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Leucobacter aridicollis strain SASBG215: A Novel biocontrol agent against *Colletotrichum orbiculare*

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ABSTRACT: In India, bottle gourd is a common cucurbitaceous vegetable that is sensitive to a variety of fungi. *Colletotrichum* sp., a common anthracnose disease on various crops, was discovered in Coimbatore, Tamil Nadu, causing unusual symptoms such as leaf spots, vertical water-soaked streaks on petioles, stems, crotches and severe stem splitting at the crown area. Farmers apply fungicides indiscriminately to control the disease, raising the expense of cultivation and leaving residues in the produce. The goal of this work was to find a successful bacterial endophyte against the disease that addresses the two challenges of high input cost and residues in a reasonable manner. An endophyte, *Leucobacter aridicollis* strain SASBG215, reduced the mycelial growth of *Colletotrichum orbiculare* by 73.51 percent in dual culture assays. GC-MS analysis of excised agar from the inhibition zone revealed the real metabolites suppressing the pathogen. Eleven distinct metabolites were profiled in GC-MS analysis, including Clindamycin; 4-Octanol, 4,7-dimethyl-; Pentanoic acid, propyl ester; 2,3,4,4-Tetramethyl-pentane-1,3-diol; Sucrose; 1-Pentanol, 2-methyl-, acetate; Cyclohexanol, 2-(2-propynyloxy)-, trans-; Cyclohexanol, 2-(2-propyn. Suitable organic formulations of *Leucobacter aridicollis* can be included in the management schedule of this pathogen.

Keywords: *Colletotrichum orbiculare*, *Leucobacter aridicollis*, Biocontrol, GC-MS, dual culture.

INTRODUCTION

Bottle gourd (*Lagenaria siceraria* (Molina) Standl.) is a cucurbitaceous vegetable used in many tropical and temperate countries. Fruit contains vitamins, proteins, choline, minerals, terpenoids, flavonoids, etc. Triterpenoids, sterols, cucurbitacins, flavones, C-glycosides, and -glycosides have been identified from L. siceraria. Researchers have studied the plant's antianxiety, antidepressant, diuretic, antimicrobial, cytotoxic, antihyperlipidemic, cardioprotective, analgesic, anti-inflammatory, anti-helminthic, anti-hyperglycaemic, antihepatotoxic, anti-urolithiasis, antistress, antiulcer, anticancer, hepatoprotective, anthelmintic, immunomodulatory, and antioxidant properties (Zahoor *et al*., 2021). Despite its economic and medicinal importance, the crop is susceptible to a variety of fungal, bacterial, and viral diseases, including downy mildew, powdery mildew, leaf spots, Cucumber mosaic virus (*CMV*) etc., (Zitter *et al.,* 1998; Saha, 2002).

Microbial communities are used as biocontrol agents, biofertilizers, bioinoculants, and stress modulators in agriculture and horticulture. Endophytes live in host tissues without generating infection. Endophytic communities have great potential as biocontrol agents and promote plant growth, making them a non-toxic choice for managing plant diseases. Endophytes are favoured over other plant growth promoting rhizobacteria for their better survival and adaption. Endophytic bacterial population from bottle gourd twigs was isolated and tested against stem splitting pathogen *Colletotrichum orbiculare*. Non-volatile compounds expressed in inhibitory zone were detected by GC-MS.

MATERIALS AND METHODS

Colletotrichum orbiculare was isolated from symptomatic bottle gourd samples in Coimbatore, Tamil Nadu, during *kharif* season, 2020-21. The pathogen was isolated according to (Nuraini and Latiffah, 2018) with slight modifications. 5mm pieces from affected regions were cut with a sterile surgical blade and surface sterilized in 1% sodium hypochlorite solution for three minutes. Five minutes were spent drying surface-sterilized specimens on pre-sterilized blotting paper. These air-dried parts were cultured at room temperature on PDA plates and single hyphal tips were sub cultured for obtaining pure cultures.

For fungus identification, DNA was isolated using the CTAB method according to Mishra *et al.* (2014) and the nrITS region was amplified using primers *viz*., ITS1 (5' CTTGGTCATTTAGAGGAAGTAA 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White *et al*., 1990). The reaction was carried out in Eppendorf® mastercycler with a 40µl reaction mixture consisting of 20µl Taq DNA polymerase master mix Red (Ampliqon®), 4µl forward primer, 4µl reverse primer, 4µl double distilled water and 8µl of genomic DNA. The PCR conditions include an initial denaturation at 95°C for 4 minutes followed by 35 cycles of denaturation at 95°C for 4 minutes; primer annealing at 62°C for 1 minute; extension at 72°C for 2 minutes and final extension at 72°C for 8 minutes. The PCR result was sequenced using ABI-3730 Prison automated DNA analyzer EBT Ver. 3.1. (Barcode Bio Science, Bangalore, India). The sequencing result was compared with NCBI-GenBank database using BLASTn algorithm (www.ncbi.nlm.nih.gov) to acquire accession number. The bottle gourd bacterial endophytes were extracted from young leaves according to Sriskandarajah *et al*., (1993). Aseptically cut bottle gourd leaves were surface sterilized in 1% sodium hypochlorite for 3 minutes and rinsed in sterile distilled water. The pieces were then soaked in 70% ethyl alcohol for 1 minute and rinsed in sterile distilled water three times. Using sterile blotting paper, surface-sterilized plant fragments were dried and 1g of surface-sterilized leaf tissues were crushed with 5 ml of peptone salt buffer. 1ml of solution was poured plated with nutrient agar and incubated at 35°C for bacterial growth. Pure bacterial colonies were streaked steaked in fresh nutrient agar plates. Final wash water was poured plated in nutrient agar plates and cultured at 35°C for 24 hours to check sterility (Zhan-Bin *et al*., 2013).

Dual culture test was conducted in PDA plates with a 5mm fungal pathogen disc in the centre and 5µl of 8-hour-old actively growing bacterial endophyte culture on four sides of fungal disc 5mm away from petri plate rim. Control plates contained solely fungal discs. Once control plates were full, the diameter of fungal discs in dual culture plates was measured. Formula for calculating % inhibition is as follows.

$$\text{PDI} = \frac{\text{Growth in control} - \text{Growth in treatment}}{\text{Growth in control}} \times 100$$

The treatments were replicated three times, and the PDI was assessed and compared with CRD in OPSTAT software.

For establishing the identity of potent bacterial endophyte, total DNA was extracted by CTAB method of Doyle and Doyle, (1990). One ml of 24 h old actively growing bacteria in nutrient broth was put into 1.5 ml Eppendorf tube and centrifuged at 7000rpm for 10min to retain pellet. One ml of broth was added to same Eppendorf tube and centrifuged at 7000rpm for 10min to retain pellet. One ml of sterile water was added to pellet and centrifuged at 7000rpm for10 min to eliminate residues of broth and the pellet was suspended in 675µl of Genomic DNA buffer (CTAB) and incubated at 37°C for 30 min with vortexing at every 10 min interval. 75 µl of 10 per cent SDS was added and incubated at 65°C for two hours with vortexing at every 10 min. The tube was centrifuged at 11000 rpm for 10 min at 4°C and the supernatant was collected in fresh Eppendorf tube to which equal volume of Phenol: Chlorophorm: Isoamylalcohol (25:24:1) was added. The tube was inverted multiple times and centrifuged at 11000 rpm for 10 min at 4°C that led to creation of three phases. Aqueous phase was transferred to another Eppendorf tube and the DNA was precipitated by adding 0.6 volume of Isopropanol with incubation for one hour at -20°C. The tube was centrifuged at 12000 rpm for 15 min at 4°C to retain DNA pellet which was washed in 70 per cent ethyl alcohol twice. The DNA pellet was air dried and suspended in double sterilized distilled water for subsequent usage.

The extracted bacterial endophyte DNA was amplified in PCR with universal 16S rRNA primers 27F (5 - AGAGTTTGATCCTGGCTCAG-3) and 1492R (5 - GGTTACCTTGTTACGACTT-3) with following conditions: initial denaturation at 95°C for 5min, denaturation at 94°C for 30 seconds, primer annealing at 58°C for 30 seconds, extension at 72°C for 40 seconds and final chain extension at 72°C for 10min (Watanabe *et al*., 2001). The PCR was run with a 40µl reaction mixture containing 20µl Amplicon® oligonucleotide mixture, 4µl forward primer, 4µl reverse primer, 4µl double sterilized distilled water and 8 µl of bacterial DNA of 54ng/µl concentration. 5 µl of amplified PCR product was loaded on to 1.2% (w/v) ethidium bromide-stained agarose gel and electrophoresed at 75 V for 1.5 hours. The gel was visualized under UV light (UVITECH, Cambridge Inc.) for amplification of said region between the primers. The Amplified PCR product was sequenced in ABI-3730 Prison automated DNA analyser EBT Ver. 3.1 (Barcode Bio Science, Bangalore, India). The resulting sequence was edited with BIOEDIT software to obtain full length sequence of 16S rRNA of the endophyte. The sequence was compared with NCBI-GenBank database using blastn algorithm (www.ncbi.nlm.nih.gov) and was submitted to obtain accession number. The nucleotide sequence was searched for sequence homology using BLAST search against Gen Bank database (http://www.ncbi.nih.gov/BLAST). The related

bacterial 16S rRNA gene sequences retrieved from the GenBank database were used for phylogenetic analysis. The phylogenetic tree was constructed with bootstrap for 1000 times using the neighbor-joining method (Saitou and Nei, 1987; Tamura *et al*., 2011) and compared in MEGA XI software.

GC-MS analysis was used to estimate secondary metabolites responsible for real pathogen inhibition. The approach of Cawoy *et al*., (2014) for extracting samples from inhibitory zones was followed with minor adjustments. 10g of Agar samples from the inhibitory zone were combined with 100ml of acetonitrile: water (1:1; v/v) in a 100ml flask. This mixture was sonicated twice for 30 seconds at 30% of the device's power (Bandelin Sonoplus HD 2070). To remove any agar particles, the samples were homogenized (vortex), centrifuged, and filtered. The obtained filtrates were analysed using GC-MS on a PerkinElmer Claurus SQ8C instrument. This instrument had a DB-5 MS non-polar capillary column with the following dimensions: 30mts length × ID 0.25 mm × film 0.25 mm IM. The carrier gas was helium, and the injection rate was 1 microliter per minute. The compounds particularly expressed in successful endophyte fungal inhibition zone were computed using peaks obtained from PDA where fungus alone was grown, interaction zone, and PDA where endophyte alone was cultivated. The data system's inbuilt libraries for searching and matching the spectrum were the NIST ms/ms database, mainlib, and replib EI libraries. Compounds with spectral fit values of 700 or higher were considered for identification using the MS data library and comparison of the spectrum acquired through.

Plant growth promotion parameters of bacterial endophyte (Qualitative tests) were conducted following standard protocols. Production of IAA, catalase, amylase, cellulase, nitrogen fixation and phosphate solubilization were carried out according procedures laid down in Digar *et al*. (2013). For assaying IAA production, endophytic bacteria was inoculated in YMD broth tube aseptically and incubated at 35°C. 1ml of supernatant was mixed with 2ml of Salkowski's reagent (2% 0.5 $FeCl_3$ in 35% $HClO_4$ solution) and maintained in dark. The optical density (OD) was recorded at 530nm after 30min and 120min to determine IAA production. For assaying catalase production, endophytic bacteria was streaked on nutrient agar slant and incubated at 35°C for 24 hrs. 1 % Hydrogen Peroxide was flooded on the actively growing bacteria to observe bubble formation. For assaying amylase production, endophytic bacteria was streaked on nutrient agar media enriched with starch@ 2g/l and incubated at 35°C for 24 hrs. Next day the plates were flooded with Iodine reagent to observe clear zone around streaks. For assaying cellulase production, endophytic bacteria was streaked on CMC agar media plates and incubated at 35°C for 24 hrs. Production of clear zone around colonies and degradation of Cango Red indicated a positive reaction. Similarly, production of clear zone around bacterial streaks on YEMA plates (yeast extract mannitol agar) and on Pikovaskya agar media plates after incubation at 35°C for 24 hrs indicated bacterial production of nitrogen and phosphorous from the media respectively.

Ammonia production was assayed according to (Hansen, 1930). endophytic bacteria was inoculated in nutrient broth and incubated at 35°C for 24 hrs. Development of yellow colour up on addition of Nessler's reagent indicated ammonia production. Production of Acetoin and 2,3 butanediol were done according to procedures of Shanmugaraj *et al*., (2021). The endophytic bacteria was inoculated in sterilized MRVP broth tubes and incubated at 35°C for 24 hrs. Development of cherry red colour after addition of VP-1 reagent (alpha naphthol) and VP-2 reagent (40% potassium hydroxide) reagent indicated positive reaction

Production of chitinase was assayed according to Chernin *et al*., (1995). The endophytic bacteria was streaked on colloidal chitin agar medium and incubated at 35°C for 24 hrs. Production of clear zone around streaked colonies indicated a positive reaction. Zinc solubilization was assayed according to (Fasim *et al*., 2002). Endophytic bacteria was streaked on Tris-minimal medium supplemented separately with zinc oxide (ZnO) [1.244 /l] = 15.23mM and zinc phosphate Zn_3 $(PO4)_2$[1.9882 g/l] = 5.0mM at a concentration equivalent to 0.1% Zn. After incubation at 35°C for 24 hrs, production of clear zone around colonies indicated a positive reaction. Production of protease was assayed according to Denizci *et al*. (2004). Endophytic bacteria was streaked on Skim Milk agar (SMA: 100% sterile of 900 mL media tryptic soy agar (TSA), 10% concentration of 100 mL of sterile skim milk) plates and incubated at 35°C for 24 hrs. Production of clear zone around colonies indicated a positive reaction

The design of the experiment and the statistical analysis was performed using OPSTAT software package. The comparative analysis of volatile compounds was followed using ClustVis online (https://www.biit.cs.ut.ee/clustvis/).

RESULTS AND DISCUSSIONS

The fungal growth from the infected twigs was sub cultured on to fresh PDA plates. The growth of the fungus on PDA medium was whitish for seven days. Later, turned to olivaceous green and then brown colour. Dark brown to orange colour spore masses produced as ooze are seen after 30days of growth. The conidia were hyaline, single celled, oval with rounded ends (Fig. 1 a-e). The pathogen produced all the typical symptoms *viz*., leaf spots, vertical water-soaked streaks on petioles, stems, crotches and splitting of stem at crown region. Subsequently, same pathogen had been reisolated fulfilling Koch's postulates. The fungal DNA was clearly amplified with ITS1 and ITS4 primers, the amplified product was sequenced and compared in NCBI blastn search to establish the molecular identity of the pathogen as *Colletotrichum orbiculare.* The sequence was submitted in NCBI database with accession number ON398802.

Fig. 1. Conidia of *Colletotrichum orbiculare* (a); Leaf spots (b); Stem splitting at field level (c); Pathogenicity test showing leaf spots (d) and stem splitting (e).

Now a days indiscriminate use of plant protection chemicals has increased many folds which can cause health hazards, on the other hand biological control of the plant pathogens is a healthier solution where microorganisms or their products will be deployed against plant pathogens (Bahadir torun *et al*., 2018; Ved Ratan *et al*., 2018). Endophytes have dual advantage of quick adaption in host tissues and resist invading plant pathogens through encouraging plant defences and plant growth promotion.

Fifteen bacterial endophytes were isolated from bottle gourd and tested for their efficacy against *Colletotrichum orbiculare* in dual culture tests. The bacterial endophytic strains SASBG201, SASBG203 and SASBG215 recorded 73.29, 71.08 and 73.51 per cent reduction of radial mycelial growth over control respectively (Table 1 and Fig. 2).

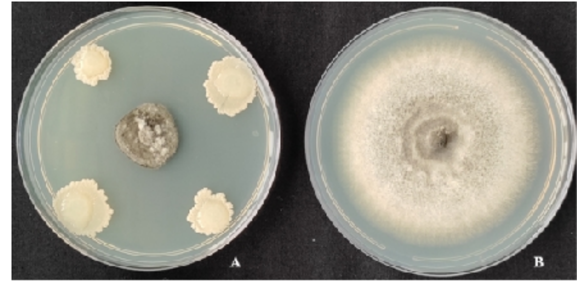

Fig. 2. Mycelial growth inhibition of *Colletotrichum orbiculare* by *Leucobacter aridicollis* strain SASBG215 in dual culture experiments. A- SASBG215 in dual confrontation assay; B-Control.

Table 1: *In vitro* per cent inhibition of mycelial growth of *Colletotrichum orbiculare* by *Leucobacter aridicollis* strain SASBG215

Treatment No	Treatment (endophytes)	Mean of highest diameters of fungal growth in petri plate (cm)			PDI
		R1	R2	R3	
1	SASBG 201	2.45	1.95	1.65	73.29
2	SASBG 203	2	2.7	1.85	71.08
3	SASBG 206	2.2	2.3	2.4	69.54
4	SASBG 208	7	6	4.5	22.74
5	SASBG 214	2.55	2.5	2.5	66.67
6	SASBG 215	2	1.75	2.25	73.51
7	SASBG 217	2	2.2	2.2	71.74
8	SASBG 221	3.35	3.2	3.35	56.29
9	SASBG 223	3	3.35	3.4	56.95
10	SASBG 202	5.1	5	5.35	31.79
11	SASBG 204	5.1	4.9	5.2	32.89
12	SASBG 205	4.4	4.9	4.35	39.74
13	SASBG 207	5.65	5.65	6	23.62
14	SASBG 209	6	6.1	6.1	19.65
15	SASBG 210	6.1	6.1	5.7	20.97
16	Control	7.65	7.5	7.5	
	C.D.	0.914			
	SE(m).	0.316			
	C.V.	4.800			

The DNA from bacterial endophytic strain SASBG215 was amplified with 16S rRNA universal primers 27F and 1492R which showed a clear band at 1500bp in agarose gel electrophoresis (Fig. 3). The BLASTn search of sequenced product NCBI database showed a close similarity with *Leucobacter aridicollis.* The sequence was submitted in NCBI database with accession number ON171370 with strain name SASBG215. The phylogenetic tree was built by neighbor joining method with 16 other *Leucobacter* spp. sequences showed a close cluster(s) formation in MEGA XI software. The strain SASBG215 showed close lineage with HE858276.1 *Leucobacter aridicollis* strain BO2 reported previously from Nigeria (Fig. 4).

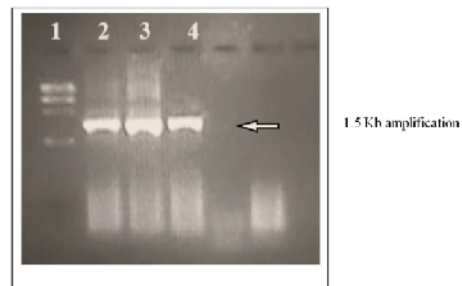

Fig. 3. Amplification of strain SASBG 215 with 16s rRNA universal primers 27F and 1492R: Well1-1Kb ladder, 2-SASBG201, 3-SASBG203, 4-SASBG215.

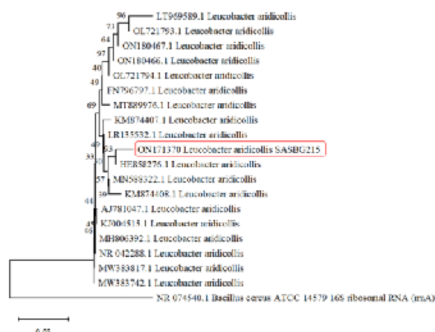

Fig. 4. Phylogenetic tree based on the nucleotide sequences of 16s rRNA of gene of *Leucobacter aridicollis.* The strain SASBG215 with accession number ON171370 grouped with strain HE858276.1 reported from Nigeria.

The *Leucobacter aridicollis* strain SASBG215 was further analyzed for plant growth promoting characteristics where, it showed positive for production of IAA, amylase, catalase, acetoin, nitrogen, cellulase, and for 2, 3, Butane diol. The bacterial strain was negative for production of ammonia, chitinase, protease, Zn solubilization and phosphate solubilization. An endophyte equipped with antifungal abilities along with plant growth promotion activities will be more advantageous and easily incorporated in integrated disease management strategies. Similarly, plant growth promotion of endophytes was previously discussed by many authors (Digar singh *et al*., 2013; Shanmugaraj *et al*., 2021).

The antifungal activity of *Leucobacter aridicollis* strain SASBG215 against *Colletotrichum orbiculare* was validated in a dual culture bioassay, and it was almost probably attributable to soluble non-volatile biomolecules produced by the endophytic bacteria. Gas chromatography and mass spectrometry (GC-MS) profiling was used to further determine the chemical composition of the metabolites. The NIST library identified most of the metabolites by similarity index of 85% and most probable hits. A total of 2069 compounds were profiled from different treatment combinations *viz*., *Leucobacter aridicollis* strain 215 alone, *Colletotrichum orbiculare* alone and their interaction in dual culture assay.

The interaction of *Leucobacter aridicollis* strain SASBG215 and *Colletotrichum orbiculare* in dual culture assay produced 58 hits with 1302 compounds and the unique compounds profiled were (1) Procyclidine. It was reported as anticholinergic and antiparkinsonian drug (Suryawanshi *et al*., 2009) (2) 2-furanmenthanol. (Alijani *et al*., 2021) reported 2-furanmenthanol (30.305%) from methanol extracts as one of the main bioactive compounds produced by the endophyte, *Bacillus atrophaeus* strain DM6120 and it inhibited the growth of *Colletotrichum nymphaeae*.

Antibacterial properties of 2-furanmethanol were discussed (Fatma *et al*., 2020); (3) 2-Thiazolidine Carboxamide, 2-methyl-. Thiazolidine derivatives have been widely discussed for their anticonvulsant, hypnotic, antitubercular and anthelmintic activity (Amit and Shailendra, 2008); (4) Acetic acid, hydroxy[(1-oxo-2-propenyl) amino]-. It constituted 8.88% in *Brucea antidysenterica* leaf oil and exhibited antibacterial and antifungal properties in dual culture experiments (Guluma *et al*., 2020); (5) Clindamycin. Saravanan et al. 2022 reported nematicidal properties of clindamycin against banana burrowing nematode, *Radopholus similis.* (6) 4-Octanol, 4,7-dimethyl-; (7) Pentanoic acid, propyl ester. Huang *et al*., (2011) reported the Pentanoic acid, 4-methyl, - ethyl ester highly inhibitory to conidial germination and mycelia growth of *Botrytis cinerea*. (8) 2,3,4,4-Tetramethyl-pentane-1,3-diol. This compound was earlier reported as responsible for bitterness in caramel colours (Li *et al*., 2021). (9) Sucrose. (Alexandra and Thierry, 2014) discussed the role of sucrose plant defence by activating plant immune responses against pathogens. (10) 1-Pentanol, 2-methyl-, acetate. (Nighat *et al*., 2016) attributed the antifungal activity of leaf extracts of *Acasia nilotica* subsp. *indica* against *Sclerotium rolfsii* to the presence of 1-Pentanol, 2-methyl-, acetate (14.80 per cent) in the leaf extracts. (11) Cyclohexanol, 2-(2-propynyloxy)-, trans-. (Huan *et al*. 2021) reported formation of cyclohexanols as essential in aged fragrance of Qingzhuan tea (QZT) during the whole post-fermentation process.

The said compounds *viz*., Procyclidine; 2-Furanmethanol; 2-Thiazolidinecarboxamide, 2-methyl-; Acetic acid, hydroxy[(1-oxo-2-propenyl) amino]-; Clindamycin; 4-Octanol, 4,7-dimethyl-; Pentanoic acid, propyl ester; 2,3,4,4-Tetramethyl-pentane-1,3-diol; Sucrose; 1-Pentanol, 2-methyl-, acetate; Cyclohexanol, 2-(2-propynyloxy)-, trans-. were not present individually either in endophyte or in pathogen but were profiled only during their interaction as represented in the chromatogram (Fig. 5).

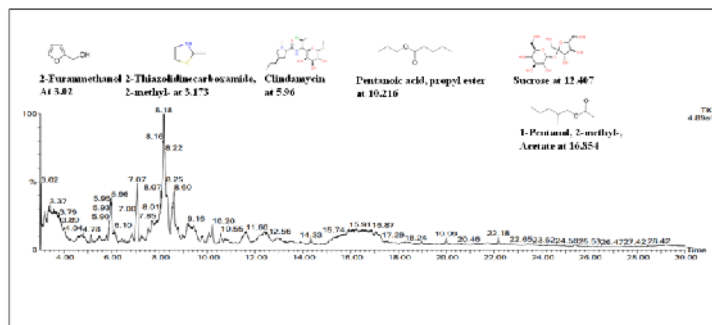

Fig. 5. Chromatogram showing the peaks of soluble metabolites in the interaction of *Leucobacter aridicollis* strain SASBG215 with *Colletotrichum orbiculare* in dual culture assay.

Two compounds *viz*., dl-Glyceraldehyde dimer and 1,2,3-Propanetriol, 1-acetate were present in both endophyte and the pathogen but were over expressed during their interaction. A clear disparity observed between the bio molecules expressed in the interaction of *Leucobacter aridicollis* strain SASBG215 with *Colletotrichum orbiculare* when compared with individual organisms alone. This pattern was well represented in the heat map (Fig. 6).

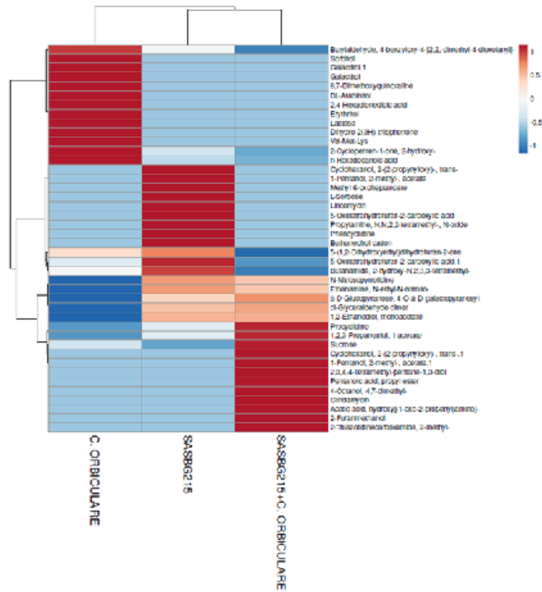

Fig. 6. Heat map of soluble metabolites from individual growth of *Leucobacter aridicollis* strain SASBG215, *Colletotrichum orbiculare* and their interaction in dual culture.

CONCLUSION

In this study, 15 bottle gourd bacterial endophytes were isolated and tested in dual confrontation tests for their efficiency against the bottle gourd stem splitting pathogen, *Colletotrichum orbiculare*. Using the 16s rRNA universal primers 27F and 1492R, one of the effective endophytes was identified as *Leucobacter aridicollis*. Additionally, metabolic profiling of biomolecules using gas chromatography and mass spectrometry found a number of antifungal chemicals. The prominent compounds were *viz*., Procyclidine, 2-furanmethanol, 1-Pentanol, 2-methyl-, acetate and Acetic acid, hydroxy[(1-oxo-2-propenyl) amino]- with antibacterial and antifungal properties, Clindamycin with nematicidal properties, Pentanoic acid, propyl ester known to inhibit conidial germination and fungal growth and sucrose known to be involved in plant defence responses.

This is the first report of a bacterial endophyte, *Leucobacter aridicollis*, being used as a biocontrol agent against *Colletotrichum orbiculare* and the soluble biomolecules it produces. More research is needed to understand the specific effects of these biomolecules against the test pathogen and to develop an organic-based spray composition for cucurbits in general.

FUTURE SCOPE

This study reported *Leucobacter aridicollis* SASBG215 as novel biocontrol agent against *Colletotrichum orbiculare.* The profiled compounds in GC-MS can further be evaluated against the pathogen and the new bioagent, *Leucobacter aridicollis* can be further evaluated for novel delivery systems via formulations at field level.

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Conflict of interest. None.

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