Impact of IAA producing and Phosphate solubilizing bacteria on the germination and cytology of *Lens culinaris*

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Abstract— Pathogenic microorganisms affecting plant health are a major threat to sustainable agriculture and ecosystem stability worldwide. The chemical fertilizers used in the agriculture to increase yields, kill pathogens, pests, and weeds, have a big harmful impact on the ecosystem. Because of current public concerns about the side effects of agrochemicals, there is an increasing interest in improving the understanding of cooperative activities among plants and rhizosphere microbial populations. *Lens culinaris* is a leguminous crop of family Fabaceae. It is an ideal crop for basic research as it is sexually propagated, amenable for pollination, limited in number of chromosomes and has an ideal growing season. Rhizobacteria that exert beneficial effects on plant growth and development are referred as Plant Growth Promoting Rhizobacteria (PGPR). Some rhizospheric microorganisms have capability to convert the insoluble phosphorous to an accessible form that exhibit increase in the plant growth, nodulation and yield of the plants. The present study documents about the growth efficacy of PGPR on the morphological, microbiological and cytological association patterns and behaviour in *Lens culinaris*. This study is based on the effects of treatment of PGPR on *Lens culinaris* and on observation, findings gave emphasis on the treatment of PGPR that raised the various parameters regarding growth, development and chromosomal pattern of the seeds upto a great extent. These findings showed the importance of PGPR in the growth and development of plant and can lead to many improvements in crop yield.

Index Terms—leguminous, chromosomes, PGPR,rhizospheric, rhizobacteria, Fabaceae, *Lens culinaris*

1 INTRODUCTION

Over the last few decades, the agriculture policy in India has undergone a major change through diversification and emphasis on sustainable production system. Rhizosphere researches have been throwing up surprise and interesting ideas for research ever since the pleasant environment of microorganisms around plant root called rhizosphere. The term rhizosphere was introduced for the first time by Hiltner. In recent years considerable attention has been paid to PGPR to replace agrochemicals (fertilizers and pesticides) for the plant growth promotion by a variety of mechanisms that involve soil structure formation, decomposition of organic matter, recycling of essential elements, solubilization of mineral nutrients, producing numerous plant growth regulators, degrading organic pollutants, stimulation of root growth, crucial for soil fertility, biocontrol of soil and seed borne plant pathogens and in promoting changes in vegetation.

Legumes are of great value in agriculture, both in arable and grassland farming. Legumes are important source of proteins, carbohydrates including fibres, minerals and beta complex vitamins. Lens culinaris is self-pollinating crop with 2n=14. Its common name is Masoor. Lens culinaris is the native of Southwestern Asia. It is now cultivated in most subtropical and northern hemisphere. Major states producing Lens culinaris are Uttar Pradesh, Madhya Pradesh, Bihar, Rajasthan, West Bengal, Maharashtra and Haryana. Lens culinaris can be described as annual bushy herb, slender almost erect or suberect, much-branched, angular with 15-75 cm height (Duke, 1981; Muehlbauer et al., 1985). The leaves are alternate, compound, pinnate, usually ending in a tendril, leaflets 4-7 paired, alternate or opposite; stipules absent; pods oblong, flattened or compressed, smooth, 1-2-seeded, seed biconvex, rounded, small, lens-shaped. Flowers are small, pale blue, purple, white or pink, in axillary 1-4-flowered racemes (Muehlbauer et al., 1985). Germination is hypogeal (Muehlbauer et al.1985). It can be grown on sandy soils to clay loams but will grow optimally in well-drained soil with a pH of 4.5 to 8.2. A soil pH of close to 7 is ideal. The optimum temperature for their growth is approximately 24°C (75°F). Lentils require an average rainfall of 10 to 12 inches per year (Ford et al., 2007; Bejiga, 2006). It has symbiotic relationship with certain soil bacteria and fix atmospheric nitrogen. It may be intercropped with barley, castor, mustard and rice. Planting rate is 132 seeds per square meter. Canada is the largest export producer in the world.
Soil contains narrow regions called as rhizosphere which directly influences root secretions and the associated microbial activity. These PGPRs are present in the rhizosphere of various plant species and lead to beneficial effects of host plant. The term, PGPRs was first used by Kloepper in the late 1970s. PGPRs are root colonizing Bacteria that form symbiotic relationships with many plants. The two major classes of relationships are rhizospheric and endophytic. Rhizospheric relationships consist of PGPRs that colonize surface of root or superficial intercellular spaces of host plant, often forming root nodules. Endophytic relationships involve PGPRs residing and growing within the host plant in apoplastic space (Kloepper,1980). Plant Growth Promoting Rhizobacteria (PGPR) are a heterogeneous group of bacteria that can be found in the rhizosphere, at root surfaces and in association with roots, which can improve the extent or quality of plant growth directly and or indirectly. PGPRs offer an improved

way to replace the use of chemical fertilizers, pesticides and other components (Glick,2012). For the plant to be able to benefit from added available nutrients provided by rhizobacteria, it needs to provide place and proper conditions for the rhizobacteria to live. Legumes are often able to colonize early succession environments due to unavailability of nutrients. Once colonized, rhizobacteria make the soil surrounding the plant more rich that can lead to competition with the other plants. PGPRs increase the availability of nutrients through solubilization of unavailable forms of nutrients and by production of siderophores which aids in facilitating of nutrient transport. PGPRs are present in the rhizosphere of various plant species and lead to beneficial effects on host plant. The term, PGPRs was first used by Kloepper in the late 1970s. PGPRs are root colonizing Bacteria that form symbiotic relationships with many plants. The two major classes of relationships are rhizospheric and endophytic. Rhizospheric relationships consist of PGPRs that colonize surface of root or superficial intercellular spaces of host plant, often forming root nodules. Endophytic relationships involve PGPRs residing and growing within the host plant in apoplastic space (Kloepper, 1980). PGPRs are used as Biofertilizers, biocontrol, production of phytohormones and siderophores.

Paenibacillus polymyxa is a Gram-positive bacterium capable of fixing nitrogen. The species may also be known as *Bacillus polymyxa.* It is found in soil, plant roots, and marine sediments. *P. polymyxa* is used as a soil inoculant in agriculture and horticulture. Biofilms of *P. polymyxa* growing on plant roots have been produce exopolysaccharides which protect the plants from pathogens. The interactions between this bacterial species and plant roots also cause the root hairs to undergo physical changes. Some strains of *P. polymyxa* produce polymyxin antibiotic compounds. *Paenibacillus polymyxa* is an endospore-forming bacterium that is non-pathogenic and found in environments such as plant roots in soil and marine sediment. The wide range of capabilities of this bacterium are to fix nitrogen, produce hormones that promote plant growth, produce hydrolytic enzymes, and to produce antibiotics against harmful plant and human microorganisms. It can also help plants in absorption of phosphorus and enhance soil porosity. This microbe has a role in ecosystem function and potential role in industrial processes. In addition, soil fluctuation and porosity is improved due to organic compounds released from *P. polymyxa* into the soil.
Indole Acetic Acid (IAA) is the most common, naturally occuring plant hormone, best known for auxins and has been subject of extensive studies. It is chemically carboxylic acid in which carboxyl group is attached through methylene group to C3 position of Indole ring. It is colourless solid. Ernstsen et al. (1987) reported that Rhizobia can synthesize significant levels of IAA symbiotically in nodules as well as in free living conditions. Indole Acetic Acid (IAA) is one of the most physiologically active phytohormone in several microorganisms as bacteria promoting plant growth (Ahmad et al. 2005). IAA is known to play very important role in microbe and plant interactions and its signaling is also associated with the plant defence mechanisms (Spaepen and Vanderleyden,2011). Glick (2012) observed that IAA is one of the important phytohormones affecting plant physiological processes such as plant cell division, extension and differentiation, stimulates seed and various tuber germination, controls processes of vegetative growth by initiating lateral and adventitious root formation, photosynthesis, pigment formation and is involved in the biosynthesis of various metabolites.
Phosphate Solubilizing Bacteria (PSB) are the group of beneficial bacteria capable of hydrolyzing organic and inorganic phosphorus forms from insoluble compounds. It creates ability in microorganisms which are considered to be one of the most important traits associated with plant phosphate nutrition. The mechanism of mineral phosphate solubilization by PSB strains is associated with the release of low molecular weight organic acids. PSB have attracted the attention of agriculturists as soil inoculums to improve plant growth and yield. The principal mechanism for mineral phosphate solubilization is the action of organic acids synthesized by soil microorganisms. Inorganic P is solubilized by the action of organic and inorganic acids secreted by phosphate solubilizing bacteria. Release of root exudates such as organic acids can also alter the concentration of P in the soil solution (Hinsinger, 2001). According to Khan et al., 2009, PSB excrete organic acid and they dissolve phosphatic minerals or chelate cationic partners of the phosphate in ions and directly release phosphorus in soil. Hariprasad and Niranjana (2009) also noticed a drop in pH of the culture broth with the increase of soluble orthophosphate which indicated the significance of organic acid production in phosphate solubilization process.

2 MATERIAL AND METHODS

2.1 Preparation of soil

The collection of the soil sample was from the Roxburgh Garden of Botany Department, University of Allahabad. The unwanted debris other small plant parts were removed and clear soil was obtained. Soil was kept in oven and then under room temperature to cool. Pots were then filled with soil and autoclaved to obtain complete sterility.

2.2 Sterilization Procedure

Sterilization process: Sterilization is the process which kills all the living organisms in order to avoid any contamination. It is the basic process without which isolation of microorganisms is impossible leading to larger amount of contamination.

2.3 Rhizospheric Isolation

Isolation of a particular microbe in the form of pure culture is highly necessary to separate it from its mixed population and to study its characteristics or other specific role, in its environment. Pure culture also helps in maintaining the identity of a particular microbe and growth of their progenies.

2.4 Serial Dilution Method

Serial dilution method: It is the simplest and the most widely used method of isolation. The organism that is dominant in its mixed culture can be easily obtained in the form of pure culture by carrying out serial dilution in tubes with suitable

sterile medium.

2.5 Detection of Phosphate Test

Pikovskaya Agar Medium was used for the cultivation of phosphate solubilizing microorganisms. Bacterial cultures were inoculated on centre of agar plate through inoculation loop under aseptic condition. Inoculated plates were incubated for 3 days at 30°C.

2.6 Detection of Indole Acetic Acid Test

Luria Bertoni Broth was used for the bacterial cultures and enrichment. An inoculae was inoculated in trypton broth and kept at 37˚C for 24-48 hours. After inoculation 1ml of Kovac's reagent were added to broth.

2.7 Preparation and Treatment of *Lens culinaris*

Seeds of *Lens culinaris* were washed with distilled water followed by Sodium hypochlorite solution and then soaked for 5-7 hours. Muslin cloth was used as seed bed so that germination shall occur easily. 16 petri plates were used for the whole experiment. These were washed by distilled water then air dried and sterilized by autoclave. Distilled water was used along with 50 seeds for the seed control treatment. Seeds were treated with Adhesive (AD) and bacteria named *Paenibacillus polymyxa* separately. Seeds were also treated with the combined mixture of adhesive and *Paenibacillus polymyxa*. Measurement of treatment was based on the level of germination of seeds, shoot growth and chromosomal studies. Observations were carried out within a period of 1 to 15 days.

2.8 Morphological Studies

Gram staining of the isolated plant growth promoting rhizobacteria (PGPR) strain was done for the morphological observations of microbes and also to determine about the isolates, whether gram positive or gram negative in nature.

2.9 Cytological Studies

Fixation: It prevents autolysis, bacterial decay, shrinkage and distortion of the material. Most rapidly penetrating and quickly active fixative used was made from 3 parts of Absolute alcohol with 1 part of Glacial acetic acid.

Hydrolysis: It is used to soften the tissue or material so that cells can easily get separated during squash preparation. The chemical used for hydrolysis is N-HCl.

Staining: Staining may be vital or non vital. It is stained with 2% acetocarmine stain.

Squash Preparation: Squash is prepared by placing slide in between 2-4 folds of filter paper and tapping with flat end of glass rod. Slide is observed under microscope.

3 RESULT AND DISCUSSION

3.1 Morphological Identification of Bacteria

Gram Staining: The isolated bacterium was found to be gram positive as it gave positive result on gram staining. It took the crystal violet stain used in the test and appeared purple coloured under microscope. Purple colour appears due to thick peptidoglycan layer in the bacterial cell that retains stain after it is washed away from rest of the sample.

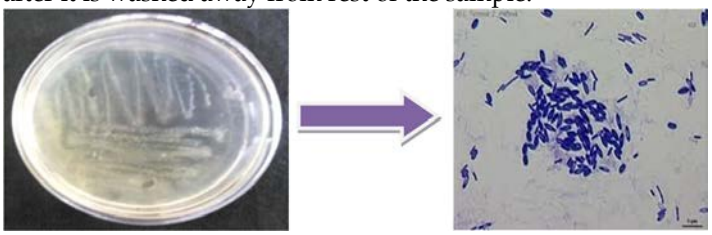

Figure (a)Gram staining of *Paenibacillus polymyxa*

3.2 Biochemical Identification of Bacteria

(i) Phosphate Solubilizing Test: PGPR strain was found to be very good phosphate solubilizer. The clear halozone or growth of rings are seen in the medium plate because of the efficiency of *Paenibacillus polymyxa* to solubilize inorganic phosphorus into solubilized form of phosphate which can be used by plant. Phosphorus is taken as macronutrient.

Figure (b) Positive Phosphate Solubilizing Test

(ii) Indole Acetic Acid Test: PGPR strain was found to be very good Phytohormone for auxin. The clear visibility and appearance of pink or red colour in the medium in ring form is visible due to the efficiency of *Paenibacillus polymyxa* to enhance the plant microbe interactions and to regulate the physiological processes in plant. Indole Acetic Acid is taken from auxin.

Figure (c) Positive Indole Acetic Acid Test

Table 1. Biochemical tests

Bacteria	Catalase Test	IAA test	HCN production	Phosphate Solubilization	Salt Stress
P.polymyxa	+	+ + +	+	+ + +	+ +

From the table above, it was easily seen that the best biochemical results of *P.polymyxa* with Indole acetic acid test and phosphate solubilization test were obtained. Salt stress also came to be prominent. The least significant results were

with Catalse and HCN production.

3.3 Morphological Study of *Lens culinaris*

(i) Growth Parameters: The treatment of plant Lens culinaris with PGPR *Paenibacillus polymyxa*(BPL-4), Silver nanoparticle (AD) and its combined interactions showed positive results and effects on the development of plant parts as roots and shoots as compared to the control plant.

(a) Root growth: The germination of seed and root development was observed for a week and the progress was taken for first three days in tabular form :

Table 2. Effect of treatment on the seed germination and root growth of Lens culinaris :

Duration	Seed Control	Seed + AD	Seed+ *P.polymyxa*	Seed+AD+*P.polymyxa*
Day 1	78%	75%	87%	92%
Day 2	85%	87%	90%	95%
Day 3	87%	90%	92%	97%
Mean	83.4%	84.2%	90.2%	94.5%

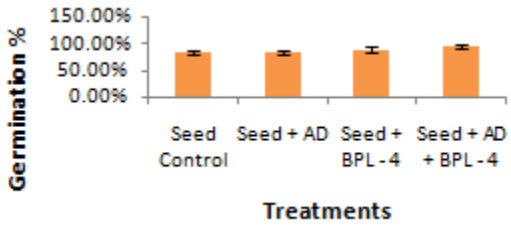

Figure (d). Chart showing germination % in the seed treatments

From the graph, the effects of *P.polymyxa,* AD and *P.polymyxa* + AD on the development of seed germination and root development are visible. It is clearly observed that effect of combination of P.polymyxa and AD showed a significant level of root development followed by treatment of *P.polymyxa* . Much slower rate of root development was seen in case of AD and seed control.

c. Shoot growth: The germination of shoot development was observed for a week and the progress was taken for gap of five days as in tabular form:

Table 3. Effect of treatments on shoot growth of *Lens culinaris*

Duration	Seed Control	Seed + AD	Seed + *P.polymyxa*	Seed+AD+ *P.polymyxa*
Day 5	85%	70%	85%	82%
Day 10	89%	75%	92%	85%
Day 15	95%	89%	97%	92%
Mean	89.6%	78.2%	92.5%	86.4%

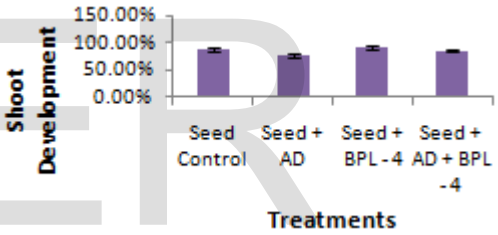

Figure 1(e) Chart showing shoot development in seed treatments

From the graph, it is clearly visible that effect of *P.polymyxa* showed a significant level of shoot development followed by treatment of the combination of P.polymyxa and AD. Much slower rate of shoot development was seen in case of AD.

Figure 1(f) Morphological shoot increament after 15 days

3.4 Cytological Studies

Cytological observations clearly show that the control was normal with 2n=14 with regular arrangements of chromosome at metaphase and regular separation at anaphase.

Different types of abnormalities were found in mitotically dividing cells of Lens culinaris root tips treated with adhesive, bacteria and a combination of both. The spectra of various metabolic abnormalities and mitotic indices have been summarized as follows :

Table 4. Active Mitotic Index (AMI) of seed treatments

Serial No.	Treatments	Active Mitotic Index (AMI)
1.	Seed Control	13.20%
2.	Seed + AD	10.9%
3.	Seed + *P.polymyxa*	12.5%
4.	Seed + AD + *P.polymyxa*	11.7%

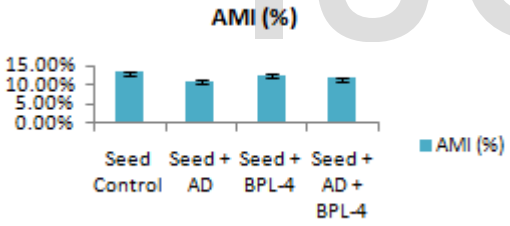

Figure 1.F. Chart showing AMI % in different seed treatments

On the basis of observations, following trends were observed in the chromosomes of Lens culinaris:

1. Seed Control clearly showed normal prophase, normal metaphase and normal anaphase.
2. Seed + *P.polymyxa* or seed with treatment of bacteria showed scattering of chromosomes, precocious movement at metaphase and unorientation of chromosomes.
3. Seed + AD or seed with treatment of adhesive showed stickiness at metaphase as well as anaphase and unorientation of chromosomes.
4. Seed + AD + *P.polymyxa* or seed with treatment of adhesive as well as bacteria showed laggered movement , forward movement of chromosomes and bridge formation at anaphase.

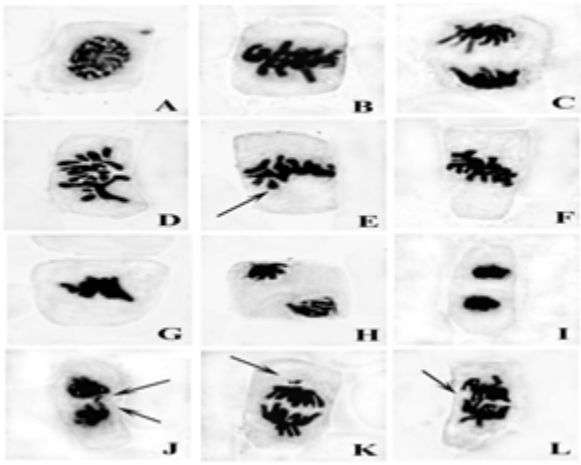

Figure (g): Prophase; H: Normal Metaphase (2n=14); I: Normal Anaphase (14:14 Separation); J: Scattering; K: Precocious movement at Metaphase; L: Unorientation at Metaphase; M:Stickiness at Metaphase; N: Unorientation at Anaphase; O: Stickiness at Anaphase; P: Laggard formation at Anaphase; Q: Forward movement at Anaphase; R: Single bridge formation at Anaphase.

Seed control is showing normal cell stages of prophase, metaphase and anaphase. Seed with treatment of AD shows stickiness as its prominent abnormality and it occurs due to defective functioning of proteins involved in chromosome organization, which are needed for chromosomal separation and segregation. Seeds with treatment of *P.polymyxa* show abnormalities as unorientation due to unequal separation of chromosomes, scattering due to loss of microtubules of spindle fibres and precocious movement due to the early terminalization, stickiness of chromosomes or due to rest at anaphase.

Seeds with treatment of mixture of AD along with *P.polymyxa* show abnormalities as laggered and forward movement of chromosomes as well as bridge formation due to the stickiness of chromosomes at metaphase and their failure to separate freely at anaphase or due to breakage and reunion of chromosomes.

4 Conclusion

In present, *P.polymyxa* individual as well as with AD i.e. Silver Nanoparticle combination proved to be most significant for the plant growth as compared to the other treatments and hence, an approach can be developed towards this combination of treatment as biofertilizer after evaluating the ecological and nutritional study of treated lens. The cytological studies might effect the fertilization or can result reduced number of viable seeds. Cytological abnormalities in chromosomes were observed significantly on the treated seeds. Treatment of Bacteria and Silver Nanoparticles resulted into increased growth and abnormalities in chromosomes which initiates proper growth and chromosome function in the plant. Lentil is an important legume crop and plays an important role in human nutrition, animal feeding and soil

fertility improvement. The crop can be grown in various agro-ecological zones and is useful for rotations with cereals. The promotion of multi-crop threshers would also help to reduce post-harvest loss significantly, as well as increase lentil grain quality and reduce post harvest loss. The use of plant growth promoting Rhizobacteria (PGPR) is a better alternative to solve this problem. They play an important role to increase in soil fertility, plant growth promotion, and suppression of phytopathogens for development of ecofriendly sustainable agriculture.

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