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Screening of Lactic Acid Bacteria as Biocontrol against *Colletotrichum musae*

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In the preliminary study, three hundred and seventy six Lactic Acid Bacterial (LAB) isolates obtained from the various natural sources were morphologically characterized and screened against the fungal pathogen *Colletotrichum musae*, causal agent of anthracnose of banana. The dual culture assay was employed to determine *in vitro* antagonistic activity of the LAB isolates. Among these twenty four, LAB isolates inhibited the mycelial growth of the pathogen. Out of twenty four, only two isolates *viz.* LS-36 and LS-44 exhibited maximum zone of inhibition. Based on 16S rRNA gene sequencing, LS-36 and LS-44 revealed maximum similarity to *Lactobacillus brevis*.

Key words: Biocontrol, Lactic acid bacteria, Anthracnose, *Colletotrichum musae*.

Banana (*Musa* sp.) is one of the nutritive and most popular fruit crop[1]. For international trading, banana fruits are usually harvested before ripening and stored at relatively low temperature during transportation and market process. Long distance transport and extended storage period in the market make banana sensitive to disease incidence. It is estimated that 20 to 25% of harvested fruits are decayed by pathogens during post harvest handling[2].

Anthracnose caused by *Colletotrichum musae (*Berk. and Curt.) Arx. considered as one of the most deadly disease of ripened and ripe bananas and produces major constraints to banana production. *C. musae* is the most vital pathogen on wounded green and ripened banana fruits[3]. Banana anthracnose usually starts as quiescent infections when conidia contaminate the banana fruits after flowering[4]. These conidia quickly germinate and form appresoria which are quiescent structures of the pathogen. On ripening fruits, sunken lesions develop with orange acervuli[5]. It deteriorates the quality and nutritive value of the fruits and renders them unfit for marketing and consumption; there by causing severe loss to farmers and traders. Fungicides are the prime means of controlling post harvest diseases[6]. However the use of chemical fungicides to control post harvest rots and deterioration has been limited due to their potential carcinogenicity and environmental pollution. Therefore, the exploitation of biocontrol agents could be an alternate way for controlling plant diseases.

The Lactic acid bacteria (LAB) form an ecologically heterogeneous group of Gram-

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positive bacteria which has gained interest as biocontrol agent against wide range of pathogenic organisms. The LAB excretes lactic acid as major end product and generally recognized as safe (GRAS) organisms. The antagonistic activity of LAB is linked with the inhibiting effects of the compounds they produce. It produces a variety of antimicrobial compounds and effective substances such as lactic, acetic, antibiotics, bacteriocins as well as hydrogen peroxide and carbon dioxide[7].

In the present study, attempt was initiated to isolate the LAB from different sources and to identify the efficient LAB isolates for their antifungal activity against the pathogen *C. musae* that infect banana fruits.

MATERIALS AND METHODS

Isolation of fungal pathogens

Banana fruits with typical anthracnose disease symptoms were collected from local market of Dharwad. The fruits were cleaned with sterile distilled water, the infected area were cubed into small pieces (3 to 5 mm in diameter) and pieces of lesion tissue were surface-sterilized with 70% ethanol for 1 minute, rinsed with sterile distilled water and then air-dried on a clean bench. The dried samples were placed on potato dextrose agar (PDA) amended with streptomycin 100 mg/ml. Plates were then incubated at 25±1^{0}C and after 4 days of incubation growing mycelial tips were cultured on new PDA plates and the conidial morphology of the isolates was examined under the Phase contrast microscope (Chippon, Japan).

Pathogenicity assay[8]

The pathogenicity test was carried on healthy green banana fruits. The fruits were surface sterilized with 70% ethanol, injured (pin pricked) with sterilized needle and the spore suspension (5 x10^5 spores per ml) of the pathogen was prepared and inoculated to the injured fruits. The uninoculated fruit was served as control. The inoculated fruits were wrapped with cover and incubated at 25±1^{0}C and the infection was recorded after seven days. The fungus was reisolated from the artificially inoculated fruits showing typical anthracnose symptoms.

Isolation of lactic acid bacteria (LAB) from different sources

Fresh fruits and vegetables were collected from different retail market of Dharwad. The samples were wrapped separately in sterile polyethylene bags and transported to the laboratory for analysis.

Ten gram each of fresh fruits and vegetables samples were soaked in 90 ml of normal saline solution homogenized for 10 min, appropriately diluted in normal saline, pour plated onto De Man, Rogosa and Sharpe agar (MRS) and incubated at 35ºC for 48-72h. Distinct colonies were sub-cultured twice and pure cultures were maintained on MRS agar slants.

Screening for the antagonistic activity of lactic acid bacteria against post harvest fruit pathogen

The freshly grown pathogenic fungus on PDA agar and lactic acid bacteria (LAB) grown on MRS agar plates were used in this experiment. The spore suspension (5x10^5 spores in 1ml) of the pathogen was prepared using a ten days old PDA culture.

The LAB isolates were screened against the pathogenic *C. musae*. The fungal pathogen and LAB isolates were co-inoculated on PDA plates. The 0.1% of fungal spore suspension of *C. musae* was spread uniformly on the PDA plates and allowed to dry for 8 or 10 min. Later, the LAB isolates were spot inoculated on the fungal pathogen in PDA plates and incubated at 25^{0}C for four to five days. The zone of inhibition (ZOI) of the growth of the pathogen by the LAB isolates was measured by an antibiotic disc measurement scale and the results were tabulated.

Molecular characterization of the LAB isolates

The genomic DNA was isolated from the efficient LAB isolates viz., LS-36 and LS-44 by the Sambrook and Russell method[9]. The 16S rRNA gene specific primers were standardized for annealing temperature by gradient PCR.

PCR amplification, sequencing of 16s rRNA gene and phylogenetic analysis

The 16S rRNA gene was amplified from the genomic DNA obtained from the LAB isolates by PCR with a set of universal primers 27F 5‘AGAGTTTGATCCTGGCTCAG3‘ and 1492R 5‘TACGGYTACCTTGTTACGACTT3‘. PCR was performed with 100 ng template DNA, 3U Taq DNA polymerase, 1 mM dNTP, 5 pM primer each, 25 mM $MgCl_2$ in a final volume of 100 ìl. Amplification was done in an Eppendorff Thermal Cycler under the

following conditions: 5 min of denaturation at 94°C followed by 35 cycles of amplification with 1 min denaturation at 94°C, 1 min of annealing at 50°C, 2 min of extension at 72°C, final extension step of 90 min at 72°C.

The PCR products were purified using a QIAgen gel extraction kit (QIAgen, Germany) and sequenced with an ABI 3730 xl sequencer (Applied Biosystems, USA) with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems, USA). The forward and reverse 16S rRNA gene sequences of LS-36 and LS-44 were assembled individually in BTI Gene Tool Lite software v. 1.0 to produce contiguous DNA sequences. The resulting contiguous DNA sequences were compared with the reference sequences available in the NCBI database using the Basic Local Alignment Search Tool (BLAST). The closest 16S rRNA gene sequences of the reference strains were retrieved from the www.ncbi.nlm.nih.gov database aligned using Clustal_W and phylogenetic tree was constructed using the UPGMA method with MEGA 4.1. Bootstrap resampling analysis for 1000 replicates was performed to estimate the confidence of tree topologies.[10]

Nucleotide sequence accession numbers

The 16S rRNA gene sequences of the potent LAB isolates LS-36 and LS-44 are submitted to NCBI Genbank under the accession no KM366784.1 and KM366785.1 respectively.

RESULTS AND DISCUSSION

Identification of the isolated fungi from post harvest diseased fruits

Fungus was isolated from the anthracnose lesions on bananas and identification of the fungal isolates was done based on conidial morphology (Fig. 1). It grew well on PDA plate and formed white aerial mycelium, which turned pinkish orange in color with age. Several dark orange structures developed abundantly on the culture surface incubated at 25°C after 10 to 12 days, which were mostly acervuli including dark orange conidia. The fungal culture derived from the conidium of the pure isolate was maintained on PDA agar for further studies. In the present study, the pathogen isolated from banana was found morphologically analogous to the description of banana pathogen *C. musae*[11]. The morphological characteristics of appressoria of fungal isolates were almost the same as that of *C. musae* including host species, color of conidium mass and colony shape on PDA. In the similar study, the *C. musae* producing acervuli like structures from dark-brown anthracnose lesions on commercial banana[12].

Pathogenicity test

The inoculated banana fruits showed typical anthracnose symptoms after 5 to 7 days of incubation. Lesions were black necrotic, circular with sunken lesions (Fig. 2). The pathogen showed sunken lesion colour, apparent growth texture which was similar to the descriptions reported by[13]. *C. musae* and *C. gloeosporioides* have been found as endophytes in banana but these fungi also cause anthracnose of banana fruits[13]. Similar symptoms were observed while performing pathogenicity test on banana[1, 12].

Antifungal activity of LAB isolated from different fruit samples

Out of 376, only 24 LAB isolates exhibited antagonistic activity against *C. musae* (Table 1). Among these 24, only two LAB isolates viz. LS-36

Table 1. Antifungal activity of lactic acid bacteria (LAB) against *C. musae*

S. No.	LAB isolates	Inhibition Zone (mm)
1.	LAB-01	17
2.	LAB-04	17
3.	BF-14	20
4.	BF-14	21
5.	BF-15	16
6.	G-5	16
7.	G-7	18
8.	Gr-4	17
9.	Gr-5	21
10.	Gr-6	21
11.	Gr-7	18
12.	Gr-8	21
13.	LS-3	24
14.	LS-8	21
15.	LS-13	19
16.	LS-35	20
17.	LS-36	25
18.	LS-39	16
19.	LS-40	19
20.	LS-41	19
21.	LS-44	25
22.	LS-52	17
23.	LS-53	19
24.	LA-14	20

and LS-44 showed highest zone of inhibition (25 mm) followed by LS-3 isolate (24 mm). (Fig. 3). However, the LAB isolates showed varying degree of antifungal activity. For the remaining isolates the zone of inhibition ranged from 17 mm to 21 mm. Several researchers have reported the antifungal activity of lactic acid bacteria against various fungal pathogens. The LAB inhibited the phytopathogenic fungi due to the production of indol acetic acids (IAA) and phenolic substances[14]. The efficacy of Lactic acid bacteria (LAB) isolated from fresh fruits and vegetables as bio-control agents against the phytopathogenic fungi viz., *Xanthomonas campestris, Erwinia caratovora, Penicillium expansum, Colletotrichum, monilia laxa* and *Botrytis cinerea* were evaluated [15]. In the similar study, The LAB strains isolated from wheat semolina shown strong inhibitory activity against *Aspergillus niger, Penicillum roqueforti* and *Endomyces fibuliger*[16]. The secondary metabolites produced by *L. plantarum* IMAU10014 revealed high antifungal activity[17]. The capability of LAB to act as plant growth promoting bacteria and biocontrol agent against *Fusarium oxysporum* (phytopathogenic fungi) has been reported [18].

Fig. 1. Microscopic features of *C. musae* with fruiting body (A) and spores (B)

Fig. 2. Banana fruit treated with sopre suspension of pathogenic *C. musae* showing sunken lesions

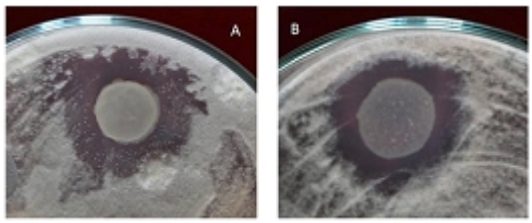

Fig. 3. LS-36 (A) and LS-44 (B) showing the inhibition of pathogen *C. musae*

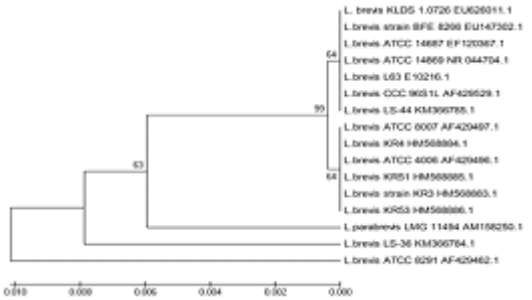

Fig. 4. Phylogenetic tree showing the similarity of LS-36 and LS-44 with reference strains

Molecular characterization and phylogenetic analysis of LAB isolates

Based on the 16S rRNA gene sequence analysis, the promising LAB isolates viz., LS-36 and LS-44 belonged to *Lactobacillus brevis* with 99% similarity. The phylogentic relationship and the similarity index of 2 LAB isolates to the closet sp. procured from the NCBI databse are presented in Fig. 4. The phylogenetic tree showed the high similarity and the evolutionary distance with several *Lactobacillus* sp. The evolutionary history was inferred using a UPGMA method[10] and the bootstrap values are indicated at the nodes.

CONCLUSION

In conclusion, potent strains LS-36 and LS-44 identified as *Lactobacillus brevis* can be used to prevent the post-harvest anthracnose disease of banana. This biological agent could be an alternative to the synthetic fungicides used in packing house. Further research work is needed to determine mechanisms of inhibition and testing the efficient isolates as biopreservative agents under *in vivo* conditions.

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