PLANT SCIENCE TODAY
Vol XX(1): 1-XX
https://doi.org/10.14719/pst.14810

eISSN 2348-1900
OPEN ACCESS

Research Article | Agriculture
2026, Volume XX, Issue X, pp. 1-XX

Plant growth-promoting and antimicrobial activities of endophytic fungus *Colletotrichum gloeosporioides* RRB2 isolated from *Rhynchostylis retusa* (L.) Blume, a medicinal orchid of North-East India

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Abstract

Orchids are known to harbour diverse communities of endophytic fungi capable of producing a wide array of bioactive secondary metabolites. In this study, endophytic fungi isolated from healthy leaf tissues of *Rhynchostylis retusa* (L.) Blume, a medicinal orchid native to Northeast India, were evaluated for their plant growth-promoting (PGP) traits and antimicrobial potential. A total of 57 fungal isolates were obtained from surface-sterilised leaf fragments, with *Colletotrichum* and *Rhizoctonia* identified as the dominant genera. Among these, *Rhizoctonia* exhibited the highest colonisation frequency, while all *Colletotrichum* isolates demonstrated positive PGP activity, including indole-3-acetic acid (IAA) production and phosphate solubilisation, alongside antimicrobial effects. Notably, *Colletotrichum gloeosporioides* (RRB2) emerged as a particularly potent strain, exhibiting strong dual functionality. Gas chromatography–mass spectrometry (GC-MS) analysis of crude ethyl acetate extracts from *C. gloeosporioides* (RRB2) resulted in the tentative identification of several compounds based on mass spectral matching, including IAA, pyrrolo[1,2-a]pyrazine-1,4-dione, benzeneacetic acid derivatives and 2-Coumaranone compounds known for their antimicrobial, antioxidant, anticancer and cytotoxic properties. These findings suggest that the endophytic fungi associated with *R. retusa* possess promising *in vitro* plant growth-promoting and antimicrobial properties, with potential for further agricultural and biotechnological investigation.

Keywords: antimicrobial activity; *Colletotrichum gloeosporioides*; endophytic fungi; phosphate solubilisation; plant growth-promoting activity; *Rhynchostylis retusa* (L.) Blume

Received: 01 April 2026. **Accepted:** 29 September 2026. **Available online:** Version 1.0: 27 November 2026.

Cite this article: Sudem W, Subham S, Prabhali D, Satya ND, Kumananda T. Plant growth-promoting and antimicrobial activities of endophytic fungus *Colletotrichum gloeosporioides* RRB2 isolated from *Rhynchostylis retusa* (L.) Blume, a medicinal orchid of North-East India. Plant Science Today. 2026;XX(X):1-XX. https://doi.org/10.14719/pst.14810

Introduction

Orchidaceae represents one of the most diverse families of flowering plants, encompassing a vast range of epiphytic species that have adapted to thrive in complex ecological niches. Among them, *Rhynchostylis retusa* (L.) Blume, locally known for its medicinal and ornamental value, is a monopodial epiphytic orchid distributed throughout the Indo-Malayan region and widely revered in the Northeastern states of India, especially Assam (1). Its characteristic racemose inflorescence, composed of densely packed pinkish-white flowers, not only adds aesthetic appeal but also contributes significantly to its horticultural importance. Medicinally, various parts of *R. retusa*, including roots, leaves and flowers have been traditionally employed in indigenous healthcare systems for managing conditions such as respiratory ailments, nervous disorders, inflammation, rheumatism and skin diseases, underlining its pharmacological potential (2).

The importance of fungal associations in orchids is well documented, particularly with orchid mycorrhizal fungi, which facilitate germination and early seedling development. A mycorrhizal association has also been reported in *R. retusa*, highlighting the close relationship between this orchid and its associated fungi. However, a growing body of research emphasises the ecological and functional significance of non-mycorrhizal endophytic fungi, which reside asymptomatically within plant tissues and have demonstrated a broad spectrum of beneficial attributes (3, 4). These endophytes are known to promote plant growth through mechanisms such as the biosynthesis of phytohormones like indole-3-acetic acid (IAA), phosphate solubilisation, siderophore production and nitrogen fixation (5, 6).

Furthermore, their ability to produce secondary metabolites, with antimicrobial, antioxidant and anticancer properties has attracted considerable attention in the field of natural product discovery.

Orchid-associated fungi exhibit diverse ecological relationships with their hosts, ranging from mycorrhizal and endophytic associations to interactions involving disease-associated fungi. Among the fungal groups reported from orchids, *Colletotrichum* and *Rhizoctonia* have attracted considerable attention because of their association with orchid tissues and their potential roles in host–fungus interactions. Studies of orchid-associated fungi have demonstrated that different fungal taxa may exhibit distinct ecological and functional characteristics, emphasising the need to investigate fungal communities beyond their established mycorrhizal associations. In particular, studies on endophytic fungi from orchids have revealed considerable fungal diversity together with plant growth-promoting and antimicrobial properties, indicating that asymptomatic endophytes may represent an important but comparatively less explored component of orchid-associated fungal communities (4–8).

Despite extensive studies on terrestrial orchids, the endophytic fungal diversity and functional potential of *R. retusa* remain underexplored. While previous work has documented the mycorrhizal association of *R. retusa*, comparatively limited information is available on its non-mycorrhizal endophytic fungi, particularly those inhabiting leaf tissues (8). Moreover, their functional characteristics, including plant growth-promoting and antimicrobial activities and their potential to produce bioactive metabolites, have not been sufficiently investigated. Thus, a specific knowledge gap exists regarding the diversity and functional potential of leaf-associated endophytic fungi in *R. retusa*. Given its conservation significance and its inclusion under CITES Appendix II since 1975, there is a need to investigate symbiotic associations that may contribute to its conservation and sustainable utilisation (7). In this context, endophytes not only offer insights into the plant's adaptive strategies under environmental stress but also serve as promising sources of bioactive compounds with applications in agriculture and pharmaceuticals.

Accordingly, the present study was undertaken to address this knowledge gap by isolating and characterising endophytic fungi from the leaf tissues of *R. retusa* and evaluating their potential for plant growth promotion and antimicrobial activity. The study further sought to determine whether the most promising endophytic isolate could serve as a potential source of bioactive metabolites by profiling its major compounds using gas chromatography-mass spectrometry (GC-MS), while internal transcribed spacer–ribosomal DNA (ITS-rDNA)-based molecular analysis was used to establish its phylogenetic affiliation. By combining isolation and characterisation with functional screening, antimicrobial evaluation, metabolite profiling and molecular identification, the present study seeks to provide a more comprehensive understanding of the functional potential of endophytic fungi associated with *R. retusa*. These findings could enhance the understanding of endophyte–host interactions and contribute to bioprospecting efforts for novel microbial metabolites with ecological and commercial relevance.

Materials and Methods

Collection of samples

Mature and healthy leaves of *R. retusa* were collected from three locations—Nalbari, Baihata Chariali and Guwahati—situated in the central region of Assam, at altitudes ranging from 42 to 56 m above sea level. The orchid was found growing epiphytically on *Mangifera indica* L. trees. From each location, five individual plants were selected and five healthy leaves were randomly collected from each plant. The samples were placed in sterile zip-lock polypropylene bags and transported to the Mycology and Plant Pathology Laboratory, Department of Botany, Gauhati University. The collected plant specimens were submitted to Gauhati University Botanical Herbarium (GUBH) for authentication and the authentication accession number was obtained. All isolations were carried out within 48 hr of sample collection to ensure sample integrity.

Isolation and identification of endophytic fungi from leaves of *Rhynchostylis retusa* (L.) Blume

The collected leaves were initially washed with 10 % teepol solution under running tap water to remove surface debris. After thorough cleaning, they were cut into small segments (approximately 0.3 cm^2) and surface sterilised following the method, which involved sequential immersion in 70 % ethanol for 3 min, 3 % sodium hypochlorite (NaOCl) for 2 min and three rinses with sterile distilled water, each lasting 1 min (4). The sterilised fragments were air-dried on sterile blotting paper and inoculated onto freshly prepared potato dextrose agar (PDA) medium at its standard formulation pH, without further pH adjustment and supplemented with 50 µg/mL streptomycin to inhibit bacterial contamination. The plates were incubated at 25 ± 2 °C for 3–4 days until fungal colonies emerged. To check the effectiveness of surface sterilisation, separate sterilised leaf fragments were pressed onto sterile PDA plates and incubated at 25 ± 2 °C for 3–4 days. These plates were used as sterility controls. The absence of fungal growth on the control plates confirmed the effectiveness of the surface sterilisation procedure. Fungal colonies that developed from the inoculated segments were subcultured to obtain pure isolates, which were maintained on PDA slants at 4 °C for

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further analysis. A total of 150 leaf segments, 50 from each sampling site, were processed for fungal isolation. Morphological identification of the isolates was based on colony characteristics such as colour, diameter and pigment production after 7 days of incubation on PDA at 28 ± 2 °C. Micromorphological features, including conidiophores and conidia, were examined by staining specimens with lactophenol cotton blue and observing them under a Primo Star® optical microscope (ZEISS, Göttingen, Germany) at 400× magnification, with measurements taken from 20 samples each.

Colonising frequency

The relative colonisation frequency (CF %) of endophytic fungi was calculated to determine the extent of fungal colonisation within the leaf tissues. The colonisation frequency was expressed as a percentage using the following formula:

CF % = (NS / TS) × 100

Where, NS represents the number of leaf segments colonised by endophytic fungi and TS denotes the total number of segments examined (8).

Determination of plant growth promoting activity

Determination of indole-3-acetic acid activity

Endophytic fungal isolates were cultivated in potato dextrose broth (PDB) medium, both with and without supplementation of 0.1 % L-tryptophan, which serves as a precursor for auxin biosynthesis. The cultures were incubated in a shaker incubator at 25 ± 2 °C and 120 rpm for 7 days. Following incubation, the cultures were centrifuged at 6000 rpm for 30 min and the resulting supernatant was collected for IAA estimation. The Salkowski reagent, prepared by mixing 50 mL of 35 % perchloric acid ($HClO_4$) with 1 mL of 0.5 M ferric chloride ($FeCl_3$), was added to the supernatant at a 2:1 ratio (2 mL reagent to 1 mL supernatant) (9). The mixture was incubated in the dark at room temperature for 15 min, allowing the development of a pink coloration indicative of the presence of IAA. The absorbance was then measured at 530 nm using a UV-Vis spectrophotometer to quantify the IAA concentration.

Determination of phosphate solubilising activity

The endophytic fungal isolates were cultured on Pikovskaya's (PVK) agar medium supplemented with 5 g of tricalcium phosphate (TCP) as the sole phosphorus source. The pH of the medium was adjusted to 7.0 before autoclaving. The inoculated plates were incubated at 25 ± 2 °C for 4 days to allow for phosphate-solubilisation. The phosphate solubilising activity (PSA) of the isolates was evaluated based on the formation of clear halo zones surrounding the fungal colonies along their growth lines. The presence of a distinct clear zone indicated a positive phosphate solubilisation response. The diameters of the fungal colonies and the total diameters of the halo zones were measured and the halo size was calculated by subtracting the colony diameter from the total halo diameter (10, 11). The phosphate solubilisation index (SI) was then calculated using the following formula:

Solubilisation index (SI) = (Colony diameter + Halo zone diameter) / Colony diameter

Determination of siderophore producing activity

The siderophore-producing ability of the endophytic fungal isolates was assessed using Chrome Azurol S (CAS) agar, following the previously described method, with slight modifications (12). The CAS agar was prepared by combining four separately sterilised solutions. Solution 1 (Fe-CAS indicator solution) consisted of 10 mL of 1 mM $FeCl_3 \cdot 6H_2O$ (prepared in 10 mM HCl), 50 mL of an aqueous solution of CAS (1.21 mg/mL) and 40 mL of a solution of hexadecyltrimethylammonium bromide (HDTMA) (1.82 mg/mL). Solution 2 (basal medium) comprised 0.5 % succinic acid, 0.4 % K_2HPO_4, 2.3 % $(NH_4)_2SO_4 \cdot 7H_2O$, 2.3 % PDB and 2 % agar, adjusted to pH 5.3 ± 0.2. Mixing the Fe-CAS indicator solution with the basal medium resulted in a blue to dark blue medium. The fungal isolates were inoculated onto CAS agar plates and incubated at 25 ± 2 °C for 7 days. No positive control was included in the siderophore assay. The development of yellowish-orange, purple, or dark purplish-red zones along the line of fungal growth indicated positive siderophore production.

Determination of antimicrobial activity

The endophytic fungal isolates were cultivated in PDB and incubated in a rotary shaker at 120 rpm at 28 ± 1 °C for 7 days. After incubation, secondary metabolites were extracted from the culture broth using ethyl acetate as the organic solvent. The organic phase was separated, vacuum-dried and concentrated to obtain crude extracts, which were then dissolved in 10 % dimethyl sulphoxide (DMSO) for antimicrobial assays. The antimicrobial activity of the crude secondary metabolites was evaluated against *Escherichia coli* (MTCC 443), *Staphylococcus aureus* (MTCC 737) and *Candida albicans* (MTCC 227) using the agar cup diffusion method (13). Briefly, bacterial strains were cultured overnight in nutrient broth and the turbidity of the cultures were adjusted to the 0.5 McFarland standard before inoculation. The standardised bacterial suspensions were uniformly spread onto fresh nutrient agar plates using sterile cotton swabs. *C. albicans* was similarly cultured in PDB and the culture was standardised to the required inoculum density before spreading onto PDA plates. Agar wells (7 mm in diameter) were created using a sterile cork borer and each well was filled with 100 µL of the crude extract. The plates were incubated at 35 ± 2 °C for 24 hr for bacterial strains and at 27 ± 2 °C for 48 hr for the fungal pathogen. Antimicrobial activity was determined by measuring the diameter of the zone of inhibition formed around the wells.

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Molecular characterisation and phylogenetic analysis

For molecular identification, total genomic DNA was extracted using the Quick-DNA™ Fungal Miniprep Kit (Zymo Research, USA). Mycelial samples were collected by scraping the surfaces of 7-day-old colonies cultured on PDA. The internal transcribed spacer (ITS) region of rDNA was amplified using the universal primer pair ITS1/ITS4 (14). The PCR amplification was carried out in a 25 µL reaction mixture containing 10 pmol of each primer, 2.5 mM magnesium chloride ($MgCl_2$), 200 µM of each deoxyribonucleotide triphosphates (dNTP), 0.5 U of Taq DNA polymerase, 1× PCR buffer (Invitrogen, Life Technologies, Brazil) and 100 ng of template DNA. The thermal cycling conditions included an initial denaturation at 95 °C for 5 min, followed by 39 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 45 sec and extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min.

Amplified PCR products were visualised on a 1.5 % agarose gel using 0.5× Tris-acetate-EDTA (TAE) buffer and observed under ultraviolet (UV) illumination. The purified PCR products were sent to Heredity Biosciences (Bhubaneswar) for sequencing. The obtained sequences were analysed through a sequence-comparison tool, BLASTn to determine the closest sequence similarity. For phylogenetic analysis, the ITS sequences, along with reference sequences retrieved from GenBank, were aligned using the sequence-alignment, MUSCLE algorithm in MEGA version 11 (15). A phylogenetic tree was constructed using the maximum likelihood (ML) method based on the Kimura 2-parameter model. Bootstrapping was performed with 1000 replicates and bootstrap values equal to or greater than 50 % were displayed on the tree.

Characterisation of crude secondary metabolites using gas chromatography-mass spectrometry analysis

The crude ethyl acetate extract obtained from a selected endophytic fungal isolate, which demonstrated significant IAA production and antimicrobial activity, was analysed using a PerkinElmer Turbo Mass Spectrophotometer (USA), model Clarus 680 gas chromatograph coupled with a Clarus 600 Mass Spectrometer. The GC-MS analysis was performed using a PerkinElmer Elite-5 MS capillary column (60 m length × 0.25 mm internal diameter × 0.25 µm film thickness), composed of 5 % diphenyl and 95 % dimethyl polysiloxane (low bleed). Helium was used as the carrier gas at a flow rate of 1 mL/min. One microlitre of the diluted sample was injected in splitless mode, with the injector temperature set at 250 °C. The oven temperature was initially held at 60 °C for 5 min, then ramped to 180 °C at a rate of 10 °C/min with a hold time of 5 min, followed by a final increase to 280 °C at a rate of 20 °C/min, again held for 5 min. The ion source temperature was maintained at 180 °C and the total run time was 37 min. The GC-MS analysis was conducted using electron impact ionisation at 70 eV. The relative percentage of each separated compound was calculated from its peak area relative to the total peak area in the total ion chromatogram (TIC). Compound identification was based on comparison of the mass spectra with the National Institute of Standards and Technology (NIST) Mass Spectral Library database (16).

Statistical analysis

All experiments were conducted in triplicate and the results are expressed as mean ± standard deviation (SD) (n = 3). Statistical analyses were performed using IBM SPSS Statistics version 32.0.0. Differences between paired groups were evaluated using a two-tailed paired t-test and differences were considered statistically significant at $p < 0.05$.

Results

Isolation, characterisation and identification of endophytic fungi

The plant material, *R. retusa* (L.) Blume, was authenticated and a voucher specimen was deposited at the GUBH where an accession number (GUBH11567) was obtained. The endophytic fungi were isolated from surface-sterilised leaf fragments of *R. retusa*, collected from three distinct locations in Assam: Baihata Chariali (BC), Nalbari (NB) and Guwahati (GU) (Fig. 1A−E). A total of 57 fungal isolates were obtained from 150 leaf segments, with the highest number of isolates recorded from Baihata Chariali-28, followed by Nalbari-15 and Guwahati-14, resulting in an overall colonisation frequency of 38.01 % (Table 1).

Most of the isolates belonged to the phylum Ascomycota and were assigned to the genus *Colletotrichum*, which was consistently recovered from all three sampling locations. Several isolates of *Colletotrichum* (RRB1, RRN4, RRB5, RRN1, RRG8, RRB2, RRB3, RRB6–RRB8, RRN3, RRN6–RRN7 and RRG1–RRG2) were obtained, indicating the genus as the dominant endophytic group associated with the studied host. In addition, isolates belonging to the genus *Rhizoctonia* were also recovered, including *Rhizoctonia* sp. RRB12 and RRG3. Notably, *Rhizoctonia* sp. RRB12 exhibited the highest colonisation frequency (6.00 %) and was exclusively recovered from Baihata Chariali samples. Four non-sporulating morphotypes (RRN8, RRN9, RRB10 and RRG11) were also observed, albeit at lower frequencies (0.67–1.33 %), indicating their minor representation in the foliar endophytic community (Fig. 1C).

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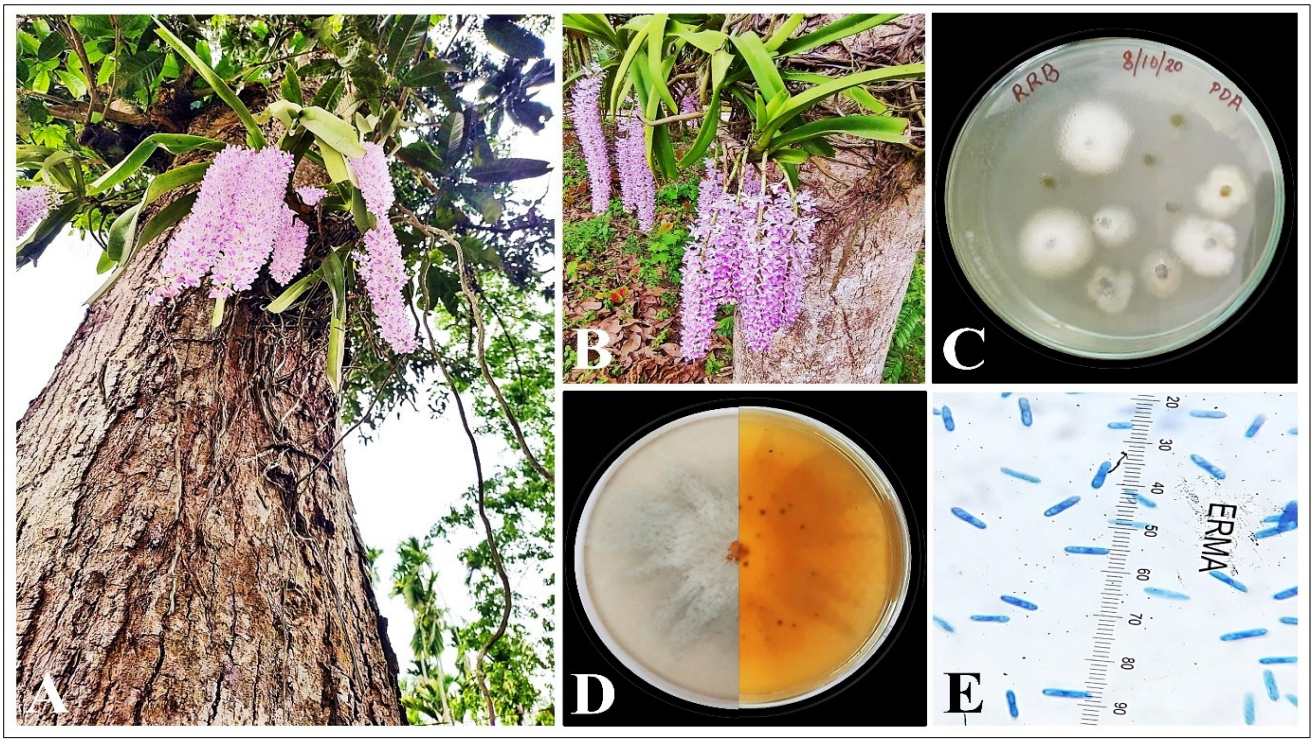


Fig. 1. Collection, isolation and identification of endophytic fungi from *Rhynchostylis retusa.* A: Orchid growing epiphytically on a tree trunk in its natural habitat; B: Close-up view of the plant showing its characteristic flowers and leaves; C: Mixed endophytic fungal culture isolated on PDA, including *Colletotrichum gloeosporioides* RRB2; D: Colony morphology and sporulation pattern of *Colletotrichum gloeosporioides* RRB2 on PDA; E: Microscopic view of conidia of *Colletotrichum gloeosporioides* RRB2 with scale bar in micrometers.

Table 1. Endophytic fungal isolates recovered from surface-sterilised leaf tissues of *Rhynchostylis retusa* (L.) Blume collected from three different locations in Assam

Isolate number	Number of isolates			Total isolates	Colonisation frequency (%)
	BC	NB	GU		
RRB1	4	–	–	4	2.67
RRN4	–	2	–	2	1.33
RRB2	2	–	–	2	1.33
RRB5	3	–	–	3	2.00
RRN1	–	4	–	4	2.67
RRG8	–	–	3	3	2.00
RRB6	1	–	–	1	0.67
RRB3	2	–	–	2	1.33
RRB7	3	–	–	3	2.00
RRB8	3	–	–	3	2.00
RRN3	–	2	–	2	1.33
RRN6	–	3	–	3	2.00
RRN7	–	2	–	2	1.33
RRG1	–	–	2	2	1.33
RRG2	–	–	1	1	0.67
RRB12	9	–	–	9	6.00
RRG3	–	–	7	7	4.67
RRN8	–	1	–	1	0.67

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RRN9	−	1	−	1	0.67
RRB10	1	−	−	1	0.67
RRG11	−	−	1	1	0.67
Total	28	15	14	57	38.01

*BC: Baihata Chariali; NB: Nalbari; GU: Guwahati; n: 50 leaf fragments inoculated per location (total n = 150).

Among all isolates, *Colletotrichum* sp. (RRB2), obtained from Baihata Chariali, was examined in detail due to its well-defined cultural and morphological features. On PDA, colonies of RRB2 initially appeared white to grey, turning olive-grey to yellowish-white on the reverse side upon maturation (Fig. 1D). The colony was characterised by dense, cottony aerial mycelium and attained a diameter of approximately 80–85 mm after 7 days of incubation at 28 ± 2 °C. Colony margins were smooth, regular and well-defined.

Microscopically, the conidia of RRB2 were observed to be hyaline, single-celled, cylindrical and straight with rounded apices (Fig. 1E). Conidiophores were short, simple and arose directly from the hyphae, bearing conidia. Measurements from 50 conidia revealed a length range of 13.50–18.78 µm (mean = 15.19 ± 1.52 µm) and a width range of 3.56–5.99 µm (mean = 4.48 ± 0.62 µm) (*n* = 50). These morphological features conformed well to standard descriptions of *C. gloeosporioides*, supporting its identification based on cultural and microscopic characteristics.

Determination of plant growth promoting activities

The isolated endophytic fungi were evaluated for their plant growth-promoting traits, namely IAA production, phosphate solubilisation and siderophore production. All isolates belonging to the genus *Colletotrichum* exhibited positive activity for both IAA production and phosphate solubilisation. In contrast, isolates of the genus *Rhizoctonia* and the non-sporulating morphotypes did not show any activity for IAA production or phosphate solubilisation. However, it was observed that none of the isolates, including those from *Colletotrichum*, displayed any siderophore production activity (Table 2).

The isolates that tested positive for IAA production were further subjected to quantitative analysis using a UV-VIS spectrophotometer and the results were compared with a standard IAA calibration curve. The results revealed that two endophytic fungi, RRB2 and RRB1, produced considerable amounts of IAA when cultured in PDB supplemented with L-tryptophan. The recorded IAA concentrations were 2.76 ± 0.026 and 2.55 ± 0.035 mg/mL, respectively. It is noteworthy that IAA production was comparatively lower when the isolates were cultured in media without L-tryptophan supplementation (Fig. 2), indicating that the presence of L-tryptophan significantly enhances IAA biosynthesis.

Furthermore, phosphate-solubilising isolates were analysed for their solubilisation index (SI) by measuring the halo zone formation on Pikovskaya's agar (Fig. 3). Among the isolates, RRG8 exhibited the highest phosphate solubilisation index of 3.63 ± 0.14, followed by RRN4, RRB2 and RRB8, with SI values of 3.34 ± 0.12, 3.20 ± 0.08 and 3.00 ± 0.12, respectively. The remaining *Colletotrichum* isolates showed relatively lower solubilisation indices, ranging from 2.30 ± 0.06 to 2.97 ± 0.11 (Fig. 4A–C).

Determination of antimicrobial activity

The antimicrobial activity of crude ethyl acetate extracts derived from various endophytic fungal isolates was evaluated against three clinically significant human pathogens namely *E. coli* (MTCC 443), *S. aureus* (MTCC 737) and *C. albicans* (MTCC 227). The agar well diffusion method was employed, with the diameter of the inhibition zone (in mm) used to quantify antimicrobial efficacy. Streptomycin (1 %) and fluconazole (1 %) were used as positive controls, while 10 % DMSO served as the negative control (Table 3).

Among the tested isolates, RRB2 exhibited the most pronounced and broad-spectrum antimicrobial activity, producing inhibition zones of 16.07 ± 0.13 mm against *E. coli*, 17.33 ± 0.34 mm against *S. aureus* and 18.33 ± 0.58 mm against *C. albicans*. These inhibition levels were comparable to those of the standard antimicrobial agents, indicating strong inhibitory activity against both gram-positive and gram-negative bacteria, as well as fungal pathogens (Fig. 4C).

In addition to RRB2, isolates from the genus *Colletotrichum* such as RRN1, RRG8, RRG1, RRG2, RRN6 and RRB3 also demonstrated considerable antimicrobial activity, though with slightly lower or variable inhibition profiles. Moderate activity was observed in the case of RRB1, RRN4, RRB5 and RRB6, whereas RRB8 and RRN3 showed comparatively weaker inhibition. In contrast, isolates from the genus *Rhizoctonia* and the non-sporulating morphotype fungi exhibited minimal or no antimicrobial activity. The assay controls validated the results, with Streptomycin (1 %) producing inhibition zones of 18.73 ± 0.38 mm (*E. coli*) and 18.33 ± 0.57 mm (*S. aureus*), whereas fluconazole (1 %) showing 17.33 ± 0.57 mm inhibition against *C. albicans*. However, no inhibitory activity was observed for the negative control (10 % DMSO).

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Table 2. Plant growth-promoting activities of endophytic fungi isolated from *Rhynchostylis retusa* (L)

Endophytic fungal isolate	IAA production	Phosphate solubilisation	Siderophore production
RRB1	+	+	–
RRN4	+	+	–
RRB2	+	+	–
RRB5	+	+	–
RRN1	+	+	–
RRG8	+	+	–
RRB6	+	+	–
RRB3	+	+	–
RRB7	+	+	–
RRB8	+	+	–
RRN3	+	+	–
RRN6	+	+	–
RRN7	+	+	–
RRG1	+	+	–
RRG2	+	+	–
RRB12	–	–	–
RRG3	–	–	–
RRN8	–	–	–
RRN9	–	–	–
RRB10	–	–	–
RRG11	–	–	–

*'+' indicates presence of activity; '–' indicates absence of activity; IAA: indole-3-acetic acid.

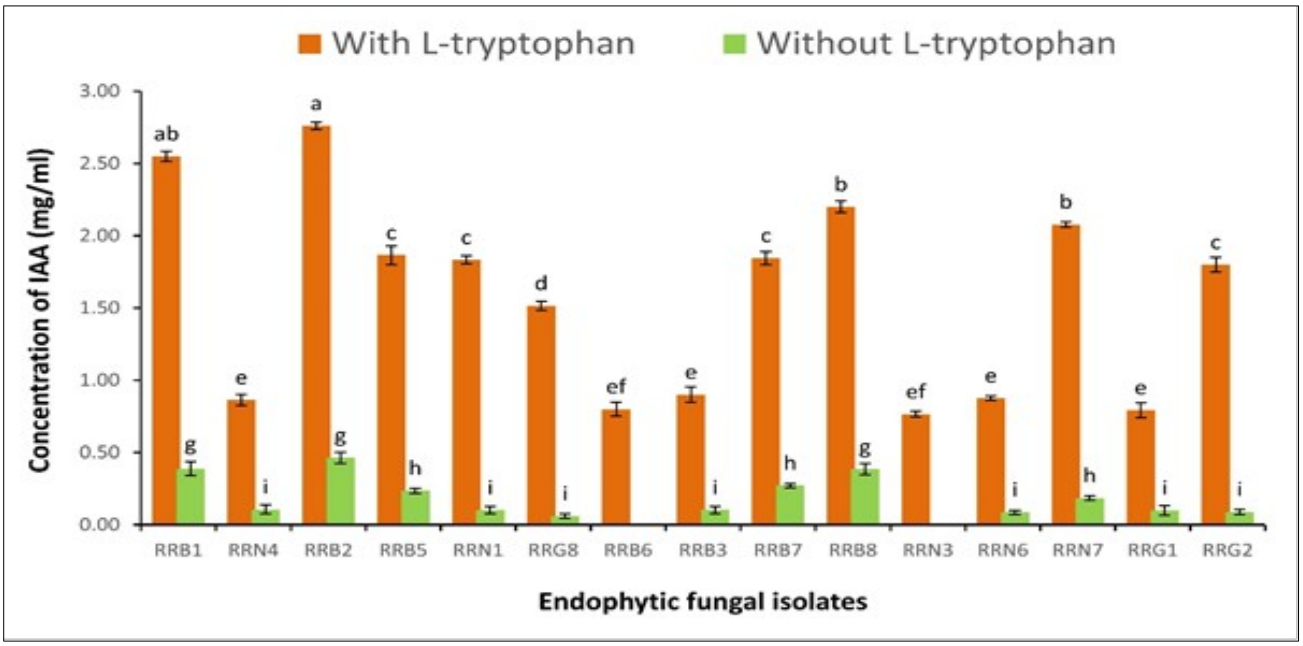


Fig. 2. Indole-3-acetic acid (IAA) production (mg/mL) by selected potent endophytic fungi isolated from *Rhynchostylis retusa* (L.) Blume, cultured with and without L-tryptophan supplementation for 7 days. Values are mean (n=3) and error bars indicate standard deviation (SD). Different letters indicate statistically significant differences (paired t-test, $p < 0.05$, two-tailed).

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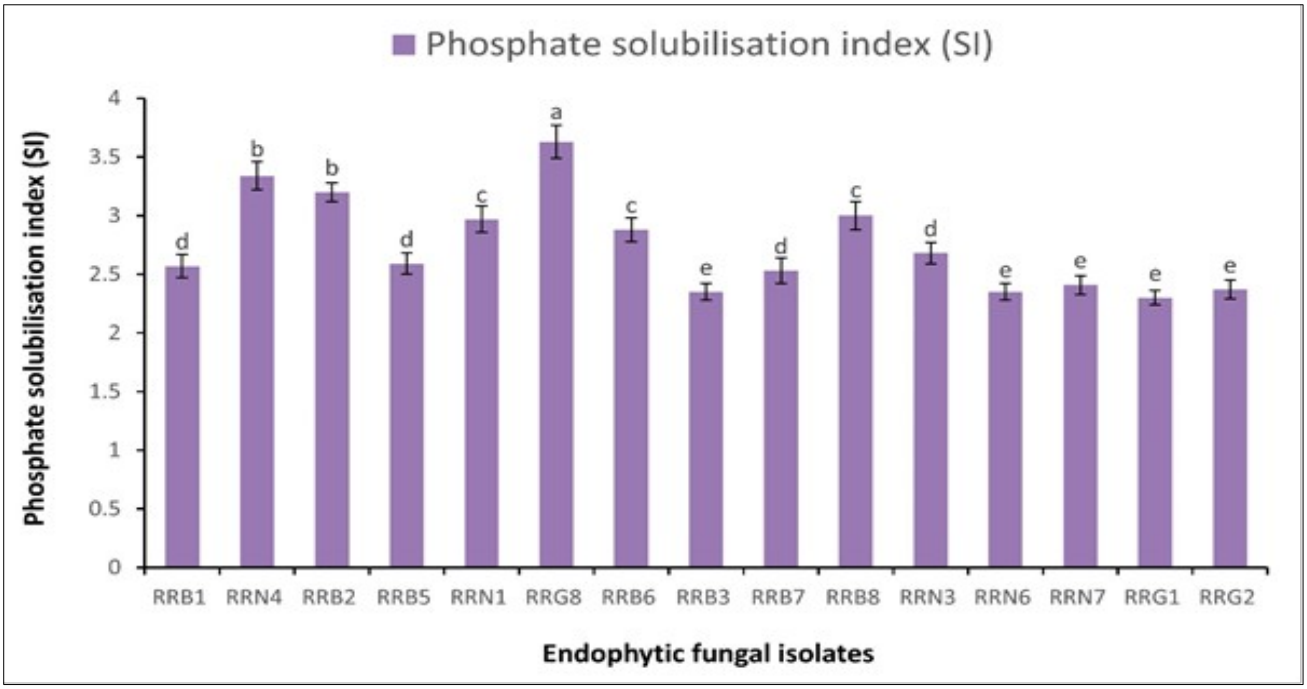


Fig. 3. Phosphate solubilisation index (SI) of selected potent endophytic fungi isolated from *Rhynchostylis retusa* (L.) Blume after 4 days of incubation. Values are mean (n=3) and error bars indicate standard deviation (SD). Different letters indicate statistically significant differences (paired t-test, $p < 0.05$, two-tailed).

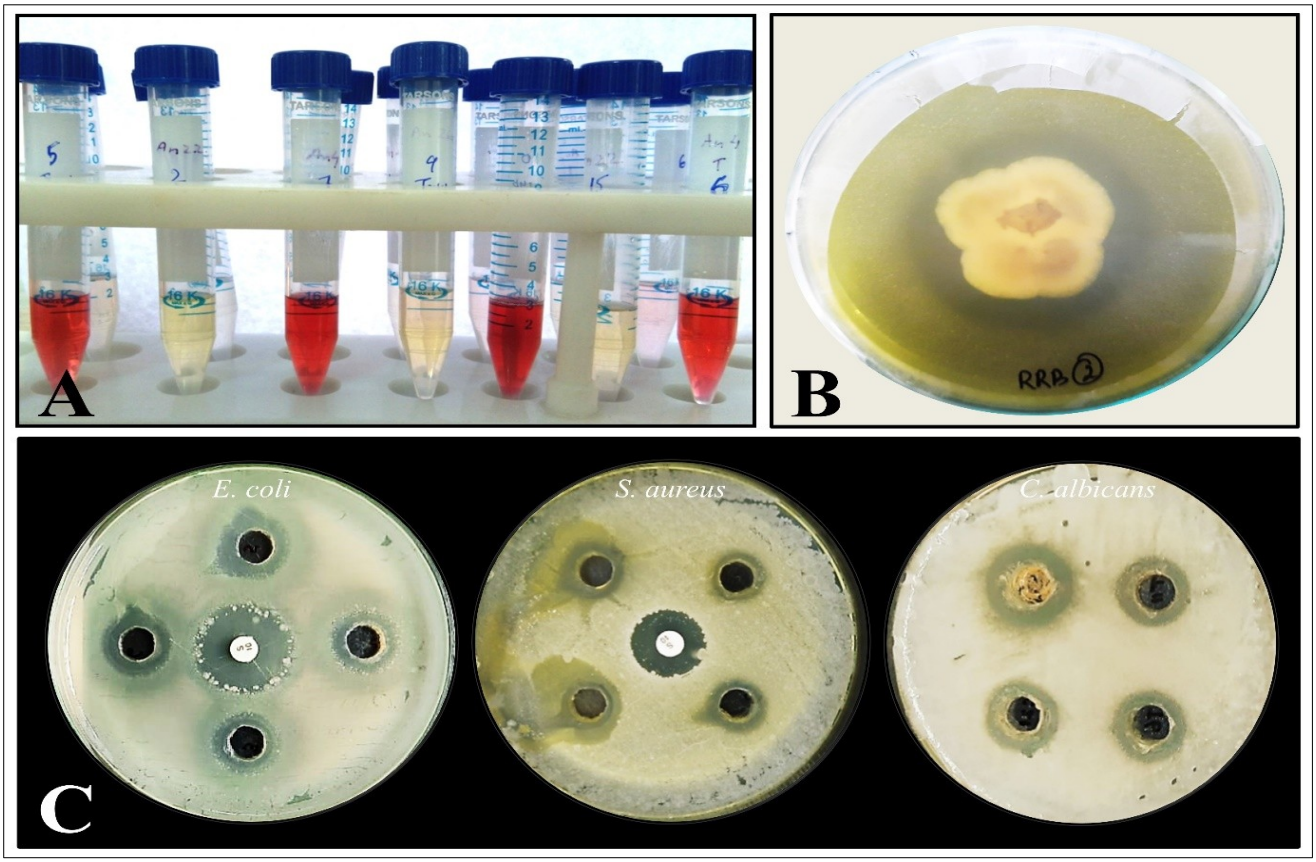


Fig. 4. Plant growth-promoting and antimicrobial activities of fungal isolate *Colletotrichum* sp. RRB2. A: Indole-3-acetic acid (IAA) production; B: phosphate-solubilising activity; C: antimicrobial activity of ethyl acetate extract of secondary metabolites from isolate RRB2 against *Escherichia coli* (MTCC 443), *Staphylococcus aureus* (MTCC 737) and *Candida albicans* (MTCC 227).

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Table 3. Antimicrobial activity of endophytic fungi isolated from *Rhynchostylis retusa* (L.) Blume against selected test pathogens

Endophytic fungal Isolate	Zone of inhibition (in mm)		
	Candida albicans	Staphylococcus aureus	Escherichia coli
RRB1	15.18 ± 0.17	14.33 ± 0.34	16.67 ± 0.58
RRN4	15.67 ± 0.58	14.29 ± 0.34	16.00 ± 0.00
RRB2	18.33 ± 0.58	17.33 ± 0.34	16.07 ± 0.13
RRB5	16.19 ± 0.17	15.25 ± 0.22	16.59 ± 0.52
RRN1	18.00 ± 0.00	17.15 ± 0.25	14.33 ± 0.34
RRG8	17.13 ± 0.12	16.26 ± 0.36	16.22 ± 0.22
RRB6	15.41 ± 0.36	15.25 ± 0.23	15.23 ± 0.20
RRB3	16.26 ± 0.23	16.18 ± 0.17	15.33 ± 0.58
RRB7	15.52 ± 0.50	16.30 ± 0.34	16.22 ± 0.19
RRB8	12.30 ± 0.34	14.33 ± 0.29	10.17 ± 0.15
RRN3	14.04 ± 0.06	15.00 ± 0.00	12.22 ± 0.23
RRN6	17.07 ± 0.12	12.15 ± 0.26	16.37 ± 0.34
RRN7	14.07 ± 0.13	13.22 ± 0.23	14.26 ± 0.28
RRG1	17.24 ± 0.28	16.37 ± 0.34	16.07 ± 0.12
RRG2	17.41 ± 0.36	16.00 ± 0.00	15.70 ± 0.61
RRB12	10.04 ± 0.06	09.05 ± 0.06	09.25 ± 0.36
RRG3	12.22 ± 0.19	12.02 ± 0.03	13.30 ± 0.34
RRN8	11.18 ± 0.17	10.11 ± 0.19	10.22 ± 0.19
RRN9	14.11 ± 0.20	13.07 ± 0.12	11.06 ± 0.05
RRB10	12.33 ± 0.34	11.48 ± 0.42	11.40 ± 0.53
RRG11	11.54 ± 0.47	10.33 ± 0.57	10.33 ± 0.58
Streptomycin (1 %)	–	18.33 ± 0.57	18.73 ± 0.38
Fluconazole (1 %)	17.33 ± 0.57	–	–
DMSO (10 %)	–	–	–

*Isolate showing potent activity is highlighted in bold; values indicate mean diameter of inhibition zones (mm) ± standard deviation (n = 3); DMSO: dimethyl sulphoxide.

Based on its strong and consistent antimicrobial activity, along with previously confirmed plant growth-promoting traits such as phosphate solubilisation and IAA production, *Colletotrichum* sp. (RRB2) was selected for molecular identification via ITS-rDNA sequencing to validate its taxonomic species characterisation. Additionally, GC–MS analysis was also conducted to characterise the bioactive metabolites responsible for its antimicrobial effects. These investigations aim to enhance the understanding of its functional potential and support its development as a multifunctional microbial agent in sustainable agriculture and natural antimicrobial applications.

Molecular characterisation and phylogenetic analysis

Molecular identification and phylogenetic characterisation of the potent isolate *Colletotrichum* sp. RRB2 were performed using the ITS-rDNA region. The obtained ITS sequence was analysed, annotated and submitted to the GenBank database under the accession number OQ186905. A BLASTn search revealed that the sequence shared 99−100 % similarity with *C. gloeosporioides* accessions, confirming its identity at the species level.

The phylogenetic tree was constructed using MEGA11 software, employing the ML method based on the Kimura 2-parameter model with the GTR+I+G nucleotide substitution scheme. The robustness of the tree topology was assessed with 1000 bootstrap replicates and bootstrap support values ≥ 50 % are

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displayed at the respective nodes. The resulting ML tree (Fig. 5) showed that isolate RRB2 clustered closely with other known endophytic strains of *C. gloeosporioides*, including isolates MR2 (MN565959.1), CR-5 (KC493156.1), JX-1 (HQ645079.1), JX-19 (HQ645082.1) and C37A (MW080959.1), with high bootstrap support (up to 100 %). This close association further confirms the endophytic nature of RRB2 and supports its taxonomic placement within the *C. gloeosporioides* species complex. The outgroup taxon *Didymella bryoniae* strain FG58 (EU030365.1) was used to root the tree.

Gas chromatography-mass spectrometry characterisation of crude secondary metabolites of the isolate RRB2

The potent isolate *C. gloeosporioides* (RRB2), which exhibited notable plant growth-promoting traits, including IAA production, phosphate solubilisation and significant antimicrobial activity, was further subjected to GC-MS analysis to characterise and identify the potential bioactive compounds.

The GC-MS chromatogram of the crude ethyl acetate extract of *C. gloeosporioides* (RRB2) resulted in the tentative identification of several compounds based on their mass spectral matches at different retention times (Fig. 6). The major compounds identified included 2-coumaranone, 2,4,6-Cycloheptatrien-1-one, Benzeneacetic acid, Benzaldehyde 3-benzyloxy-2-fluoro, Undec-2-enal, Pyrrolo[1,2-a]pyrazine-1,4-dione and Indole-3-acetic acid.

These metabolites have been reported in literature for their wide range of pharmacological properties, such as antimicrobial, antitubercular, antifungal, antioxidant, cytotoxic, anticancer and anti-inflammatory activities (Table 4). The identification of such potentially bioactive compounds from *C. gloeosporioides* (RRB2) suggests promising applications in pharmaceutical development, cancer therapeutics and novel antimicrobial agent discovery.

Discussion

Orchids are known to harbour diverse fungal endophytes within their tissues, which can influence plant health, physiology and resilience to environmental stress (4). In the present study, 57 endophytic fungal isolates were obtained from the leaves of *R. retusa* collected from three different regions of Assam—Baihata Chariali, Nalbari and Guwahati. The highest colonisation frequency was recorded in samples from Baihata Chariali. The isolates were predominantly from the phylum Ascomycota, with *Colletotrichum* emerging as the most dominant genus, followed by *Rhizoctonia* and a few non-sporulating forms. Previous reports have similarly highlighted *Colletotrichum* as a dominant orchid endophyte (27). However, this is the first detailed study documenting such a diverse community of *Colletotrichum* species as endophytes in *R. retusa*. Interestingly, while a previous study reported *Rhizoctonia* from the roots of *R. retusa*, this study also recovered *Rhizoctonia* from leaf tissues, suggesting that this fungal genus may colonise both below- and above-ground tissues of the host (28). The absence of detectable PGP traits in the *Rhizoctonia* isolates may indicate that their association with *R. retusa* is not primarily related to the tested growth-promoting mechanisms. Their ecological role may instead involve other functions, such as nutrient exchange, tissue colonisation, or interactions with other members of the endophytic community, which were not assessed in the present study.

An important outcome of this study is the demonstration of PGP traits among the *Colletotrichum* isolates. All *Colletotrichum* species showed positive activity for both IAA production and phosphate solubilisation, two crucial traits that support root development and nutrient uptake. Quantitative IAA analysis confirmed significant auxin production by RRB2 and RRB1, especially in the presence of L-tryptophan, a known precursor of IAA biosynthesis. The enhanced production in the presence of L-tryptophan suggests that IAA biosynthesis in these isolates may be influenced by precursor availability. The IAA yields recorded for RRB2 and RRB1 (2.55–2.76 mg/mL) were relatively high compared with those reported for other orchid-associated fungi. For instance, an orchid-associated *C. gloeosporioides* isolate was reported to produce approximately 0.244 mg/mL IAA under L-tryptophan-supplemented conditions (29). Thus, the IAA production observed in the present study was substantially higher than that reported in the previous study. However, direct comparison should be made with caution, as IAA production can vary considerably depending on the culture medium, L-tryptophan concentration, incubation period and assay conditions. Additionally, phosphate solubilisation was evident through halo zone formation on Pikovskaya's agar, with RRG8 showing the highest solubilisation index, followed by RRN4, RRB2 and RRB8. These observations are consistent with earlier studies showing that orchid-associated endophytes can significantly enhance plant nutrient acquisition and development (27, 30). In contrast, the absence of PGP activity in *Rhizoctonia* suggests functional variation among endophytic taxa, indicating that not all fungal associates necessarily contribute to plant growth through IAA production or phosphate solubilisation. The absence of detectable siderophore production may reflect strain-specific differences in iron acquisition strategies or adaptation to the nutrient conditions within the host tissue.

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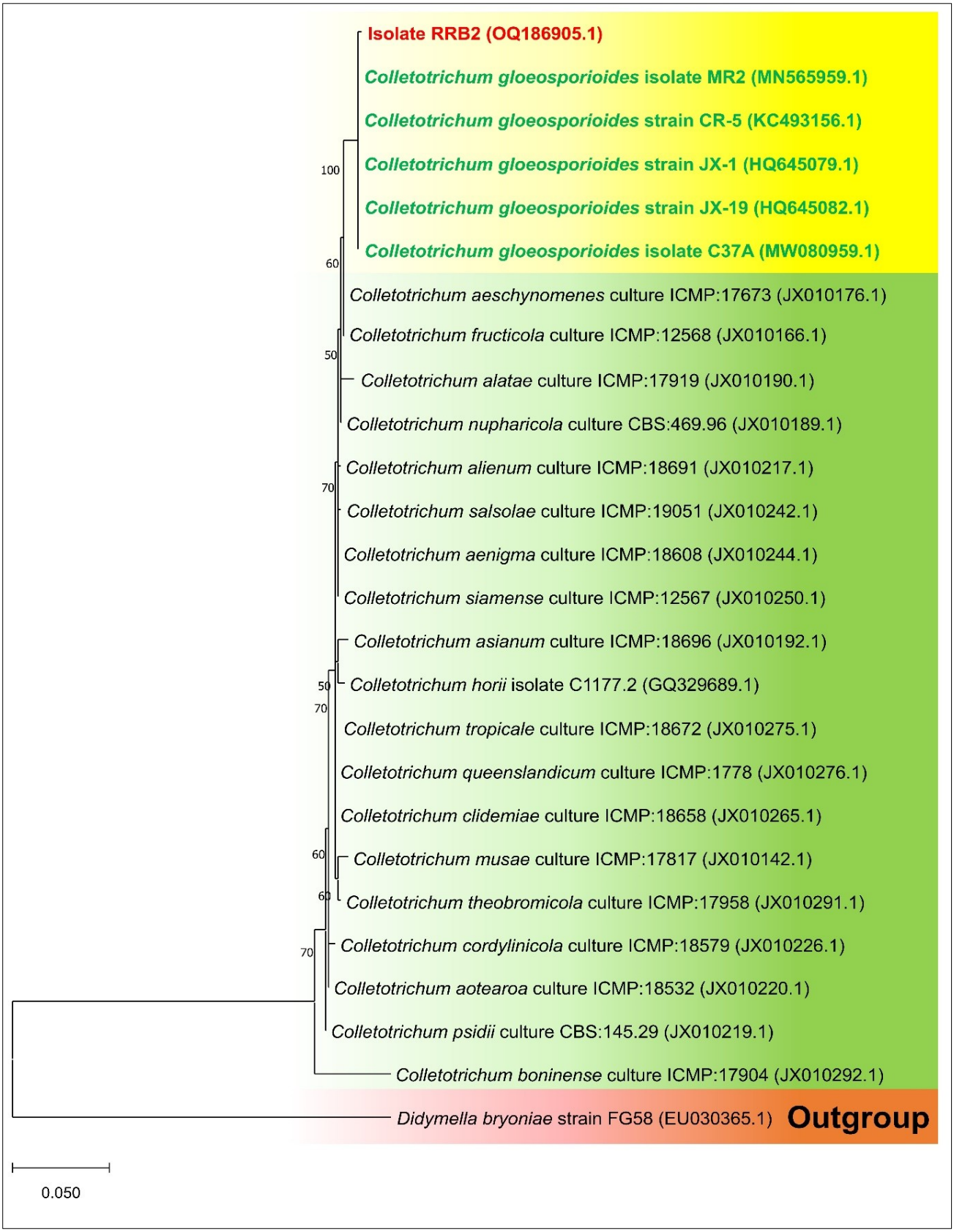


Fig. 5. Maximum likelihood phylogenetic tree of *Colletotrichum* species, including isolate RRB2, constructed using ITS-rDNA gene sequences. Bootstrap values (≥ 50 %) based on 1000 replicates are shown at the corresponding nodes. The sequence generated in this study (RRB2) is highlighted in bold red. GenBank accession numbers are provided after each taxon name. *Didymella bryoniae* strain FG58 (EU030365.1) was used as the outgroup.

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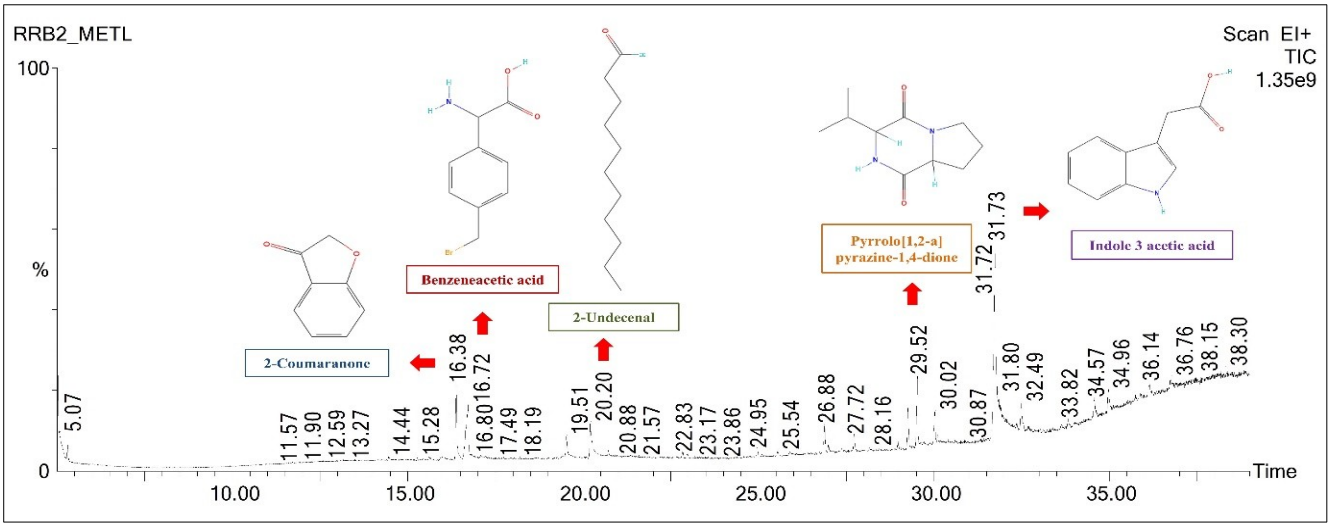


Fig. 6. GC-MS chromatogram of the crude ethyl acetate extract obtained from C*olletotrichum gloeosporioides* (RRB2).

Table 4. Major compounds tentatively identified through GC-MS analysis in the crude ethyl acetate extract of *Colletotrichum gloeosporioides* RRB2

Retention time (min)	Area	Area %	Major compounds	Reported bioactivities	References
16.378	12148496.0	3.176	2-Coumaranone; 2,4,6-Cycloheptatrien-1-one	Antimicrobial and antioxidant activities	(17)
16.723	15328933.0	4.008	Benzeneacetic acid; Benzaldehyde, 3-benzyloxy-2-fluoro	Antimicrobial; antitubercular; cytotoxic; antimycobacterial	(18, 19)
20.205	10808295.0	2.826	2-Undecenal	Antimycobacterial; antiprotozoal	(20, 21)
29.518	10772900.0	2.817	Pyrrolo[1,2-a]pyrazine-1,4-dione	Antifungal; antibacterial; antioxidant activities	(22, 23, 24)
31.734	66251884.0	17.322	Indole-3-acetic acid	Antimicrobial; cytotoxic; anticancer; antioxidant; ant-inflammatory activities	(25, 26)

Endophytic *Colletotrichum* strains may rely on alternative mechanisms for iron acquisition while retaining the ability to produce IAA and solubilise phosphate. However, no positive control was included in the siderophore assay; therefore, the negative results should be interpreted with caution, as the assay was not validated using a known siderophore-producing organism. Ecologically, the absence of detectable siderophore production may indicate that siderophore-mediated iron acquisition is not a major detectable trait under the tested conditions, although alternative iron-acquisition mechanisms cannot be excluded.

Alongside growth-promoting attributes, the endophytic fungi also exhibited potent antimicrobial activity. The ethyl acetate extracts of several isolates —particularly *C. gloeosporioides* (RRB2)— demonstrated the highest inhibition against *E. coli*, *S. aureus and C. albicans*, with inhibition zones comparable to those produced by standard antibiotics such as streptomycin and fluconazole. This broad-spectrum antimicrobial activity of *C. gloeosporioides* (RRB2) is particularly noteworthy, as it complements its strong PGP performance, positioning it as a multifunctional endophyte of significant biotechnological interest. Other *Colletotrichum* isolates also showed moderate antimicrobial effects, whereas isolates belonging to *Rhizoctonia* and the non-sporulating morphotypes displayed minimal or no inhibitory activity.

However, to understand the bioactive basis of its dual functionality, *C. gloeosporioides* (RRB2) was further subjected to molecular identification and GC-MS characterisation. Phylogenetic analysis based on ITS-rDNA sequences confirmed its identity, clustering it closely with established endophytic strains of *C. gloeosporioides*. Moreover, GC-MS analysis of the ethyl acetate extract resulted in the tentative identification of several compounds based on mass spectral matching, including IAA,

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pyrrolo[1,2-a]pyrazine-1,4-dione, benzeneacetic acid derivatives and 2-coumaranone. Some of these tentatively identified compounds have previously been reported to exhibit various biological activities (26, 31); however, their identities and individual contributions to the antimicrobial activity observed in the present extract require further confirmation. Therefore, the GC-MS findings provide a preliminary chemical profile of the extract and the detected compounds should not be considered definitively identified as bioactive compounds without further purification and structural confirmation.

Conclusion

The present study demonstrates that *Rhynchostylis retusa* (L.) Blume hosts a rich and functionally significant community of endophytic fungi, with *Colletotrichum* species predominating within the foliar tissues. Among these isolates, *Colletotrichum gloeosporioides* (RRB2) emerged as a particularly promising strain, exhibiting plant growth-promoting (PGP)-related traits, including indole-3-acetic acid (IAA) production and phosphate solubilisation, while also displaying broad-spectrum antimicrobial activity against clinically relevant human pathogens.

The gas chromatography-mass spectrometry (GC-MS) analysis of the ethyl acetate extract of *C. gloeosporioides* (RRB2) resulted in the tentative identification of several compounds based on mass spectral matching. Although some of these tentatively identified compounds have previously been reported to exhibit various biological activities, their identities and individual contributions to the antimicrobial activity observed in the present extract were not confirmed in this study. Therefore, the GC-MS findings provide a preliminary chemical profile of the extract and further purification and structural characterisation are required to confirm the identities and biological activities of the detected compounds. These findings indicate the potential of such multifunctional endophytes for further investigation in sustainable agriculture and antimicrobial research.

Future research should focus on field-based validation of plant growth-promoting effects, comprehensive metabolite purification and characterisation and elucidation of the molecular mechanisms underlying these beneficial interactions. The present findings are limited by the *in vitro* assessment of PGP traits and antimicrobial activity, the use of crude extracts for antimicrobial assays and the tentative nature of compound identification by GC-MS. Additionally, incorporating endophytic fungi into conservation and propagation strategies for medicinal orchids like *R. retusa* could provide an eco-friendly approach to biodiversity preservation while unlocking their broader biotechnological potential.

Acknowledgements

The authors gratefully acknowledge the Department of Science and Technology (DST), Government of India, for providing academic and laboratory support under the CURIE scheme at Handique Girls' College. The authors also express their sincere gratitude to the Head, Department of Botany, Gauhati University, for providing the necessary facilities and support to carry out this study.

Authors' contributions

SW conducted the methodology, investigation, data analyses and prepared the original draft of the manuscript. SS assisted with methodology, data processing, bioinformatics support and phylogenetic analysis and participated in writing, reviewing and editing the manuscript. PD and SND participated in the preparation, review and editing of the manuscript. KT was involved in the methodology and supervision of the study, provided resources and assisted in preparing, reviewing and editing the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest

Authors do not have any conflict of interests to declare.

Ethical issues

None

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

During the preparation of this work, the authors used ChatGPT to improve the language and readability of the manuscript. Following the use this tool, the authors reviewed and edited the content as needed and take full responsibility for the final content of the publication.

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https://doi.org/10.14719/pst.14810

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