In vitro evaluation of biocontrol potential of Lactic acid bacteria isolated from natural ecosystem against plant pathogenic fungi.

ABSTRACT

Lactic acid bacteria (LAB) are ubiquitous, Gram-positive, fermentative bacteria, which are regarded as safe for both human and environment. The present study was aimed to evaluate the biocontrol efficiency of LAB isolated from different natural ecosystem 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 was 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 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 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 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 comprises *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 comprises 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 food for human consumption is provided by 14 crop plants belonging to different families and genera. One of them is the Solanaceae family that comprises of the most important vegetable crops from genus Solanum. These solanaceous species not only fulfil the 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 like as organic acids such as lactic acid, acetic acid, 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 was aimed to study biocontrol potential of LAB against few fungal plant pathogens.

2. Materials and method

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% CaCO3 and incubated at 37°C in BOD incubator for 48-72 h [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 interval for short term storage and further evaluation. Glycerol stock of isolates were also prepared for cultured MRS broth with 50% glycerol (w/v) were 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 plant pathogens.

2.3.1 By plate assay.

The inhibitory effect of LAB isolates against fungal plant pathogens were 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 to the plates and solidified. Agar wells were scooped in solidified agar plate 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 pathogen was maintained. The diameter of 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 millimetres. Higher area of inhibition indicated greater antimicrobial activity. The inhibition area was calculated using the formula [9].

Area of inhibition = $\pi (R + r) (R - r)$

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 were kept in incubator at 30 °C under static conditions for 10 days. Control flasks without any LAB were maintained for each pathogen. After incubation period, the contents in the flasks were filtered through a pre weighed Whatman filter No.1 paper and fresh weight of contents were recorded. The filter papers along with contents were dried in 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 mycelial mat. The reduction in weight of mycelium in co inoculated flasks were determined by comparing with the control flasks [10].

2.4 Molecular characterization of LAB isolates.

Total genomic DNA of the LAB were extracted by alkaline lysis method given by [11] and the concentration of the DNA was measured using nano drop instrument and stored at -20 °C. Further

polymerized chain reaction (PCR) amplification of 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 Barcode Bioscience, Bangalore, India.

2.5 Statistical analysis

The experimental data generated in lab studies was 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 were 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 which produce 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% CaCO3. Among them 14, 9, 4 and 3 lactic acid bacteria (LAB) were isolated from rhizosphere soil & phyllosphere sample of Solanaceous crop viz., Brinjal, Capsicum, Chilli, Tomato, Whey and Sauerkraut respectively.

LAB is cosmopolitan in nature and previously it has known to be isolated from rhizosphere soil of fruit trees [15], plant surfaces [16] and dairy products like milk, curd, paneer and fermented food items [17] and were characterised 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 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 plant pathogens

3.2.1 By plate assay.

The LAB isolates that showed the 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, Figure 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 the isolate LAB 26 (61.63 mm^2) followed by isolate LAB 24 (55.39 mm^2). Isolate LAB 22 showed inhibition area of 58.48 mm^2 against *Fusarium oxysporium*. *Sclerotium rolfsii* was found to be inhibited by isolate LAB 24 (52.37 mm^2). Following the similar trend isolate LAB 24 inhibited *Rhizoctonia solani* and *Alternaria* spp., by forming 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 the antifungal activity against *Fusarium oxysporium*. They found that all the isolates showed varied 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 LAB against soil-borne pathogen 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 of soil-borne pathogens.

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}

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

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

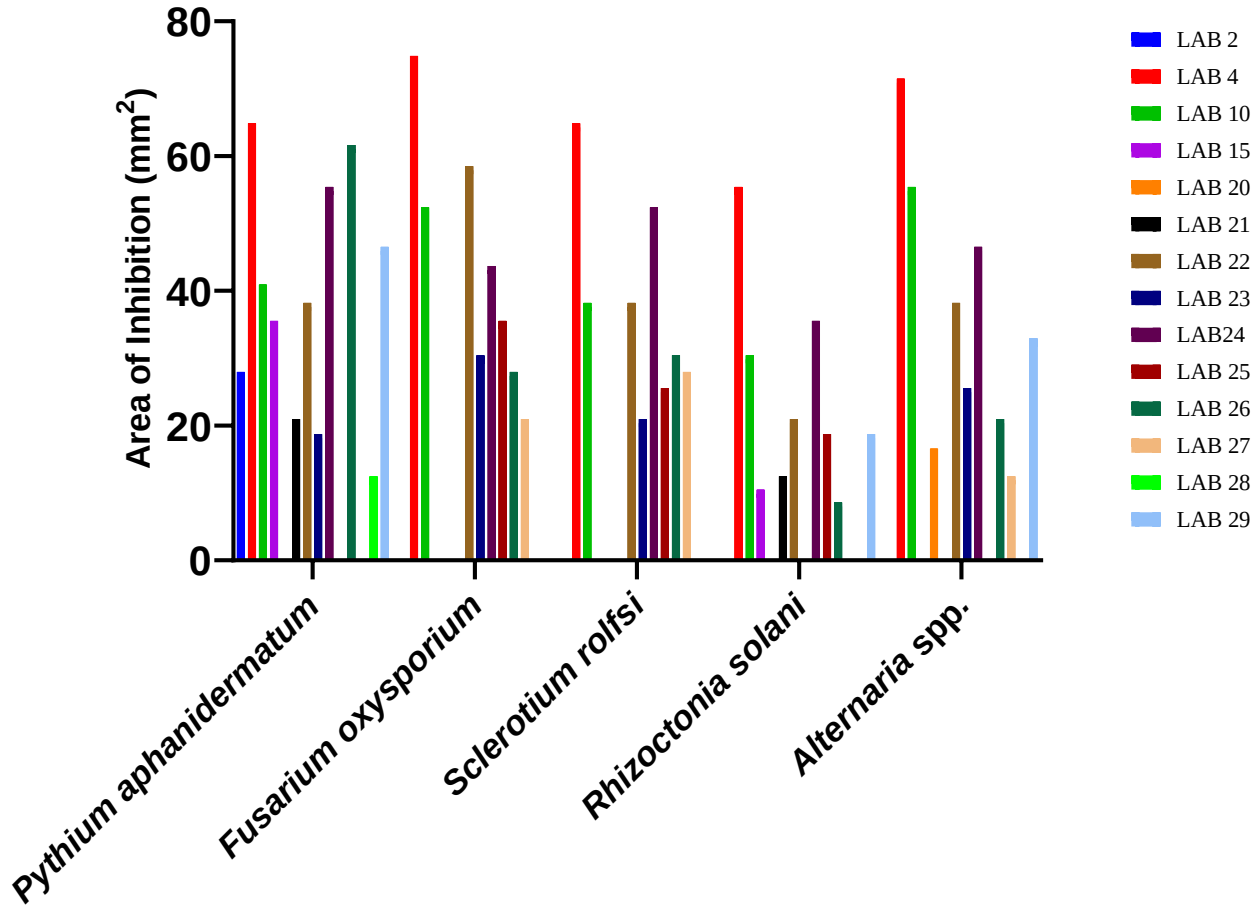

Figure 1: Area of inhibition of lactic acid bacterial isolates on growth of fungal plant pathogens by agar well diffusion assay

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, Figure 2.

Results of the liquid broth assays shows that the LAB isolates showed varied percentage reduction in mycelium dry weight of all fungal pathogen ranging from very low, moderate to high. Isolate LAB 4 was significant in inhibiting mycelial growth of all fungal pathogen even in liquid broth, whereas isolate LAB 2 inhibited 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 isolate LAB 4 mycelial dry weight of *Alternaria* spp., was reduced effectively by isolate 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 important fungal plant pathogen like *Fusarium oxysporum* in vitro conditions and recorded reduction in mycelial dry weight was recorded by 75% by isolate 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.

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 same superscript, in a column do not differ significantly at *P* ≤0.05 as per Duncan Multiple Range Test (DMRT).

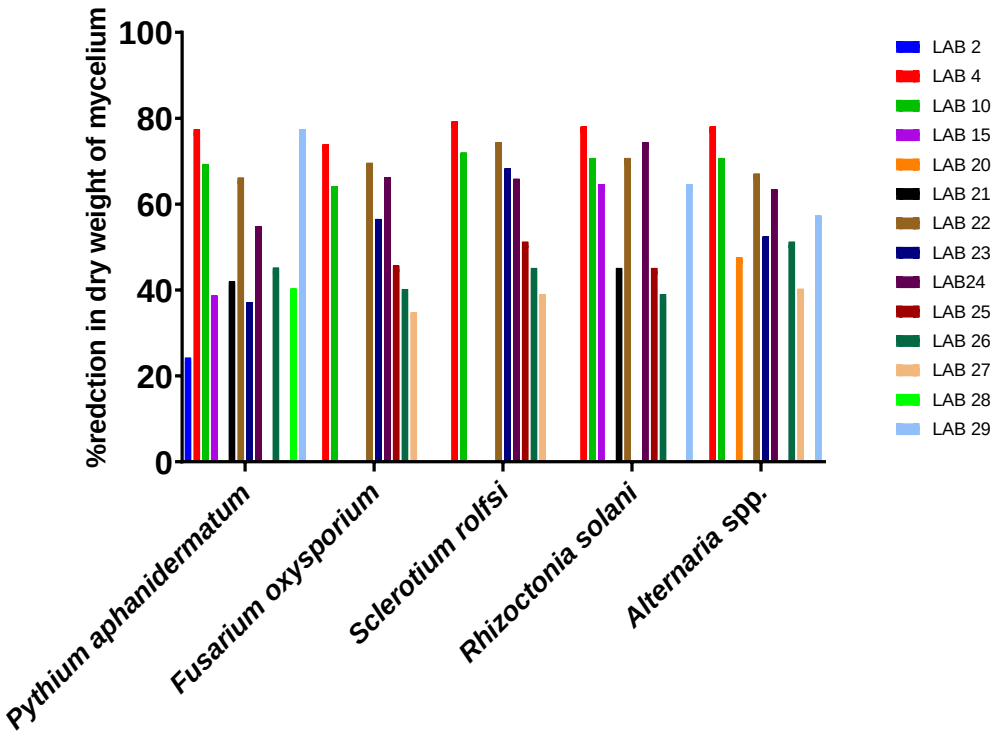

Figure 2: Effect of lactic acid bacterial isolates on per cent reduction of mycelial dry weight of fungal plant pathogens in liquid culture.

3.3 Molecular characterization of LAB isolates.

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

The sequence of 16s rRNA gene sequence of the LAB isolates was searched in 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 phylogentic tree of LAB isolates were presented in the Figure 3a and 3b.

The results of phenotypic characterization of Lactic acid bacteria isolated was 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 *Weissella* and *Leuconostoc*. Similarly, Chen et al. [24] isolated, 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*.

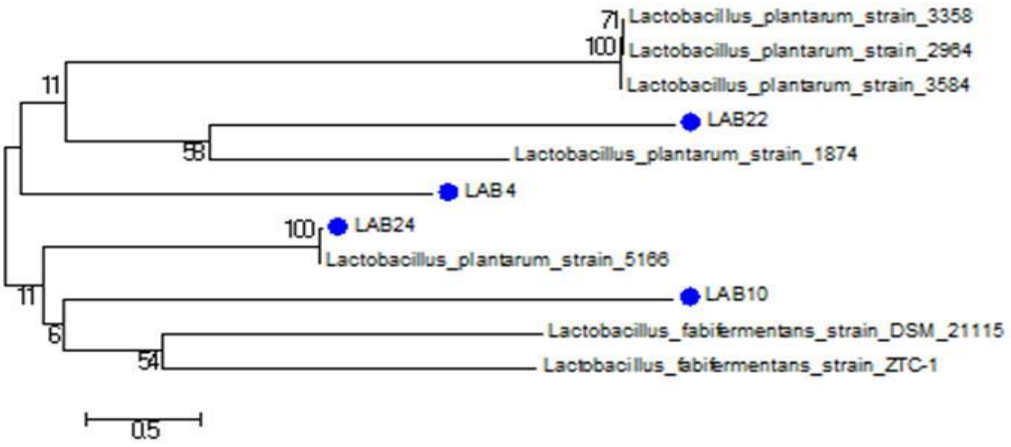

Figure 3a: Phylogenetic placement of lactic acid bacterial isolates (LAB 4, LAB 10, LAB 22, LAB 24) based on nearly full length 16S rRNA gene sequencing. Boot strap values are based on 1000 replicates.

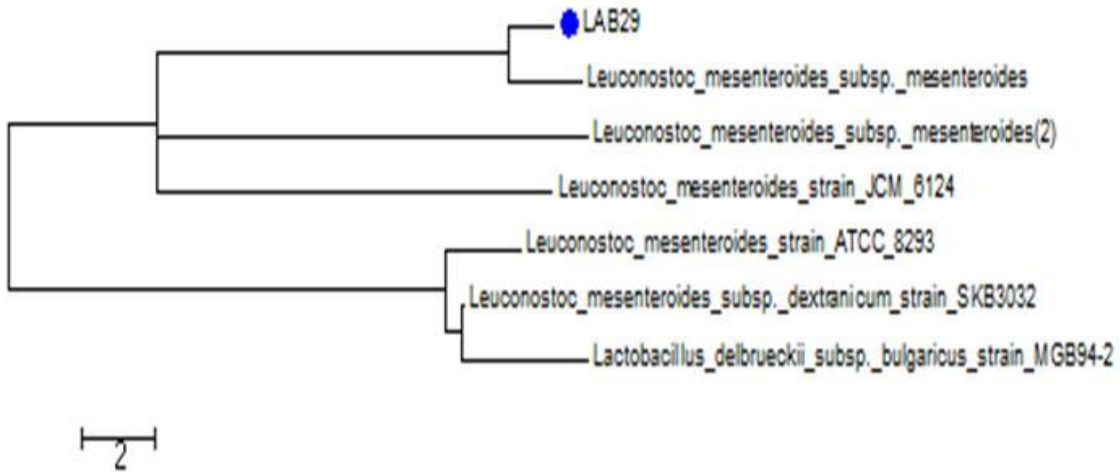

Figure 3b: Phylogenetic placement of lactic acid bacterial isolate (LAB 29) based on nearly full length 16S rRNA gene sequencing. Boot strap values are based on 1000 replicates.

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 novel biocontrol agent against them. Among LAB isolates Lactobacillus plantarum strains were found to exhibit antifungal property more profoundly than other isolates and has to further confirm by field studies.

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COMPETING INTREST

Authors have declared no competing interest.

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