Studies on the Potential of *Lactoplantibacillus plantarum* in Plant Growth Promotion

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ABSTRACT

Background: Plant growth-promoting Rhizobacteria are bacteria associated with plant roots and enhancing plant growth. Numerous compounds generated by rhizobacteria, like esterases, oxidases, bacteriocins, exopolysaccharides and conjugated linoleic acid, have been linked to the indirect enhancement of plant growth and control of pathogenic organisms. As lactic acid bacteria are present in the rhizosphere and other parts of the plants, the possible role of lactic acid bacteria in promoting plant growth is studied in this paper.

Methods: A comparison of well-established plant growth promoting bacteria (PGPR) *Azotobacter* spp. and the test organism *Lactoplantibacillus plantarum* ((MTCC 2973) was done by testing both the organisms for antimicrobial activity, gibberellic acid production, Indole acetic acid production and Siderophore production. The pot experiment studied the effect of *Lactoplantibacillus plantarum* on the growth of Cress Seeds (*Lepidium sativum*).

Result: The study found that *L. plantarum* and *Azotobacter* spp. both exhibit antibacterial activity against *Xanthomonas* spp. using *Lactoplantibacillus plantarum* to grow plants is eco-friendly and economical, offering a sustainable approach with potential applications in agriculture and environmental management.

Key words: Azotobacter, Gibberellic acid, GRAS, IAA, Lactic acid bacteria, PGPR, Siderophore.

INTRODUCTION

Recent developments in the study of plant-microbe interactions have highlighted the significance microbial populations encourage plant health and resilience (Smith *et al*., 2015). A viable approach for sustaining agricultural output in a changing environment and expanding population is to engineer the phyto-microbiome to encourage plant development. Plant growth-promoting microorganisms (PGPM) are generally found on the root surface and in the rhizosphere. PGPM enhances nutrient uptake, functions as biocontrol agents (BCAs), increases the host plant's resistance to biotic and abiotic stress and produces substances that directly stimulate plant growth.

Rhizobium, Azospirillum, Azotobacter, *etc.*, are Plant growth promoting bacteria that can fix nitrogen (Kajić *et al*., 2024). Azotobacter is one of the well-known Plant growth promoting bacteria. The presence of *Azotobacter* spp. in soils has beneficial effects on plants. Azotobacter is a genus of Gram-negative, free-living, and nitrogen-fixing aerobic bacteria mainly found in soil. *Azotobacter* spp. are characterised by nitrogen fixation, siderophore production, Indol Acetic Acid (IAA), ammonia production and gibberellic acid production (GA) (Kajić *et al.,* 2024). These characteristics help improve overall plant health (Sambul *et al*., 2020; Resmi *et al*., 2024).

Along with these characteristics, *Azotobacter* spp has a wide range of applications, such as bioremediation, pesticide degradation and heavy metal tolerance. Bioremediation is an effective method for reducing anthropogenic pollution from the environment. Bacteria that belong to the Azotobacter genus have been reported to exploit a wide range of organic substances and can form several biologically active compounds which initiate the proliferation of rhizospheric microorganisms into the soil.

Growth hormones are natural substances produced by both microorganisms and plants. These hormones alter plants' and microbes' physiological and biochemical processes by having a stimulatory and inhibitory impact (Sambul *et al*., 2020). Azotobacter releases Indole 3 acetic acid after adding tryptophan to the culture medium. Along with IAA, Azotobacter produces growth hormones like auxins and gibberellins (Zahedi and Abbasi, 2015; Resmi *et al*., 2024).

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Genus Azotobacter is reported to synthesise auxins, cytokinins, and GA-like substances directly associated with improved plant growth (Sumbul *et al*., 2020).

Lactic acid bacteria can also be considered useful plant growth promoters since they can exhibit important characteristics similar to the standard PGPR. It is a facultative anaerobe, a Gram-positive bacteria, which can be found in animal feed, fermented foods, and the human gastrointestinal tract (Raman *et al*., 2022). The pH range for the growth of Lactobacillus lies between 3.5 and 6.5, and they can thrive in environments with temperatures between 15°C and 50°C. They generally do not produce spores and are non-motile (Raman *et al*., 2022).

Lactic acid bacteria can serve as PGPR as they involve various vital applications related to the agricultural field. Due to its capacity to break down multiple contaminants, including phenols, polycyclic aromatic hydrocarbons, and heavy metals, Lactobacillus has potential applications in bioremediation. This is due to synthesising enzymes like esterases and oxidases that can degrade these contaminants (Duar *et al*., 2017). Numerous bioactive substances that Lactobacillus can produce, including cytokinin, exopolys accharides and siderophores, have potential uses in the food and pharmaceutical industries (Raman *et al*., 2022).

Auxins, cytokinins, and gibberellins, among other phytohormones that Lactobacillus can produce, can promote plant growth and development. Auxins can encourage root development, cytokines can improve nutrient absorption, and gibberellins can lengthen stems and increase plant height (Wang *et al*., 2012). Phytase and cellulase are the two enzymes that can solubilise phosphorus and break down cellulose in the soil, respectively and are produced by some strains of the Lactobacillus. This may boost the availability of nutrients to plants and encourage overall development (Khanghahi *et al*., 2024). According to reports, Lactobacillus possesses biocontrol abilities against plant diseases. For instance, it has been demonstrated that *Lactobacillus plantarum* inhibits the development of the fungi *Fusarium oxysporum* and *Botrytis cinerea*, which cause root rot and grey mould disease in plants, respectively (Gajbhiye and Kapadnis, 2016; De Simone *et al*., 2021; Khanghahi *et al*., 2024).

Lactobacillus spp. can also encourage nutrient cycling in the soil. Plants and *Lactobacillus* can coexist in symbiotic partnerships, benefiting both the host plant and the bacteria. Depending on the type of crop being grown and the surrounding environment, *Lactobacillus* can have various effects on plant development and productivity. For instance, it has been demonstrated that *Lactobacillus plantarum* promotes the growth of tomato and maize plants but not cucumber plants (Sharma *et al*., 2003).

MATERIALS AND METHODS

Sources of strains and culture conditions

The present research was carried out from June 2023 to April 2024 at the Microbiology Research Center, Haribhai V. Desai College, Pune, Maharashtra, India.

Lactoplantibacillus plantarum

(MTCC 2973) was procured from the Microbial Type Culture Collection Center in Chandigarh, India. *L. plantarum* was maintained on MRS medium (de Man-Rogosa-Sharpe medium, HiMedia, India). The culture was incubated at 37°C under stagnant conditions for 24 h. *Azotobacter* spp. was isolated from the garden soil of Haribhai V. Desai College Pune, Maharashtra, India, and was enriched in Ashby's Mannitol broth (Himedia India). The isolated colonies were studied for morphological characteristics and biochemical tests such as oxidase, catalase, sugar fermentation, amylase, and cellulose production (Patel, 2023).

Plant growth-promoting traits of *Lactoplantibacillus plantarum* and *Azotobacter* spp.

I. IAA production

The IAA production was checked by the spectrophotometric method given by (Mohite, 2013) using the Salkowaski reagent. The bacterial cultures were inoculated in the appropriate media supplemented with 500 µg/ml tryptophan and incubated on a rotary incubator shaker at 250 rpm for 2-3 days at 30°C (REMI CIS 24). The broths were centrifuged at 10,000 rpm for 10 min. The supernatant (2 ml) was mixed with Salkowski reagent (4 ml). The inoculation media supplemented with 500 µg/ml tryptophan without inoculation was kept as a control. The development of the pink colour indicated the production of IAA.

II. Gibberellin production

The *Lactoplantibacillus plantarum* and *Azotobacter* spp. were inoculated in 100 ml media MRS and Ashby's Mannitol broth for 7 days at 30°C at 100 rpm. After 7 days, the broth was centrifuged at 5000 rpm for 7 min, and the cell-free extract was used to analyse the amount of gibberellin produced. The gibberellins were estimated calorimetrically using the standard method (Graham and Thomas, 1961) with some modifications. Cell-free extract (5 ml) and ethyl acetate (5 ml) were combined in a test tube, and the mixture was shaken rapidly for 5 min to separate the ethyl acetate layer. The leftover ethyl acetate evaporated from the organic layer and dissolved in alcohol. After incubation in a water bath at 100°C for 5 min, 2, 4 - dinitrophenyl hydrazine (DNPH) (1 ml) and organic suspension (2 ml) were combined and allowed to cool. Following this, 10% potassium hydroxide (5 ml) was added to the cooled extract and observed for the development of red wine colour. The contents were diluted to 1:2.5 with distilled water, and absorbance was noted at 430 nm using a UV-VIS spectrophotometer (UV-1800 Shimadzu). The standard graph of gibberellin was made in the range of 10-100 µg/ml using distilled water as blank.

III. Siderophore Production

Siderophore production was detected using the CAS agar plate method and $FeCl_3$ method.

1. CAS method

Siderophore production was detected using Chrome Azurol S (CAS) reagent as per the method given by (Perez *et al*., 2007). CAS agar plate was prepared as *viz.* Chrome Azurol S (CAS) 60.5 mg, hexadecyl trimethyl ammonium bromide (HDTMA) 72.9 mg, Piperazine-1,4-bis (2-ethane sulfonic acid) (PIPES) 30.24 g, and 1 mM FeCl36H2O in 10 mM HCl 10 mL and agar powder (0.9%, w/v) was used as a solidifying agent. Siderophore production was detected by the overlay of the above medium onto agar plates containing the growth of test organisms. After 15-20 min of overlay, a colour change will be noted from blue to purple or blue to orange, indicating a positive result. The experiment was repeated three times. All glassware used in the experiment was rinsed with 3 mol/L hydrochloric acid (HCl) to remove iron and subsequently washed in deionised water.

2) $FeCl_3$ method

This test was performed as described by Atkins *et al*. (1970). To 1 ml of cell-free supernatant, 1 ml of 2% aqueous $FeCl_3$ solution was added and examined for the appearance of yellow or brown colour, which indicates a positive result.

IV. Phosphate solubilising activity

The test was performed per the method given by Jaini *et al*. (2022). The test cultures were spot inoculated on the Pikovaskaya's agar media supplemented with 0.5% tricalcium phosphate [$(Ca_3(PO4)_2)$] (Merck, Germany) and the plates were incubated for 30°C for 48-72 hrs. The appearance of a clear zone around the colony indicated the phosphate solubilisation ability of the isolates. The diameter of the clearance zone of more than 0.01 cm around colonies is considered a positive test.

V. Ammonia production

According to Jaini *et al.* (2022), the test cultures were tested for ammonia production. Briefly, 20 µl of test culture was inoculated into 10 ml of peptone water (Himedia, India). Then, it was incubated in a shaker incubator (REMI CIS 24) at 150 rpm for 48 hrs. at 30°C. After incubation, 0.5 ml Nessler's reagent (Himedia India) was added, and the formation of a brown to yellow colour indicated a positive test for ammonia production.

VI. Antibacterial activity

Investigating the antimicrobial potential of test cultures against plant pathogen *Xanthomonas* spp., a lab isolate was performed using the method described by Emerenini *et al*. (2014). The 24-hour-old culture of *Xanthomonas* spp. was spread uniformly on Muller Hinton agar (Himedia India) plates. The wells were then prepared using a sterile well borer (5 mm). Fresh cell-free supernatant of test cultures (50 µl) were added to the wells separately. The plates were incubated in the incubator for 48-72 hrs. and observed for the zone of inhibition. The diameter of the zone of inhibition was measured.

VII. Pot experiments to evaluate PGPR activity of *Lactoplantibacillus plantarum*

For the pot experiment, Cress (*Lepidium sativum*) seeds were selected. *Lepidium sativum* is a fast-growing, medicinally important herb rich in proteins, carbohydrates, dietary fibres, and minerals. One gram of seeds was inoculated in each pot.

Inoculum preparation

48-hour-old cultures of *Lactoplantibacillus plantarum* and *Azotobacter* spp. were grown in MRS broth, and Ashby's broth was used as an inoculant. The bacterial pellet was collected by centrifugation, dissolved in distilled water, and diluted to an optical density of OD600 = 0.250 (Lutz *et al*., 2012). 10% inoculum was used in appropriate combinations.

The PGPR potential of *Lactoplantibacillus plantarum* was compared with *Azotobacter* spp.Eight different combinations (Table 1) were respectively made using the above inoculum for the pot assay as shown in Table 1. The experiment was performed in triplicates.

RESULTS AND DISCUSSION

Morphological and biochemical characterization of *Azotobacter spp*

A total of 5 isolates belonging to *Azotobacter* spp. were isolated out of them only one was selected for further studies. *Azotobacter* spp. used in present study belong to Gram negative, spherical and motile bacteria (Table 2).

Table 1: Different combinations of soil, manure to evaluate PGPR potential of *Lactoplantibacillus plantarum* and *Azotobacter* spp.

Combination no.	*Lactoplantibacillus plantarum*	*Azotobacter* spp.
1	Soil	Soil
2	Sterile soil	Sterile soil
3	Soil + LAB	Soil + *Azotobacter* spp.
4	Sterile soil + LAB	Sterile soil + *Azotobacter* spp.
5	Soil + LAB + Manure	Soil + *Azotobacter* spp. + Manure
6	Sterile soil + LAB + Manure	Sterile soil + *Azotobacter* spp. + Manure
7	Soil + Manure	Soil + Manure
8	Sterile soil + Manure	Sterile soil + Manure

Plant growth-promoting traits of *Lactoplantibacillus plantarum* and *Azotobacter* spp.

I. IAA production

The development of a pink colour after adding the Salkowski reagent in both test cultures indicates a positive result. *L. plantarum* and *Azotobacter* spp. both showed the production of IAA. Lactic acid bacteria KLF01 produced IAA, as demonstrated in a study by Shrestha *et al*. (2014) after 48 hours of incubation in the presence of tryptophan.

Table 2: Morphological and biochemical characters of cultures used in study.

Test cultures used	*Lactoplantibacillus plantarum*	*Azotobacter* spp.
Morphological characteristics		
Gram character	+	-
Shape	Small rods	spherical
Motility	Non-motile	Motile
Biochemical characterization		
Catalase	-	+
Oxidase	-	+
Sugar fermentation		
Glucose	A	A+G
Lactose	A	A+G
Xylose	-	A+G
Sucrose	A	A+G
Mannitol	-	A+G
Enzyme production		
Amylase	-	+
Cellulase	+	+

Note: + indicates positive test, - indicates negative test, A acid production, A+G acid and gas production.

II. Gibberellin production

The amount of gibberellin produced by both test cultures *viz. L. plantarum* and *Azotobacter* spp. were found to be 84.45 and 86.88 µg/ml, respectively. In a study by Turaeva *et al*. (2021), *Lactobacillus plantarum* produced 2.286 mg/ml of gibberellin (Turaeva *et al*., 2021).

III. Siderophore Production

After adding the CAS reagent, the colour changed from blue to orange, indicating a positive result. *L. plantarum* and *Azotobacter* spp. both showed the production of siderophore, which was also confirmed by the $FeCl_3$ method, where a wine-red-coloured complex was formed for both *L. plantarum* and *Azotobacter* spp., indicating the production of a Catecholate type of siderophore.

IV. Phosphate solubilisation

L. plantarum showed the capability of phosphate solubilisation with a diameter of zone of clearance 25 mm. Lactic acid bacteria Strain KPD03 phosphate solubilising effect with 18.5-mm diameter zone clearance. While *Azotobacter* spp. did not show solubilisation of tricalcium phosphate.

V. Ammonia production

L. plantarum and *Azotobacter* spp. both showed positive results for ammonia production. Ammonia production is an essential trait of PGPR, which indirectly affects plant growth (Agbodjato *et al*., 2015; Abhyankar *et al*., 2022).

VI. Antibacterial activity

L. plantarum and *Azotobacter* spp. both showed antibacterial activity against *Xanthomonas* spp., with a diameter of the zone of inhibition of 12 mm and 14 mm, respectively. *Lactobacillus plantarum* KLF01 showed

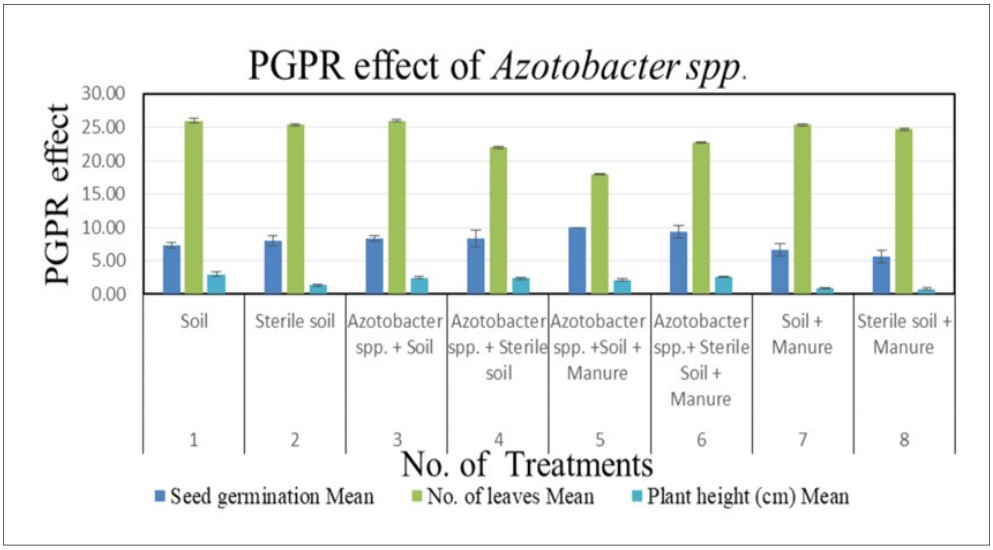


Fig 1: PGPR effect of azotobacter spp. on the growth of Cress (*Lepidium sativum*).

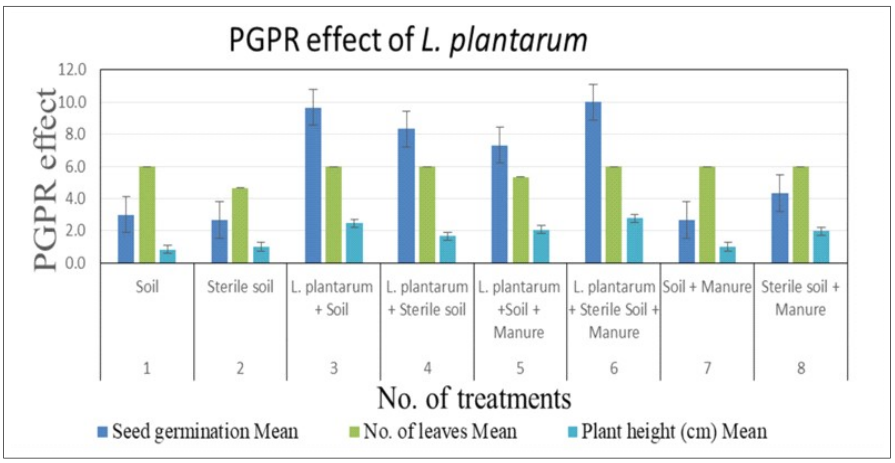


Fig 2: PGPR effect of *L. plantarum* on the growth of cress (*Lepidium sativum*).

antagonistic activity against *X. axonopodis* pv. *Citri* (Shrestha *et al*., 2009).

VII. Pot experiments to evaluate PGPR activity of *Lactoplantibacillus plantarum*

In pot studies, similar no. of seeds (10.0±2.0) were germinated using *L. plantarum* plus sterile soil and manure in comparison to *Azotobacter s*pp. treatment (10.0±00) confirming the PGPR effect of *L. plantarum* on germination (Fig 1 and 2). This could be due to the production of IAA and gibberellin by *L. plantarum*. Compared to the Azotobacter treatment, fewer leaves were developed in seeds treated with all combinations with *L. plantarum*. Plant height was increased in the treatment of seeds with *L. plantarum* plus sterile soil and manure compared to that of *Azotobacter* plus sterile soil and manure.

The increase in height was observed in pepper [*Capsicum annuum* (L.) var. *annuum*] plants treated with Lactic acid bacteria strain KLF01 from 12% to 27% (Shrestha *et al*., 2014). Gibberellic acid, or gibberellin, is a plant hormone that stimulates the growth and development of a plant. The gibberellin signalling system is critical for germinating seeds, stem elongation, meristematic tissue development, and floral organ differentiation (Gupta and Chakraborty, 2013). When seeds or roots were inoculated with *Azotobacter*, the reported impacts on plant development and yield were most likely caused by the gibberellins in *Azotobacter* cultures. The production of more gibberellins in the root zone, which occurs when the *Azotobacter* inoculum colonises growing roots, may also impact plant growth (Brown and Burlingham, 1968). Gibberellin production by *Lactoplantibacillus plantarum* and *Azotobacter* spp was studied using DNPH assay. Gibberellic acid production by *Azotobacter* spp. was found to be 86.88mg/ml. Similarly, Gibberellic acid production by *Lactoplantibacillus plantarum* was 84.45 mg/ml. From the results, it can be seen that the values of Gibberellic acid produced are very similar in both cases. Hence, gibberellic acid production by *Lactoplantibacillus plantarum* may play a similar role in plant growth as of *Azotobacter* spp.

One significant kind of PGPR is siderophilic bacteria, which release siderophores to chelate unavailable Fe^{3+} in the soil for plant growth. It has been demonstrated that siderophilic bacteria are essential for both disease prevention and plant growth promotion (Wang *et al*., 2022). Siderophore detection was studied using two methods, namely, the CAS method and the $FeCl_3$ method.

A pot assay was performed to study the practical effect on plants. Seed germination in the case of Azotobacter was seen after 3 days, while *Lactoplantibacillus plantarum* was seen after 4 days of planting. The number of plants observed in both cases is almost the same. The height of the plant was higher in the case of Azotobacter than in the case of *Lactoplantibacillus plantarum*. A significant difference was observed in no leaves produced where *Azotobacter* spp. inoculated plants showed an average of 26 leaves, whereas an average of 6 was shown by *Lactoplantibacillus plantarum* inoculated plants. One of the possible reasons for this significant difference might be due to seasonal variations.

Antimicrobial activity was performed using the agar well diffusion method. Both the organisms *Lactoplantibacillus plantarum* and *Azotobacter* spp. showed zones of inhibition against plant pathogen *Xanthomonas* spp. This indicates that *Lactoplantibacillus plantarum* may show the same antimicrobial activity as *Azotobacter* spp. Recent research shows the production of Plantaricin FB-2, a novel bacteriocin produced by *Lactoplantibacillus plantarum.* It is non-hemolytic and has a broad antimicrobial spectrum. Destruction of bacterial cell membrane structure is its mode of action (Li *et al*., 2023).

LAB strains have the potential to enhance agricultural output through multiple mechanisms, including augmenting nutrient availability, alleviating the impact of biotic and abiotic stresses and directly promoting plant development. Their extended food research history and GRAS status make them perfect for crop protection

applications. Despite being widely distributed in the phytomicrobiome, LABs' potential to stimulate plant development has not received much attention. Research from both the past and the present indicates that LAB can be used as safe, renewable agricultural inputs to help manage plant diseases and encourage plant development. Further research on LAB is necessary, though, and it should concentrate on LAB bio-production and formulations in addition to its biocontrol effectiveness in field settings. An effective strategy to boost efficacy against phytopathogens and assist in resolving the issues about the achievement of sustainable food security would be the incorporation of LAB as a biocontrol agent that could be utilised with other biocontrol approaches in an integrated control program (Jaffar *et al*., 2023)

Future research on Lactobacillus should focus on its plant growth-promoting capabilities, which could potentially be used in bioremediation and biofuel generation (Duar *et al*., 2017).

CONCLUSION

The various studies and applications of *Lactoplantibacillus plantarum* suggest that this organism has a huge potential for use as a plant growth-promoting bacteria, which would also be very eco-friendly, economical, and sustainable.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions.

Informed consent

Not applicable.

Conflict of interest

The authors declare no conflicts of interest.

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