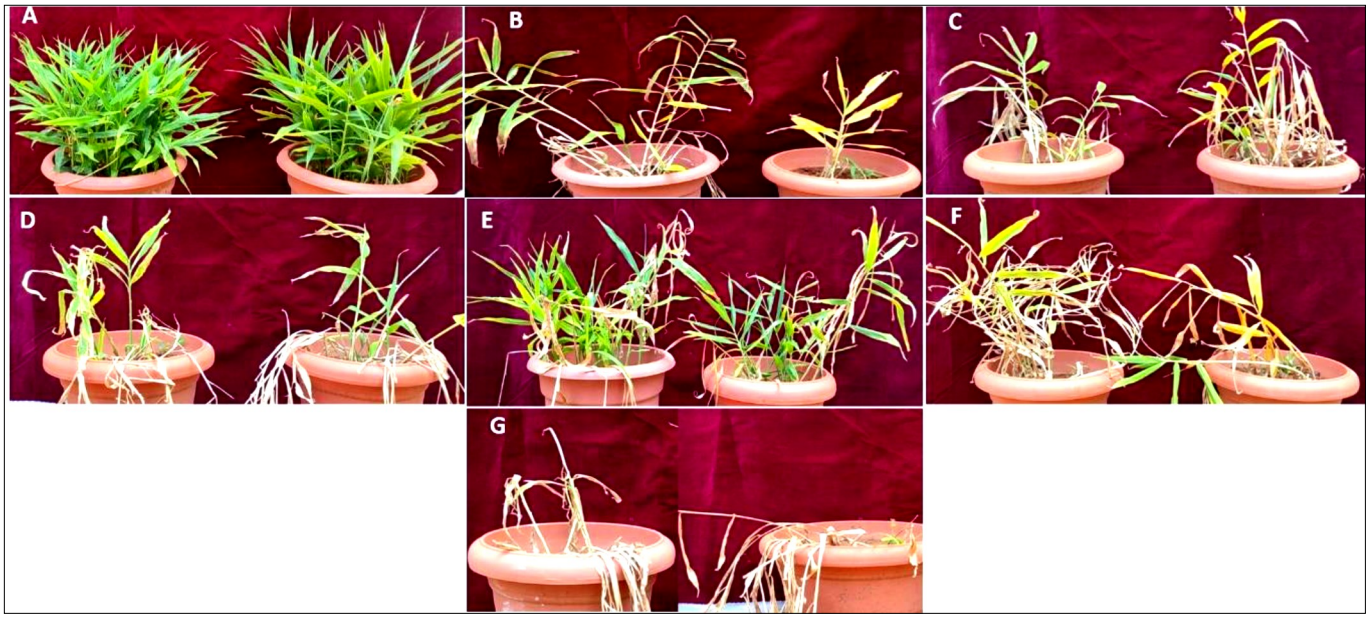

Fig. 7. Representative samples from the pot culture experiment in the third week after inoculation with the pathogen. **A**: Chemical control (Copper oxychloride)

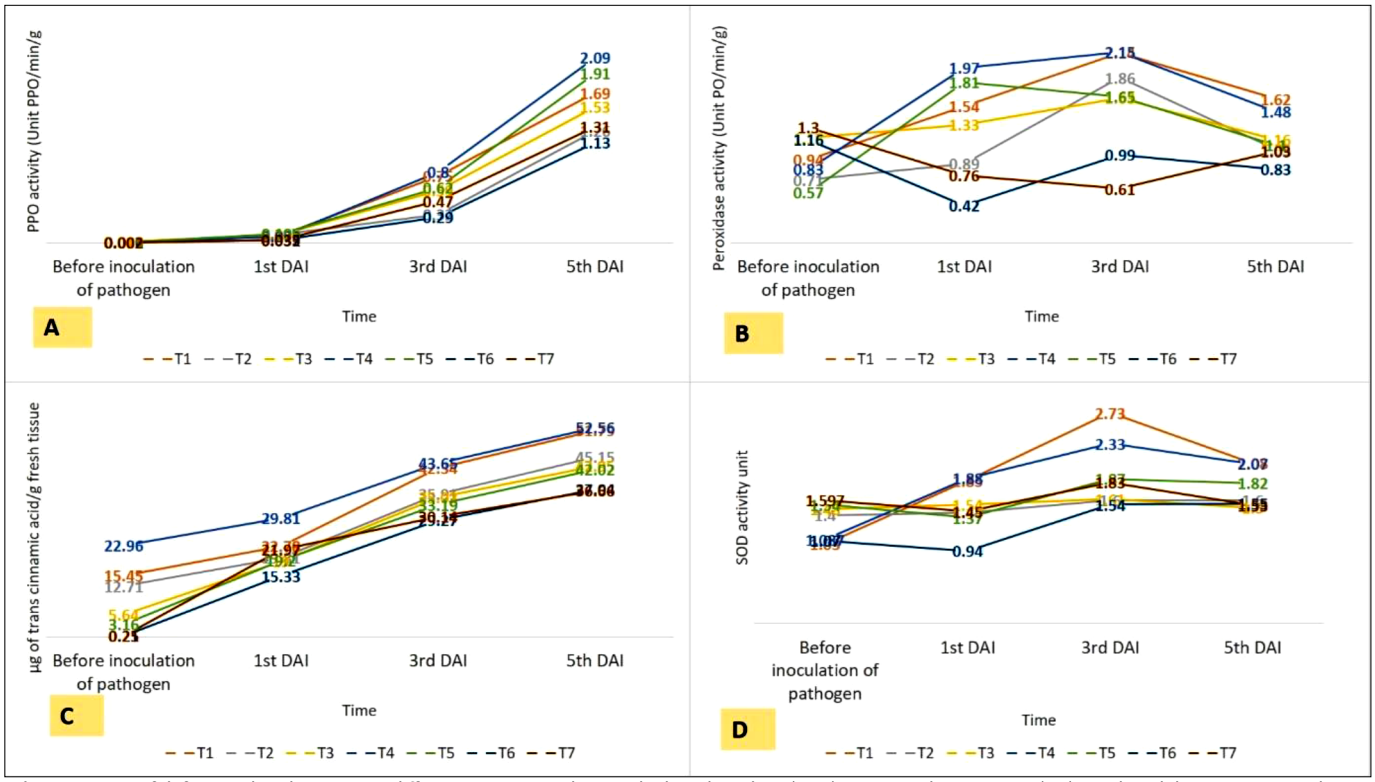

Fig. 8. Activity of defence-related enzyme at different time intervals. **A**: Polyphenol Oxidase (PPO), **B**: Peroxidase enzyme (PO), **C**: Phenylalanine ammonia-lyase (PAL) expressed in micrograms of trans-cinnamic acid/g tissue **D**: Super oxide dismutase (SOD), **T1**: Plants treated with A8, **T2**: Plants treated with B13, **T3**: Plants treated with *P. indica* , **T4**: Plants treated with combination of A8 and *P. indica,* **T5**: Plants treated with combination of B13 and *P. indica* T6: Pathogen control T7: Plants treated with copper oxychloride. DAI-days after inoculation of pathogen.

combination with *P. indica*, across all 3 intervals (Fig. 8).

In the case of PO activity, plants treated with bioagents followed same trend as that of SOD. Before inoculation there was no significant distinction between the treatments in terms of enzyme activity. The highest activity (1.97) was detected in plants treated with the isolate EEB1 A8 one day after inoculation. All the plants treated with the bioagent showed notably higher enzyme activity contrasted with control on third day and it ended up without any significant difference in the enzyme function on the fifth day.

The bio-catalytic activity of PPO in all the treatments kept on increasing up to 5th day after application of pathogen. There was no significant distinction in the activity on the day before inoculation. All the plants treated with bioagents showed statistically significant difference in activity compared to control, in which the treatment with the isolate EEB1 A8 showed the highest value in the third (0.80) and fifth day (2.09) after inoculation.

Enzyme activity of PAL was significantly elevated in the treatment with the isolate EEB1 A8 even before inoculation of pathogen. The enzyme activity in every treatment showed same trend of increasing continuously up to 5th day after inoculation. All the plants administrated with bacterial bioagent showed significant variation in the activity compared to control and chemical control. The highest activity was noted in the treatment with the isolate

EEB1 A8, either alone or in combination with *P. indica*.

Storage study

Table 7. Percentage disease index (PDI) of rhizomes under storage.

Treatment	PDI
A8	45.67 ± 1.16^{c}
B13	49.67 ± 13.20^{c}
Consortium of both isolates	27.33 ± 2.08^{b}
Pathogen control	96.89 ± 0.49^{d}
Chemical control	5.33 ± 1.52^{a}
LSD	10.986

Mean (± SD) of 3 replications each containing 5 plants. Values in a column followed by the same letters do not differ significantly ($p \leq 0.05$)

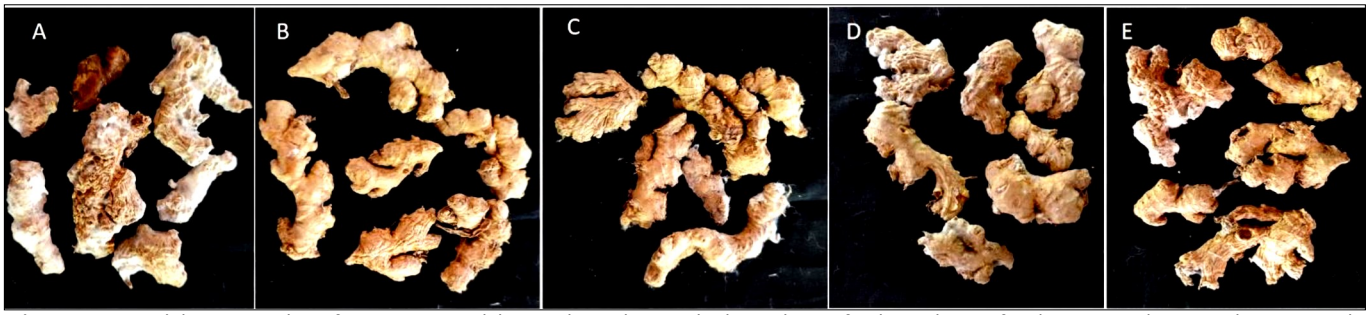

Fig. 9. Ginger seed rhizomes 10 days after treatment with bacterial inoculants and subjected to artificial inoculation of *Pythium myriotylum*. **A**: Pathogen control, **B**: Chemical control, **C**: Consortium of A8 and B13, **D**: A8 E: B13.

The lowest percentage disease index was recorded in the treatment with the consortium (27.33) of both the isolates other than chemical control (5.33). The rhizome treated with isolate EEB1 A8 and EEB2 B13 individually had a percentage disease index of 45.67 and 49.67, which was very low compared to that in the pathogen control (Table 7, Fig. 9).

Discussion

The microbial communities inhabiting within rhizomes of various plants with medicinal and aromatic properties remain largely unexplored. In this study, we focussed on isolating potential endospore-producing endophytic bacteria from ginger rhizomes and assessed their biocontrol efficacy against *Pythium myriotylum*. The capacity of endospore-producing endophytes to withstand adverse environmental conditions and nutrient limitations through endospore production makes them suitable bioagents. In a study, where bioformulation prepared using endospores of *Bacillus megatherium*, cellulose and glucose as carrier material, spore dormancy and cell viability were sustained for 6 months at room temperature. It served as an effective delivery system, in plant's environment and enhancing growth and biocontrol efficiency in various plants (30). In our research, the two endospore-forming endophytic bacteria (EEB) isolated by the double enrichment method and identified as *Bacillus* spp., showed the ability to supress the soft rot pathogen, *P. myriotylum* directly and indirectly, as reported in case of most of the *Bacillus* spp. (3, 31).

In vitro assays of various biocontrol traits allow for rapid evaluation of key attributes such as antagonistic activity, enzyme production and stress tolerance and serve as an initial screening step for identifying potential biocontrol candidates. Disease controlling mechanisms by PGPR include antibiosis, Induced Systemic Resistance (ISR), siderophore production, competition for nutrients and niches, and synthesis of lytic enzymes (32). Biocontrol traits like production of HCN, siderophore and volatile organic compound (VOC) were tested positive for the isolates. Experimental evidence supports the use of PGPR, particularly *Bacillus* and *Pseudomonas* strains, as effective biocontrol agents across a variety of plants as they have multiple biocontrol traits (33). Our results are comparable with the study, where *Bacillus subtilis* isolate S17 producing HCN, siderophore and volatile organic compound was able to control the red rot causing pathogen in sugarcane (34). The mechanism of HCN toxicity was explained in a study, where cyanide-producing strains of *P. putida* suppress the fungal pathogen *Septoria tritici* in wheat by inhibiting cytochrome C oxidase and other essential metallo -enzymes (35). Certain bacterial strains that produce siderophores engage in competition for the limited nutrient, iron in soil, thereby suppressing various fungal pathogens, particularly *Pythium* spp. (36, 37). Studies have documented that *Bacillus* spp. that produce a multitude of volatile organic compounds like Decanal and Benzothiazole could hold promise even as potential biofumigants for managing fungal phytopathogens (38).

The PDI reflects the severity of a disease, while disease incidence indicates its spread. Notably, applying a combination of EEB1 A8 with *P. indica* showed the highest level of *in vivo* disease suppression compared to chemical control. It can be assumed that the efficiency of the isolate EEB1 A8 as a biocontrol agent has been enhanced when applied together with *P. indica.* Several researches have reported that co-inoculation with *P. indica* enhances the efficiency of PGPR strains as plant growth promoters (15, 39) and biocontrol agents (11, 40), as the endophytic fungus induces the production of various metabolites by the host plant which can influence the endophytic and rhizospheric bacterial colonization. Instances of enhanced root colonization of fungal component which is supported by the co-inoculated bacteria (15), induction of production of various antifungal compound like bacillomycin by the bacteria in *in vitro* co-culturing system with fungus (41),the biofilm formation when co-inoculated *in vivo* (42), reported earlier can be attributed for the reason of better disease suppression in the co-inoculated treatment.

Plant defense enzymes like PAL, PPO, PO and SOD possess important role in pathogen suppression with various mode of actions. PAL and PPO involve in phenolic pathways, resulting in the formation of lignins and quinones which facilitates the wound healing process (43). The antioxidative enzymes like PO and SOD protect cells from oxidative damages caused by biotic and abiotic stress (44). In contrast with the control group, the plants treated with bioagents exhibited significantly higher enzyme activity associated with defense. Notably, the isolate EEB1 A8 elicited the most effective response in countering the pathogen through defense-related enzymes. Initiation of defense reaction counter to *Rhizoctonia solani* in *Cucumis sativus* by endospore producing bacterium *Bacillus thuringiensis* GS1 due to 1.5-fold enhanced production of defense related enzymes has been reported earlier (45). Similarly, the bioagents enhanced the enzyme activity and boosted up the defense system of plants resulting in lower disease severity and spread compared to control plants in the current study.

Good-quality, disease-free, large and plump rhizomes should be selected as seed for successful ginger cultivation, typically harvested between December and January and they need to be preserved for approximately four months before planting (from April to May). Several reports suggest that, rhizome rot occurring in ginger fields can be transmitted to stored seed rhizomes, causing 30-75% damage and often reducing the available planting material for subsequent ginger crops (2, 46, 47). To prevent soft-rot disease, during storage, the selected rhizomes are usually treated with fungicides, followed by drying them in the shade. Bioformulation with biocontrol agents is a sustainable alternative for the fungicides (48, 49). In this study, though PDI of the rhizomes treated with bacterial consortium is not on par with that of rhizome treated with copper oxychloride, it can be suggested as an environmentally friendly solution. While we remain uncertain about the ability of endophytes residing in ginger plants to survive within ginger seed rhizomes over long term, endospore-producing bioagents offer a distinct advantage. Their capacity to withstand environmental stresses and nutrient scarcity through endospore production makes them appropriate bioagents for seed treatment during storage. Also, environmental tolerance and suitability for long-term storage are an advantageous in bioformulations with endospore producing bacteria (5).

Conclusion

Rhizome rot incited by *Pythium* causes 20% yield loss in fields and *Pythium* in combination with other fungal and bacterial pathogen causes soft rot with a loss up to 75% both in field and storage. Endophytes from endorhizosphere of ginger with biocontrol efficiency are underexplored and their use is considered as a sustainable solution for managing the devastating disease. In this study endophytic isolates identified as *Bacillus* from ginger were proven to have the ability to produce HCN, siderophore and volatile organic compounds that supress the pathogen and induce the defense response by enhancing the defense-related enzyme activity. Co-inoculating the bacterial isolates with the beneficial fungus *P. indica* further enhanced their biocontrol efficiency. While these isolates show promise as biocontrol agents against *P. myriotylum*, further research is needed to explore their growth promotion abilities and standardize a practical formulation for field level application and during ginger seed storage.

Acknowledgements

Authors are grateful to the Kerala Agricultural University for providing facilities for carrying out the research.

Authors' contributions

MSN carried out the experiments, collected and analysed the data and drafted the original manuscript. KNA conceptualized and supervised the research, drafted, reviewed and edited the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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