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In vitro Evaluation of Biocontrol Potential of Lactic Acid Bacteria Isolated from Natural Ecosystem against Plant Pathogenic Fungi

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Lactic acid bacteria (LAB) are ubiquitous, Gram-positive, fermentative bacteria, which are regarded as safe for both humans and the environment. The present study aimed to evaluate the biocontrol efficiency of LAB isolated from different natural ecosystems against fungal pathogens. A total of 30 LAB was isolated from rhizosphere soil and phyllosphere sample of Solanaceous crop viz., Brinjal, Capsicum, Chilli, Tomato, whey, and sauerkraut out of them 14, 9, 4 and 3 lactic acid bacteria (LAB) were isolated from rhizosphere soil & phyllosphere sample of Solanaceous crops, whey and Sauerkraut respectively. The biocontrol ability of LAB isolates were tested against fungal plant pathogens such as *Fusarium oxysporum*, *Pythium aphanidermatum*, *Sclerotium rolfsi*, *Rhizoctonia solani*, *Alternaria* sp. agar well diffusion assay and mycelial growth inhibition in liquid culture. The results indicated that the isolates LAB 4, LAB 10, LAB 22, LAB 24 and LAB 29 were prominent in inhibiting the growth of most

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of the pathogens. Molecular characterization of selected LAB isolates revealed that LAB 4, LAB 10, LAB 22, LAB 24 belongs to *Lactobacillus plantarum* and LAB 29 belong to *Leuconostoc mesentroides.*

Keywords: Lactic acid bacteria; biocontrol; Lactobacillus plantarum; Leuconostoc mesentroides.

1. INTRODUCTION

Lactic acid bacteria (LAB) are a heterogeneous group of Gram-positive, asporogenous, fermentative bacteria. Lactic acid bacteria are ubiquitous in nature, found on plants, insects, soil, milk, human gut and animal gastrointestinal & vaginal tracks. LAB are widely known for their role in the production of over 3,500 types of fermented foods, beverages, and silages. LAB has the ability to produce different compounds, during the fermentation of carbohydrates, based on this characteristic lactic acid bacteria are broadly classified as homo-fermenters and hetero-fermenters. The homo-fermenters produce lactic acid as their major end product by utilizing carbohydrates comprising *Lactococcus* spp., *Pediococcus* spp., *and Streptococcus* spp. Hetero-fermenters produce other compounds like acetic acid, propionic acid, carbon dioxide, ethanol, etc., apart from lactic acid as the end product of fermentation and comprise genus *Leuconostoc* spp. and *Lactobacillus* spp. [1].

"Fungal plant pathogens, which infect all major crops, are a threat to global food security as they cause serious losses both in the field and post-harvest. Soil-borne fungal diseases are among the most important factors limiting the yield of many economically important plants, resulting in serious economic losses. They are known to attack roots and shoots of plants, causing damping-off or root rot. Estimations suggest that the major part of the food for human consumption is provided by 14 crop plants belonging to different families and genera. One of them is the Solanaceae family which comprises the most important vegetable crops from the genus Solanum. These solanaceous species not only fulfill nutritional requirements but are also a source of drugs, ornamentals, and medicines. These crop plants are attacked by major groups of plant pathogens *viz*., viruses, bacteria, fungi, nematodes, oomycetes, and parasites" [2].

"Lactic acid bacteria were recognized as producers of bioactive metabolites which are functional against a broad spectrum of pathogenic microorganisms, such as fungi, oomycetes, and other bacteria" [3]. They are known to produce antimicrobial compounds as organic acids such as lactic acid, acetic acid, and propionic acid [4]; antimicrobial peptides [5]; fatty acid [6]; bacteriocin [7], etc., In this context, they may represent an interesting tool for developing novel concepts in plant disease management the need of the hour. With this background, the present study aimed to study the biocontrol potential of LAB against a few fungal plant pathogens.

2. MATERIALS AND METHODS

2.1 Isolation of LAB and Culture Maintenance

In the present study LAB were isolated from whey, sauerkraut, phyllosphere, and rhizosphere soil of solanaceous crops like Capsicum (variety: Indra), Chilli (variety: east West company), Tomato (Variety: NS 501) and Brinjal (variety: Lalit) by enrichment culture technique on specific media De Man's Rogosa Sharpe (MRS) agar supplemented with 1% $CaCO_3$ and incubated at 37°C in BOD incubator for 48-72h [8].

The colonies that formed cleared zone were randomly picked and purified. Further, the isolates which were positive for gram staining and negative for catalase production were presumed to be lactic acid bacteria and were maintained at 4°C in MRS broth with sub-culturing at 15 days intervals for short-term storage and further evaluation. A glycerol stock of isolates was also prepared for cultured MRS broth with 50% glycerol (w/v) stored at −20°C for longer periods [8].

2.2 Source of Plant Pathogens

In the present study the following plant pathogenic fungi were used viz., *Fusarium oxysporum*, *Pythium aphanidermatum*, *Sclerotium rolfsi*, *Rhizoctonia solani*, *Alternaria* sp. were procured from the Department of Plant Pathology, University of Agricultural Sciences, Bangalore.

2.3 *In vitro* evaluation LAB isolates for biocontrol efficiency against fungal plantpathogens.

2.3.1 By plate assay

The inhibitory effect of LAB isolates against fungal plant pathogens was carried out by agar

well diffusion assay. Pathogen inoculum was added at the rate of 2.5 ml per 100 ml of potato dextrose agar media which was poured onto the plates and solidified. Agar wells were scooped in solidified agar plates using sterilized cork borers (5mm). 50 µl suspension of a day-old culture of LAB isolates (10^8 cfu/ml) were added in each well. The plates were incubated at room temperature for 96 h. Three replications for each LAB isolate and test, the pathogen was maintained. The diameter of the clear zone devoid of mycelial growth around the well was measured and the radius (R) was calculated. The area of inhibition was computed in terms of square millimeters. A higher area of inhibition indicated greater antimicrobial activity. The inhibition area was calculated using the formula [9].

Area of inhibition = π (R + r) (R - r)
Where R = is the radius of the clear zone around the well
r = is the radius of the agar well.

2.3.2 In liquid culture assay

"The LAB isolates showing high inhibition of the pathogen in plate assay were tested in liquid media (Potato dextrose broth). Mycelial disc of (5 mm size) respective pathogens were inoculated to 100 ml liquid broth along with one ml of 24-hour-old LAB cultures and was kept in an incubator at 30 °C under static conditions for 10 days. Control flasks without any LAB were maintained for each pathogen. After the incubation period, the contents in the flasks were filtered through a pre-weighed Whatman filter No.1 paper and the fresh weight of contents was recorded. The filter papers along with the contents were dried in a hot air oven at 105 °C for 48 h and reweighed along with the mycelium to get the constant dry weight values. The weight of the fungal mycelial mat was calculated by subtracting the weight of the pre-weighed filter paper from the weight of the filter paper plus the mycelial mat. The reduction in the weight of mycelium in co-inoculated flasks was determined by comparing it with the control flasks" [10].

2.4 Molecular Characterization of LAB isolates

The total genomic DNA of the LAB was extracted by the alkaline lysis method given by [11] and the concentration of the DNA was measured using a nanodrop instrument and stored at -20°C. Further polymerized chain reaction (PCR) amplification of the 16S rRNA gene for the isolated genomic DNA was done using universal primers 27F and 1492R primers as reported by NCBI. The amplified 16S rRNA gene was purified and sequenced using Sanger dideoxy sequencing method, commercially by BarcodeBioscience, Bangalore, India.

2.5 Statistical Analysis

The experimental data generated in lab studies were subjected to CRD statistical analysis keeping the significance level $P \leq 0.05$ as per Duncan Multiple Range Test (DMRT). The analysis of variance and interpretation of the data was done as per procedures given by Fisher and Yates (1963) [12], Panse and Sukhatme (1967) [13], and Gomez and Gomez (1984) [14]. Means were separated by Duncan Multiple Range Test (DMRT).

3. RESULTS AND DISCUSSION

Lactic Acid Bacteria (LAB) is a gram-positive and fermentative bacterium that produces a mixture of antimicrobial compounds like organic acid, hydroxy fatty acids, cyclic dipeptides, proteinaceous compounds, phenolic compounds, and hydrogen peroxide, which are effective against fungal plant pathogens. The present study was taken up to isolate and evaluate the bio-control efficacy of lactic acid bacteria against soil and air-borne plant pathogens.

3.1 Isolation of LAB and Culture Maintenance

A total of 30 isolates were obtained from the samples by using specific media MRS agar supplemented with 1% $CaCO_3$. Among them, 14, 9, 4, and 3 lactic acid bacteria (LAB) were isolated from rhizosphere soil & phyllosphere sample of Solanaceous crops viz., Brinjal, Capsicum, Chilli, Tomato, Whey, and Sauerkraut respectively.

LAB is cosmopolitan in nature and previously it has been known to be isolated from the rhizosphere soil of fruit trees [15], plant surfaces [16], and dairy products like milk, curd, paneer, and fermented food items [17] and were characterized to be *Lactobacillus*, *Lactococcus*, *Leuconostoc,* and *Fructobacillus*.

Fakri et al. [18] isolated LAB isolates which exhibited antifungal activity against

Colletotrichum from samples of sandy clay loam soil collected from the Rice field and Roselle cultivation area of Terengganu. They were identified to be *Lactococcus lactis* sub sp. *lactis*.

3.2 *In vitro* Evaluation LAB Isolates for Biocontrol Efficiency against Fungal PlantPathogens

3.2.1 By plate assay

The LAB isolates that showed antifungal activity against fungal pathogens (*Pythium aphanidermatum*, *Fusarium oxysporium*, *Sclerotium riolfsii*, *Rhizoctonia solani* and *Alternaria* spp.,) by forming the area of inhibition is presented in Table 1, Fig. 1. Some LAB isolates showed prominent inhibition against all the fungal pathogens as determined by agar well diffusion assay.

Isolate LAB 4 significantly inhibited the growth of all fungal pathogens. Further *Pythium aphanidermatum* was inhibited significantly by isolate LAB 26 (61.63 mm^2) followed by isolate LAB 24 (55.39 mm^2). Isolate LAB 22 showed an inhibition area of 58.48 mm^2 against *Fusarium oxysporium*. *Sclerotium rolfsii* was found to be inhibited by isolated LAB 24 (52.37 mm^2). Following a similar trend isolate LAB, 24 inhibited *Rhizoctonia solani* and *Alternaria* spp., by forming an inhibition zone of 30.41mm^2 and 46.51mm^2 respectively.

Lopez-Sejia et al. [19] isolated LAB - *Lactobacillus plantarum, Lactobacillus hilgardii*, *Lactobacillus paracasei* and *Lactococcus lactis* from malolactic fermentation. The isolates were evaluated for antifungal activity against *Fusarium oxysporium*. They found that all the isolates showed a varying degree of inhibition ranging 56% to 76%, among them *L. paracasei* LPAUV12 and *L. plantarum* LPLUV10 strains were more effectively inhibited *Fusarium oxysporum.*

A study was conducted on biocontrol efficacy of LAB against soil-borne pathogens by Lutz et al. [20]. They isolated 294 isolates of LAB from soil and the rhizosphere of maize, rye, carrots, garden soils, and compost from two origins and tested them against *Pythium ultimum*. Results obtained showed that 75% of the isolates showed an inhibitory effect and 50% suppressed Pythium growth by more than 60%. Among them, the most promising strains were isolated from maize roots, compost, or garden soil. Hence, they reported that LAB would be a novel promising bacterial group in the biological control ofsoil-borne pathogens.

3.2.2 By liquid culture assay

The antifungal activity of LAB isolates in liquid broth assay by percent reductions in mycelium mat dry weight of the fungal plant pathogens by the LAB isolates in liquid culture are presented in Table 2, Fig. 2.

Results of the liquid broth assays show that the LAB isolates showed varied percentage reductions in mycelium dry weight of all fungal pathogens ranging from very low, to moderate to high. Isolate LAB 4 was significant in inhibiting the mycelial growth of all fungal pathogens even in liquid broth, whereas isolate LAB 2 inhibited the mycelial growth of *Pythium aphanidermatum* only. Isolates LAB 22 and LAB 24 were known to reduce the mycelial dry weight *of Sclerotium rolfsii* and *Rhizoctonia solani* by 74.39%. Apart from isolating LAB 4 mycelial dry weight of *Alternaria* spp., was reduced effectively by isolating LAB 10 by 70.73%.

Similar results were obtained by Moustafa et al. [21]. Where they evaluated Lactic acid bacteria (LAB) isolates against one of the important fungal plant pathogens like *Fusarium oxysporum in vitro* conditions and recorded a reduction in mycelial dry weight was recorded by 75% by isolating LB-2, LB-3, and LB-4.

Wang et al. [22] reported that the strain *Lactobacillus plantarum* IMAU10014 among the 77 LAB isolates isolated from koumiss showed broad-spectrum antifungal activity against plant pathogens. Cell-free supernatant of *L. plantarum* IMAU10014 was tested against *Botrytis cinerea, Alternaria solani, Phytophthora drechsleri Tucker, Fusarium oxysporum* and *Glomerella cingulata* and was found to completely inhibit *Phytophthora drechsleri* Tucker, *Fusarium oxysporum* and *Alternaria solani* by 79.9% and 79.7% respectively.

3.3 Molecular characterization of LAB isolates

The genomic DNA of selected LAB isolates was extracted successfully. Likewise, the PCR amplification of the 16S rRNA gene and sequencing of the PCR product was carried out by Sanger dideoxy method (Barcode biosciences, Bangalore).

Table 1. Antagonistic activity of lactic acid bacteria against common fungal plant pathogens of solanaceous vegetables by agar well diffusion method

Sl. No.	Isolates	Area of inhibition (mm^2)				
		Pythium aphanidermatum	***Fusarium oxysporium***	***Sclerotium rolfsii***	***Rhizoctonia solani***	***Alternaria sp.***
1.	LAB 2	27.95^{ef}	ni	ni	ni	ni
2.	LAB 4	64.85^{a}	74.86^{a}	64.85^{a}	55.39^{a}	71.46^{a}
3.	LAB 10	40.89^{cd}	52.37^{bc}	38.18^{c}	30.41^{b}	55.39^{b}
4.	LAB 15	35.53^{de}	ni	ni	10.48^{d}	ni
5.	LAB 20	ni	ni	ni	ni	16.56^{g}
6.	LAB 21	20.93^{fg}	ni	ni	12.45^{d}	ni
7.	LAB 22	38.18^{cd}	58.48^{b}	38.18^{c}	20.93^{c}	38.18^{d}
8.	LAB 23	18.71^{g}	30.41^{e}	20.93^{f}	ni	25.55^{ef}
9.	LAB 24	55.39^{b}	43.67^{cd}	52.37^{b}	35.53^{b}	46.51^{c}
10.	LAB 25	ni	35.53^{de}	25.55^{ef}	18.71^{c}	ni
11.	LAB 26	61.63^{ab}	27.95^{ef}	30.41^{cde}	8.58^{d}	20.93^{fg}
12.	LAB 27	ni	20.93^{fg}	27.95^{def}	ni	12.45^{g}
13.	LAB 28	12.45^{g}	ni	ni	ni	ni
14.	LAB 29	46.51^{c}	ni	ni	18.71^{c}	32.94^{de}

***Superscripted alphabet letters show statistical groups for that column.ni- no inhibition*
Note: Means with the same superscript, in a column, do not differ significantly at P ≤0.05 as per Duncan Multiple Range Test (DMRT)

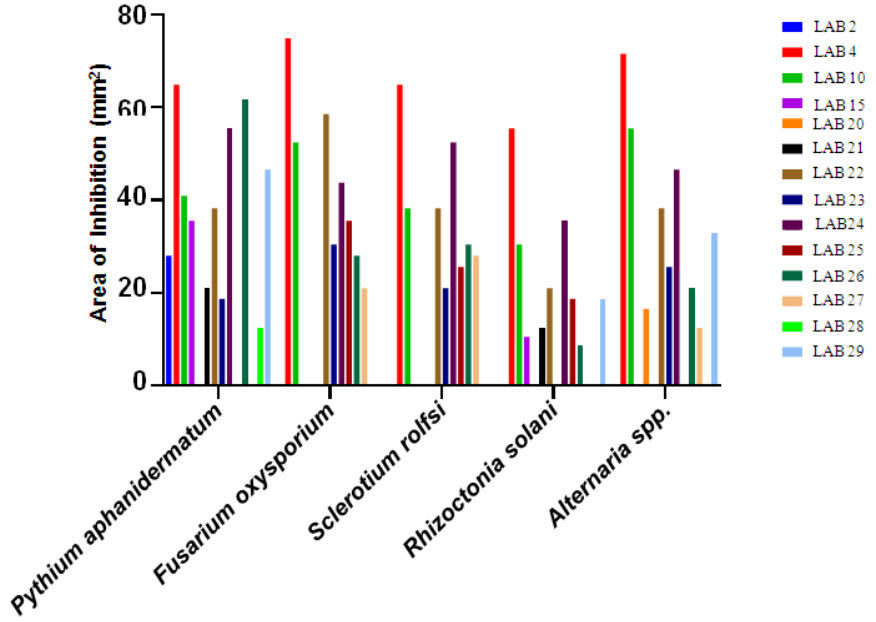

Fig. 1. Area of inhibition of lactic acid bacterial isolates on the growth of fungal plant pathogens by agar well diffusion assay

The sequence of 16s rRNA gene sequence of the LAB isolates was searched in the NCBI database. The phylogenetic analysis of the 16S rRNA gene revealed that out of five isolates, the isolates LAB 4, LAB 22, and LAB 24 were identified as *Lactobacilus plantarum* subsp. *plantarum* and LAB 10 was identified as *Lactobaciluus fabifermentans*. The isolate LAB 29 was identified as *Leuconostoc mesenteriodes* subsp. *mesenteroides*. The phylogenetic tree of LAB isolates was presented in Figs. 3a and 3b.

Table 2. Effect of lactic acid bacterial isolates against common pathogens of Solanaceous vegetables by percent reduction in dry weight of mycelium

Sl. No.	Isolates	*Pythium aphanidermatum*	*Fusarium oxysporium*	*Sclerotium rolfsii*	*Rhizoctonia solani*	*Alternaria sp.*
1.	LAB 2	24.19^{i}	ni	ni	ni	ni
2.	LAB 4	77.42^{a}	73.91^{a}	79.27^{a}	78.05^{a}	78.05^{a}
3.	LAB 10	69.35^{b}	64.13^{c}	71.95^{b}	70.73^{c}	70.73^{b}
4.	LAB 15	38.71^{gh}	ni	ni	64.63^{d}	ni
5.	LAB 20	ni	ni	ni	ni	47.56^{g}
6.	LAB 21	41.94^{f}	ni	ni	45.12^{e}	ni
7.	LAB 22	66.13^{c}	69.57^{b}	74.39^{b}	70.73^{c}	67.07^{c}
8.	LAB 23	37.10^{h}	56.52^{d}	68.29^{c}	NI	52.44^{f}
9.	LAB 24	54.84^{d}	66.30^{c}	65.85^{c}	74.39^{b}	63.41^{d}
10.	LAB 25	NI	45.65^{e}	51.22^{d}	45.12^{e}	ni
11.	LAB 26	45.16^{e}	40.22^{f}	45.12^{e}	39.02^{f}	51.22^{f}
12.	LAB 27	NI	34.78^{g}	39.02^{f}	ni	40.24^{h}
13.	LAB 28	40.32^{fg}	ni	ni	ni	ni
14.	LAB 29	77.42^{a}	ni	ni	64.63^{d}	57.32^{e}

**Mean values with superscripted alphabet letters show statistical groups for that column.ni- no inhibition*
Note: Means with the same superscript, in a column, do not differ significantly at P ≤0.05 as per Duncan Multiple Range Test (DMRT)

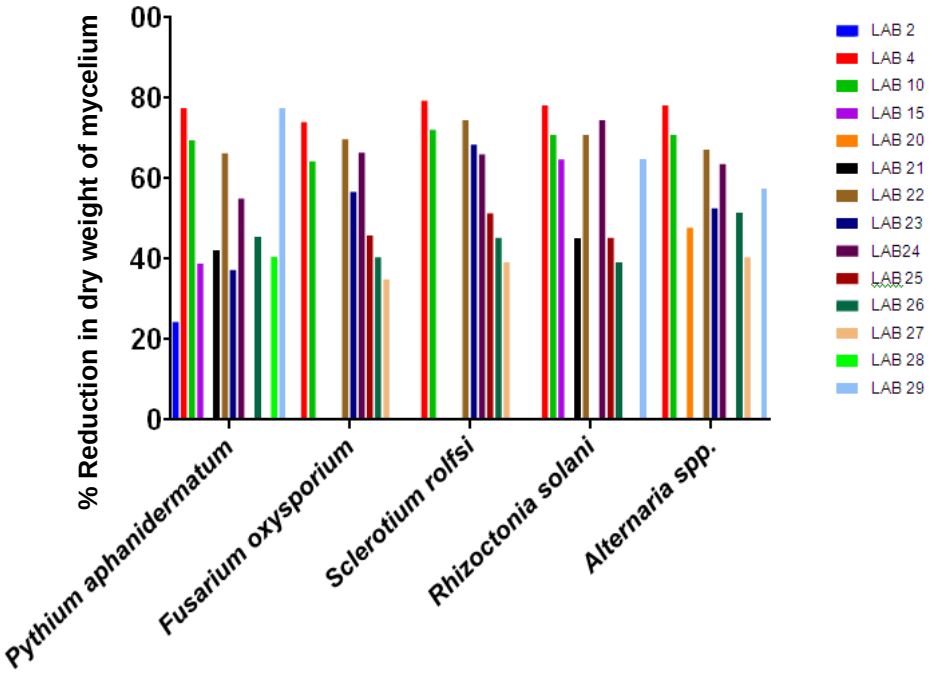

Fig. 2. Effect of lactic acid bacterial isolates on percent reduction of the mycelial dry weight of fungal plant pathogens in liquid culture

The results of phenotypic characterization of Lactic acid bacteria isolated from fermenting cassava by Kostinek et al. [23] showed that the predominant group of lactic acid bacteria consisted of *Lactobacillus Plant*arum strains, followed by the cocci belonging to the genera

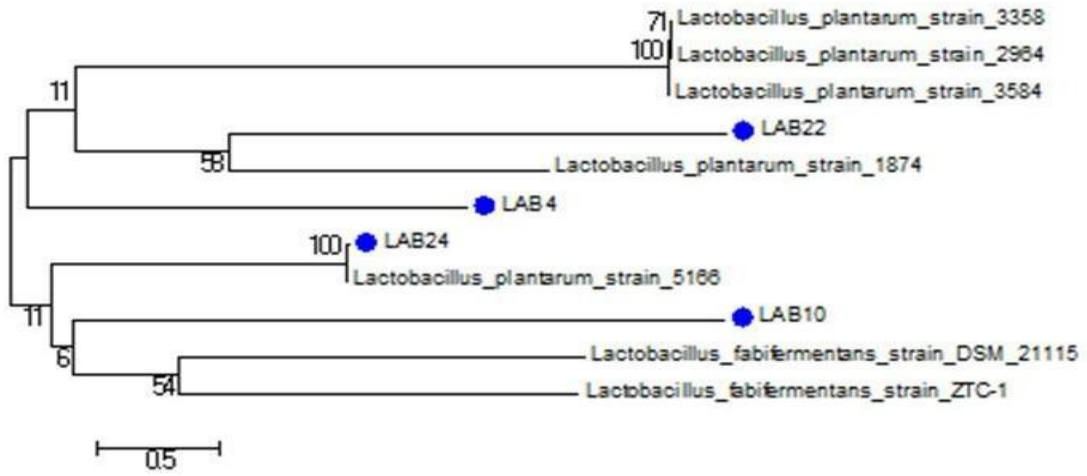

Fig. 3a. Phylogenetic placement of lactic acid bacterial isolates (LAB 4, LAB 10, LAB 22, LAB 24) based on nearly full length 16S rRNA gene sequencing. Boot strap valuesare based on 1000 replicates

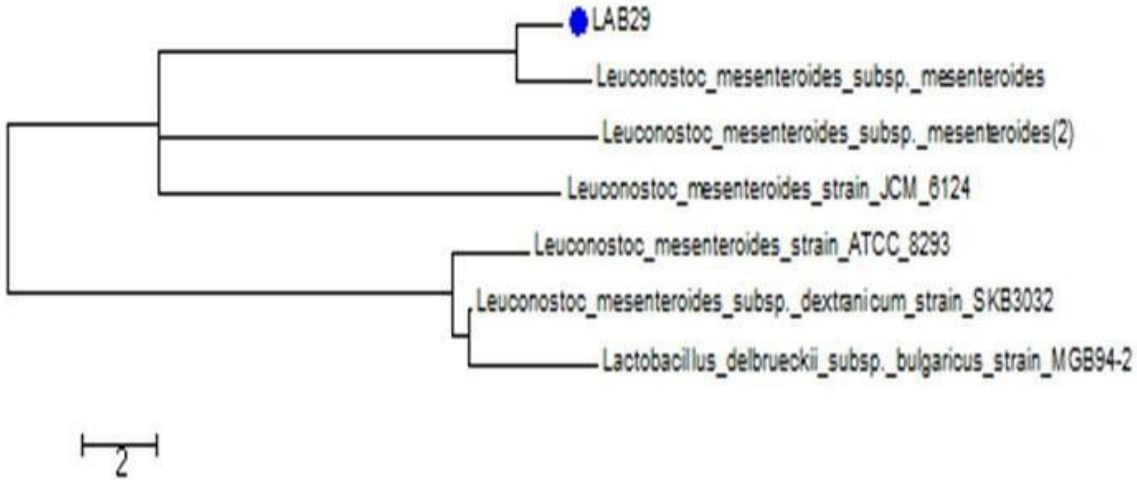

Fig. 3b. Phylogenetic placement of lactic acid bacterial isolate (LAB 29) based on nearly full-length 16S rRNA gene sequencing. Bootstrap values are based on 1000 replicates

Weissella and *Leuconostoc*. Similarly, Chen et al. [24] isolated and characterized lactic acid bacteria (LAB) from ripe mulberry samples. By sequencing of 16S ribosomal DNA (rDNA) they were identified as *Weissella cibaria* was the most abundant type of LAB followed by *Lactobacillus plantarum, Lactococcus lactis* subsp. *lactis*.

4. CONCLUSION

In conclusion, our study indicates that lactic acid bacteria have the potential to inhibit soil and air-borne plant pathogenic fungi and could be used as a novel biocontrol agent against them. Among LAB isolates Lactobacillus Plantarum strains were found to exhibit antifungal properties more profoundly than other isolates and have to be further confirmed by field studies.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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