Research Article

Antagonistic Evaluation of Bioactive Metabolite from Endophytic Fungus, *Aspergillus flavipes* KF671231

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Of the total 40 endophytic fungi isolated from foliar tissues of medicinal plant *Stevia rebaudiana* Bertoni, a fungal isolate, *Aspergillus flavipes*, was subjected to bioassay guided fractionation. The fractionation was found active against medicinal plant pathogen *Sclerotinia sclerotiorum* with an inhibition zone of 29 mm in size. Further the metabolite was extracted which shows 20% growth inhibition in 24 h and 46% after 48 h, respectively. Bioassay guided chemical compound was identified as 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester. On the basis of morphological characters and rDNA sequencing of ITS region the endophyte was identified as *Aspergillus flavipes* which showed promising plant growth promotory properties.

1. Introduction

Bioprospecting refers to the search for novel products of economic importance from animal, plant, and microbial sources [1]. Traditionally we always relied on natural remedies for treating and healing our ailments. Natural products have been exploited for human use for thousands of years and plants have been a major source of compounds of medicinal use. Plants are found to be associated with microorganisms which are capable of producing molecules possessing remarkable biological activities. Microorganisms that live in the intercellular spaces of different parts of plants showed no evident expression of their presence and are called endophytes [2]. The relationship between plant and endophyte is considered to be mutualistic, the former being protector and feeder for the latter which in return produces bioactive substances that provide protection to plant and also enhance the growth and competitiveness of the host in nature [3].

Sclerotinia sclerotiorum, a phytopathogenic fungus, occurs worldwide and infects a wide array of plants, resulting in considerable losses. Although a significant number of natural bioactive compounds have been reported to antagonize this fungus [4], *Stevia* (*Stevia rebaudiana* Bertoni; Asteraceae),

an exotic annual plant originating from Paraguay, contains glucosides of a diterpenoid nature, which are used as a low-caloric sweetener in some South American and South East Asian countries [5]. The main active ingredient of *Stevia* is a stevioside, which is 100 to 300 times sweeter than sucrose.

Traditionally, farmers use agrochemicals to protect plants from diseases and to optimise the crop yields. Prolonged and persistence use of pesticides including fungicides and herbicides made several organisms resistant to such chemicals besides causing the environmental pollution. This present study represents a step towards understanding the endophytes as a means to develop effective biocontrol agents for *Stevia rebaudiana* Bertoni with broader implications for use against other crops.

2. Materials and Methods

2.1. Sample Collection. Leaves of *Stevia rebaudiana* Bertoni (Family: Asteraceae) were randomly collected from Misrod Agricultural field, near Bhopal, India (Lg N 2309021' and Lt E 077°27'04.3). Immediately after collection, leaves were washed with tap water and processed for isolation of endophytic fungi.

2.2. Media Preparation. Potato dextrose agar (PDA, Hi-media) was used to isolate endophytic fungi. Chloramphenicol (0.2 g L^{-1}) was added to the medium to avoid bacterial contamination.

2.3. Isolation of Endophytic Fungi. Endophytic fungi were isolated from healthy leaves employing surface sterilization procedure [6]. Briefly, the tissues were surface sterilized with 70% ethanol for 2 min followed by treatment with 4% sodium hypochlorite for 2 min. Finally tissues were washed with sterile distilled water for 2 sec. Sterile leaves were placed on blotting sheet and cut into 5 mm pieces which were transferred to PDA plates. Plates were incubated at 28°C for 3–6 days. Hyphal tips of the developing fungal colonies were transferred to fresh PDA plates to get pure cultures.

2.4. Confrontation Bioassay. Primary screening for antagonism was done by a confrontation assay [7]. The endophyte and the pathogenic fungus (*Sclerotinia sclerotiorum*) were cocultured on 90 mm petri dish containing PDA and incubated at 28°C for 3–5 days to check the interaction between them. A culture of the pathogen *Sclerotinia sclerotiorum* was obtained from the Directorate of Soybean Research, Indore. Plates were observed regularly and antagonism was expressed by the presence of inhibition zone at the point of interaction.

2.5. Molecular Identification of Antagonistic Endophyte. Morphological identification of the organism was carried out at National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, Pune. For molecular identification, total genomic DNA of the endophytic fungus was isolated directly from actively growing mycelium growing in potato dextrose broth (PDB), using CTAB method [8]. DNA amplification was performed by PCR using primer pair ITS1: TCCGTAGGTGAACCTGCGG and ITS4: TCCTCCGCTTGATATGC [9]. PCR was carried out according to the following protocol: initial denaturation at 95°C for 5 min; denaturation at 95°C for 1 min; annealing at 55°C for 45 sec; extension at 72°C for 10 min; steps 2–4 were repeated 35 times. Sequencing of PCR product was carried at Xcelris Labs Ltd, Ahmedabad. The sequenced data was subjected to BLAST algorithm and submitted to Genbank for accession number.

2.6. Plant Growth Promoting Attributes. Plant growth promoting attributes of the antagonistic endophyte such as production of IAA [10] and siderophore [11] and phosphate solubilization activity [12] was assayed both qualitatively and quantitatively.

2.7. Evaluation of Bioactivity. Bioactivity of the culture filtrate against *Sclerotinia sclerotiorum* was evaluated over growth and temperature.

2.8. Influence of Growth Period. A 48 h old fungal culture grown on PDA was inoculated in Erlenmeyer flasks containing 100 mL PDB. The flasks were incubated at 28°C at 120 rpm for 10 d. Culture broth was recovered at 2, 4, 6, 8, and 10 d

after incubation by centrifugation at 12000 rpm for 20 min at 4°C . A disc diffusion assay was carried out to determine the bioactivity of the culture filtrate obtained after 6 d of fungal growth.

2.9. Influence of Temperature. The Erlenmeyer flasks containing 100 mL PDB were incubated at 22, 24, 26, 28, 30, and 32°C at 120 rpm for 6 d to assess the influence of temperature on bioactive metabolite. The culture filtrate was harvested and antagonistic activity of the filtrate was checked by disk diffusion assay against *Sclerotinia sclerotiorum*.

2.10. Extraction of Antifungal Metabolite. For extraction of antifungal metabolite, the culture filtrate was centrifuged at 12,000 rpm for 20 min at 4°C and the culture supernatant (pH 5.8) was acidified (1 N HCl) to a final pH 2.0 [13]. The acidified filtrate was subjected to organic extraction by ethyl acetate (1:1 v/v). Organic extract was dried under vacuum in a rotary evaporator. The dried crude was dissolved in 2 mL of methanol. For antagonistic assay, 40 μL of methanolic extract was impregnated on sterile paper discs; MeOH coated discs were used as control. Plates were incubated at 28°C and inhibition of fungal growth was recorded after 24 h and 48 h. Growth inhibition was calculated as per the following formula:

$$\text{GI} = \frac{(A - B)}{A} \times 100, \quad (1)$$

where A = radial diameter of test fungus; B = radial diameter of test fungus against culture.

2.11. Characterization of Bioactive Compound

2.11.1. Chromatographic Detection and Partial Purification of Bioactive Metabolite. Thin layer chromatography (TLC) was performed on crude extracted from the culture broth of the endophyte (TLC silica gel 60, 20×0.5 mm, Merk Co, Inc.). For this, the crude fraction was spotted (30 μL) on the TLC plate and chromatography was performed by employing solvent system dichloromethane: methanol (95:5 v/v). Spots were visualized by spraying with ceric sulphate; silica residue was extracted and centrifuged and the supernatant was transferred to a microcentrifuge tube. The silica-free supernatant was checked for antifungal activity. Preparative TLC was carried out to obtain the partial purified sample which showed antifungal activity.

2.11.2. GC-MS Analysis. GC-MS analysis was carried out at Central Instrumentation Facility JNU (New Delhi). Gas-chromatography mass spectrometry (GC-MS) analysis of the crude was performed on a Shimadzu GCMS-QP-2010 plus system. RTX-5 Sil MS column ($30\text{ m} \times 0.25\text{ mm id} \times 0.25\text{ film thickness}$) was used for the analysis. The operating conditions of the column were as follows: oven temperature program from 80°C to 210°C at $4^{\circ}\text{C}/\text{min}$ with holding time of 2 min and from 210°C to 300°C at $15^{\circ}\text{C}/\text{min}$ with holding time of 5 min, and the final temperature was kept for 20 min. The injector temperature was maintained at 270°C , and

the volume of injected sample was 0.3 μL , pressure 85.4 kPa, total flow 76.8 mL/min, column flow 1.21 mL/min, linear velocity 40.5 cm/sec, purge flow 3.0 mL/min, split ratio 60.0, ion source temperature 230°C, scan mass range m/z 40–600, and interface line temperature 280°C. The identification of compounds was performed by comparing the mass spectra with data from NIST05 (National Institute of Standards and Technology, US), WILEY 8, and FFNSC1.3 (Flavour and Fragrance Natural and Synthetic Compounds) libraries.

3. Results

3.1. Confrontation Assay and Identification of Potential Antagonistic Endophytic Fungi. The confrontation assay showed strong inhibition of *Sclerotinia sclerotiorum* and produced an inhibition zone of 29 mm after 48 h. The antagonistic endophytic fungus was identified by rDNA sequencing of ITS region wherein it showed 99% similarity with *Aspergillus flavipes* (accession number KF671231).

3.2. Plant Growth Promoting Attributes. *Aspergillus flavipes* was positive for siderophore production as evident by an orange halo on CAS medium. It solubilized inorganic phosphorus as determined by a clear zone around the culture on Pikovskaya's agar. IAA production was confirmed by the appearance of stable pink colour when reacted with Salkowsky reagent. This fungal strain produced $7.3 \mu\text{g mL}^{-1}$ of IAA whereas siderophore level after 2 d was estimated at 22.72 mg mL^{-1} ; endophyte was found as a promising phosphate solubilizer ($12.5 \mu\text{g mL}^{-1}$).

3.3. Extraction and Evaluation of Bioactivity of Metabolite. The culture filtrate from 48 h old culture inoculated with PDB showed no bioactivity on 2 d whereas an inhibition zone of 10 mm was observed on 4 d; maximum bioactivity was obtained on 6 d with zone size of 17 mm. There was reduced bioactivity with further incubation and no antagonism was obtained with 10 d old culture filtrate. The culture filtrate recovered after 6 d of incubation was acidified in the pH range (1–6). The filtrate showed maximum activity at pH 2.0, that is, 19 mm inhibition zone; zone size was reduced to 9 mm at pH 4.0.

The inhibitory activity of the culture filtrate was maximum at 28°C, that is, 15 mm inhibition zone; a 10 mm zone was obtained at 24°C whereas there was further reduction at 30–32°C. During the temperature dependent incubation, biomass was constantly increased up to 28°C (0.5–1.02 g dry wt/100 mL) followed by a decline (Figure 1). The metabolite was extracted through ethyl acetate. The dried extract was dissolved in methanol and subjected to disk diffusion assay. Percent growth inhibition against *Sclerotinia sclerotiorum* was 20.45% after 24 h and 46.2% after 48 h (Figure 2).

3.4. Characterization of Bioactive Compound. Thin layer chromatographic analysis and partial purification of bioactive compound: the crude extract prepared from the cell-free culture filtrates showed strong antagonistic activity against the pathogenic fungus *S. sclerotiorum*. No inhibitory activity

FIGURE 1: Effect of temperature on biomass and inhibition zone (mm). The inhibitory activity of the culture filtrate was maximum at 28°C whereas there was further reduction at 30–32°C. During the temperature dependent incubation, biomass was constantly increased up to 28°C followed by a decline as the temperature increases.

was observed in the supernatant. The crude extract was subjected to TLC analysis for the separation of the antifungal molecule. Two fractions designated as 1st and 2nd were observed when developed in dichloromethane: methanol (95:5) on silica gel TLC plates and sprayed with ceric sulphate. These were eluted out and checked for antifungal activity; only 2nd fraction having $R_f = 0.82$ exhibited the antifungal activity. The spot showed purplish colour when sprayed with ceric sulphate. Preparative TLC was carried out to obtain sufficient crude material for further analysis.

3.5. GC-MS Analysis. The crude extract was partially purified by TLC analysis. The partially purified crude was subjected to GC-MS analysis which showed one sharp peak at RT 14.648 which covers maximum % area (Table 1). This matched at 98% level in the standard library (NIST) with 1,2-benzene dicarboxylic acid, mono(2-ethylhexyl) ester. The antifungal property is likely to be due to this compound (Figure 3).

4. Discussion

Anitha et al. [14] recently reported occurrence of an endophyte from the stem of endemic medicinal plants of Tirumala hills while others have earlier reported *A. flavipes* from inner bark of *Acanthus ilicifolius* [15]. Endophytic presence of *A. flavipes* has also been recorded in flowers of *Calotropis gigantea* [16]. However, to the best of our knowledge this is the first report on *A. flavipes* from the foliar tissues of *Stevia rebaudiana* Bertoni, a medicinal plant. *Sclerotinia sclerotiorum* is the world's most successful and omnivorous fungal pathogen

TABLE 1: Percentage area covered by the antifungal compounds.

Peak	R. time	Area	Area %	Name
1	6.348	119244	1.09	1-Pentanamine, N-nitroso-N-pentyl
2	8.057	331055	3.04	
3	8.594	395652	3.63	
4	9.144	317567	2.92	10,12-Tricosadiynoic acid, methylester
5	9.558	276323	2.54	1,6-Cyclodecadiene,1-methyl-5-Met
6	10.217	156155	1.43	Hexadecanoic acid, methyl ester
7	10.549	974536	8.95	Dibutyl phthalate
8	11.337	326456	3.00	9-Octadecenoic acid (Z)-methyl ester
9	14.648	7995418	73.40	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester
10		10892406	100

FIGURE 2: Disc diffusion assay: (a) control plate, (b) inhibition after 24 hrs, and (c) inhibition after 48 hrs. (a) Control plate having discs impregnated with methanol. (b) Zone of inhibition observed after 24 h on plate having discs impregnated with methanolic extract. (c) Zone of inhibition observed after 48 h on plate having discs impregnated with methanolic extract.

FIGURE 3: Mass spectrum of 1,2-benzene dicarboxylic acid, mono(2-ethylhexyl) ester compound from NIST Library.

with a host range of more than 400 plant species. Despite decades of dedicated efforts, resistant germplasm is still lacking in economically important crops. This was an important reason for screening antagonistic endophytes against this phytopathogen for current study. Matroudi et al. [17] screened three species of *Trichoderma* spp. which showed 85% growth reduction of *Sclerotinia sclerotiorum* in dual culture assay. Rocha et al. [18] observed a reduction in the growth rate of *S. sclerotiorum* ranging from 46.7% to 50.0% by different strains of endophytic fungi isolated from Comfrey (*Symphytum officinale* L.). Powthong et al. [19] studied the fungal extracts

of endophytic fungi for antimicrobial activity against *Bacillus subtilis* and *Candida albicans*. Evaluation of the antifungal activity of *Aspergillus flavipes* from *Stevia rebaudiana* was performed wherein it was maximum on 6th day at pH 2.0. Ethyl acetate worked as the best solvent system for extraction of metabolite. GC-MS analysis of the bioactive metabolite showed that the compound is 1,2-benzene dicarboxylic acid, mono(2-ethylhexyl) ester which showed antifungal activity against *S. sclerotiorum*; a similar compound along with 1-tetradecamine, N,N-dimethyl, squalene, and phytol were reported from *Muscodora tigerii*, as novel endophyte from *Cinnamomum camphora* [20]. This showed 100% inhibition of *Alternaria alternata* and 71.67% inhibition of *Rhizoctonia solani*; the compounds were also active against *Candida albicans* and *Staphylococcus aureus*. Present investigation was carried out against *S. sclerotiorum* which showed 20% growth inhibition in 24 h and 46% growth inhibition in 48 h. We also recovered *Alternaria alternata* as endophyte from healthy leaves of *Stevia rebaudiana*. However, production of 1,2-benzene dicarboxylic acid, mono(2-ethylhexyl) ester produced by *Aspergillus flavipes* might prevent *Alternaria alternata* to cause infection in *Stevia rebaudiana* although leaf spot disease caused by *A. alternata* in *Stevia rebaudiana* was

reported [21]. A marine *Burkholderia cepacia* also produced 1,2-benzene dicarboxylic acid, (2-ethylhexyl) ester which showed potential antibacterial activity against *Aeromonas hydrophila*, *Edwardsiella tarda*, and *Vibrio ordalii* [22]. These studies showed that the compound 1,2-benzene dicarboxylic acid, (2-ethylhexyl) ester is a potential antifungal and antibacterial in endophytic fungi.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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