BIOFORTFICATION OF SPINACH (*Spinacia olaracea*) WITH PLANT GROWTH PROMOTING RHIZOBACTERIA –*Pseudomonas fluorescens*

Anmol Khare[1] Himani More[2], Sonal Madliya[3], Vrushali D. patil[4]
[1]Student, [2]Student, [3]Student, [4]Student
[1]Barkatullah University
Department of Microbiology, Career College, Bhopal, India

Abstract: Spinach, like many other vegetables, contains low levels of the essential micronutrient iron. Up to two billion people worldwide suffer from iron deficiency, particularly in regions with predominantly vegetable-based diets. Although, an attractive and more sustainable solution is biofortification, which requires enhancing the uptake of iron in spinach using plant growth promoting bacteria – *Pseudomonas fluorescens*. Our studies aimed at increasing iron uptake in spinach using natural rhizospheric bacteria which actively colonize the spinach plant. Siderophore formation by *Pseudomonas fluorescens* will chelate iron and make it accessible to spinach. Siderophore delivered was additionally turned out to be valuable for plant development advancement because of increment in root length, shoot length and number of leaves. Thus siderophore can be utilized as a part of blend with other biofertilizers to build edit profitability.

Index Terms **- Micronutrient, deficiency, biofortification, siderophore**

I. INTRODUCTION

All living organisms require essential mineral micronutrients to maintain metabolism and humans obtain these from their diet [1]. However vegetables such as spinach often contain suboptimal quantities of micronutrients, especially iron (Fe), but the quantity of iron present in the spinach is very less. In regions where the human diet consists mainly of vegetables this leads to deficiencies in micronutrients. The World Health Organization estimates that approximately 25% of the world's population suffers from anemia [2], and that Fe-deficiency anemia led to the loss of over 46,000 disability adjusted life years (DALYs) in 2010 alone [3].

There are many possible strategies to improve micronutrient intake in the human diet including dietary diversification, mineral supplementation and post-harvest food fortification. However, these strategies depend on continued investment and infrastructure, and current levels of post-harvest fortification of Fe are often inadequate [4], [5], & [6]. Biofortification goes around these issues by enhancing the micronutrient substance of the yields themselves by expanding mineral levels and bioavailability in the palatable parts. Improving crop varieties by enhancing its mineral uptake especially Iron (Fe) uptake using the rhizospheric bacterial colonization [7]. Its advantage is that once the initial research and development is completed, the benefits from these nutritionally-enhanced crops will be sustainable with little further investment [8].

Plant growth promoting rhizobacteria (PGPR) can be used for improving the Iron uptake of spinach by siderophore production. Under these circumstances, plant growth promoting Rhizobacteria (PGPR) may offer a valuable alternative to enhance the mineral and nutrient uptake of spinach. This is because PGPR live freely in soil, colonize plant roots aggressively and establish symbiotic association with plants. Utilization of microbial inoculants or plant growth promoting Rhizobacteria (PGPR) for the enhancement of sustainable agricultural production is becoming a more widely accepted practice in intensive agriculture in many parts of the world.

II. DIRECT MECHANISM OF PGPR

2.1. Siderophore production-

Iron is a crucial supplement for all types of life. All microorganisms known up to this point, except for specific lactobacilli, basically require press [17]. In the oxygen consuming condition, press happens basically as Fe^{3+} and is probably going to frame insoluble hydroxides and oxyhydroxides, in this manner making it by and large out of reach to the two plants and microorganisms [18]. Ordinarily, microscopic organisms gain iron by the discharge of low-atomic mass iron chelators alluded to as siderophores which have high affiliation constants for complexing iron. Most of the siderophores are water-soluble and can be divided into extracellular siderophores and intracellular siderophores. For the most part, rhizobacteria varies with respect to the siderophore cross-using capacity; some are capable in utilizing siderophores of similar sort (homologous siderophores) while at the same time others could use those created by other rhizobacteria of various genera (heterologous siderophores). In both Gram-negative and Gram-positive rhizobacteria, iron (Fe^{3+}) in Fe^{3+}-siderophore complex on bacterial membrane is reduced to Fe^{2+} which is further released into the cell from the siderophore by means of a gating instrument connecting the internal and external films. During this reduction process, the siderophore may be destroyed/recycled (Rajkumar et al., 2010; Neilands, 1995). Hence, siderophores go about as Solubilizing specialists for press from minerals or natural mixes under states of iron impediment. Plants absorb press from bacterial siderophores by methods for various instruments, for example, chelate and arrival of iron, the immediate take-up of

siderophore-Fe buildings, or by a ligand trade response. Various investigations of the plant development advancement versus siderophore-interceded Fe-take-up because of siderophore delivering rhizobacterial vaccinations have been accounted for (Rajkumar et al., 2010). For instance, Crowley and Kraemer uncovered a siderophore intervened press transport framework in oat plants and surmised that siderophores created by rhizosphere microorganisms convey iron to oat, which has systems for utilizing Fe-siderophore edifices under iron-constrained conditions. Also, the Fe-pyoverdine complex integrated by Pseudomonas fluorescens C7 was taken up by Arabidopsis thaliana plants, prompting an expansion of iron inside plant tissues and to enhanced plant development (Vansuyt et al., 2007). As of late, Sharma et al. (2003) evaluated the part of the siderophore-creating Pseudomonas strain GRP3 on press sustenance of Vigna emanate. Following 45 days, the plants demonstrated a decrease in chlorotic side effects and iron, chlorophyll and chlorophyll b content expanded in strain GRP3 vaccinated plants contrasted with control.

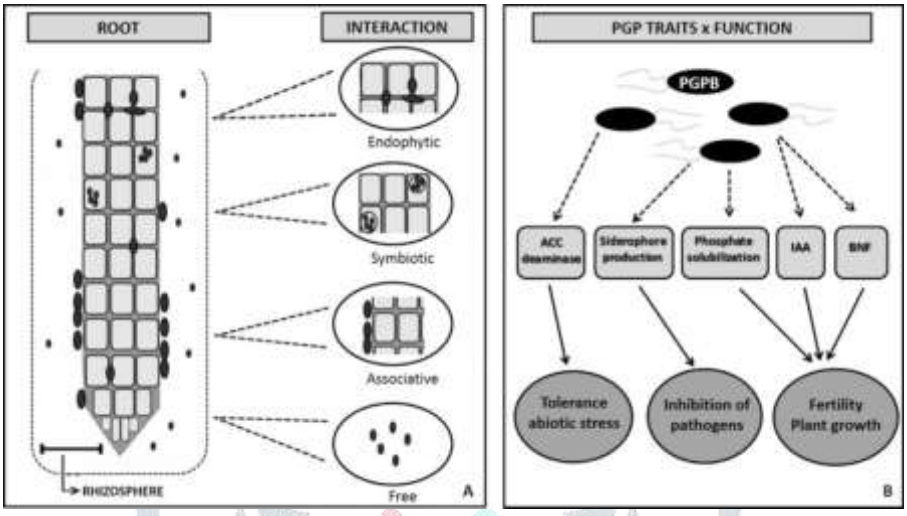

Figure 1. Direct and indirect mechanisms of Plant growth promoting rhizobacteria

III. MATERIAL AND METHOD

3.1. Isolation and screening of PGPR-

Sample collection-

The soil samples were collected from spinach fields of Bairagarh. During collection the upper 7 cm. soil were discarded and the lower soil layer beneath this were collected aseptically in neat and clean polythene packet and then brought to the laboratory and was stored in aseptic condition for further use.

3.2. Isolation of siderophore producing bacteria (*pseudomonas fluorescens*)-

These roots with its surface soil was cut from the plant and moved into tapered carafe containing 100ml of sterile refined water and brooded on shaker at RT for 6-7 hrs. 1ml of this suspension was serially diluted up to10^{-6} and about 0.1ml from last three dilutions was surface spread on King's B medium to isolate the colonies. The plates were incubated at37^{o}c for 48 hr. The colonies showing yellow pigmentation on King's B medium were picked up based on the pigment formation. This was followed by biochemical and morphological characterization such as Gram staining and motility along with Sugars fermentation, Gelatin hydrolysis, Citrate, Oxidase, Nitrate test. Organism was identified on the basis of Bergey's manuals.

3.3. Plant growth and inoculation in plants-

Seed germination test-

The efficiency of germination of the collected spinach seeds were performed by using isolated PGPR-*Pseudomonas fluorescens*. At first the spinach seeds were surface sterilized with 0.01% $Hgcl_2$ for 2 minutes followed by successive washing with sterile distilled water. Seeds were kept into respective bacterial culture medium containing 10^6 cells/ml. for 10 minutes. After that the seeds were transferred and placed on sterile soil containing pots and incubated for 2-3 days. After 3 days seed germination was recorded in comparison with control. The nature of seed germination was also checked by planting the imbibed seeds into pots.

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Exploitation of PGPR on growth of spinach seedlings-

Spinach seeds were sown in (the soil of the pot was sterilized for successive 3 days at 15 lbs pressure for 40 minutes.) pot culture after proper imbibitions in the bacterial suspension for 24 hours and the set was kept in laboratory conditions. The experiment was designed with two pots in set and soil from botanical garden added with manure and sand in proportion 2:1:1 ratio. Set 1 served as control without treatment of bacterium and in Set. 2 (set for PGPR-Pseudomonas fluorescens) sterile soil seeded with spinach seeds. The length of root and shoot of the test plant samples were measured.

IV. RESULTS

4.1. Isolation and characterization of obtained isolate-

Siderophore producing bacterial colonies were isolated from the surface of spinach root sample and it was observed that, these colonies as compare to other colonies produced diffusible yellow green florescent pigment around them on Kings B (KB) Agar (specific for pyoverdine).Pure cultures of the isolate was prepared and maintained. These were screened for siderophore production in sterile iron free Succinate medium. The result showed that, all the three isolate were able to produce diffusible yellow green siderophore but out of these three, one isolate were visually producing intense yellow green diffusible siderophore of pyoverdine type. Presence of non-pigmented colonies on Kings A (KA) agar (specific for Pyocyanin) further confirmed siderophore of pyoverdine type .Thus, this isolate obtained was selected for further study. On the basis of morphological, biochemical and structural characterization, isolate was found to be gram negative short rods which were positive for Gelatin hydrolysis, Oxidase, Citrate, Nitrate along with the growth at 4^0c. These are the trademark highlights of Pseudomonas fluorescens as per Bergey's manuals ninth version. Table 1. Demonstrates the biochemical portrayal of *Pseudomonas fluorescens*. In this way, as indicated by comes about acquired and by contrasting the got qualities and gauges for Pseudomonades in Bergey's manuals, gotten confine was distinguished as Pseudomonas fluorescens

4.2. Confirmation of siderophore produced-

Iron free Succinate medium was used for further confirmation of siderophore. Siderophore produced by *Pseudomonas fluorescens* showed florescence under UV light this was further confirmed by maximum absorbance at 410 nm. The production of reddish brown colored hasten on expansion of iron further affirms the yellow green diffusible shade as siderophore. This is because of iron acquisition by siderophore molecule (Bholay A. D *et al*, 2012).

4.3. Yield and yield parameters-

Bacterial inoculation with *Pseudomonas fluorescens* resulted in an overall increase in the morphological, physiological and biochemical parameters in spinach plant due to increase in iron uptake efficiency of the plant. The performance of the plants was better in inoculated treatments in comparison to the control.

Seed germination-

To see the effect of PGPR isolate on seed germination, spinach seeds were treated with the isolate. The PGPR isolate remarkably affected the germination of spinach seeds. It is also noted that PGPR isolate increased seed germination by 30% over control.

Root length-

The PGPR isolate significantly increased the root length of spinach. Root length ranged from 1.2 to 2.4 cm. In comparison to uninoculated controls whose root length is 1.1 cm.

Shoot length-

The PGPR isolates significantly increased the shoot length of wheat. Shoot length ranged from 2.2 to 3.4 cm. In comparison to uninoculated control (2 cm) the isolate increase the shoot length of spinach. In this study PGPR from agricultural field were isolated and enumerated their role for improvement of the growth and yield of spinach in the laboratory conditions Morphological, Biochemical, PGPR characters were determined following different techniques. Colony morphology and biochemical tests of the bacterial isolates were tested by following the standard methods of "Microbiology- a Laboratory Manual" by Cappuccino Sherman (7th edition). Effect of isolated PGPR on spinach seed germination in normal environment in garden was estimated. The outcome of the experiment denoted that the isolated PGPR strain had outstanding effects on the germination of spinach seeds. At first the surface sterilized seeds were dipped into isolated PGPR emulsions for 24 hours and then allowed those seeds to germinate in the pots kept the research garden and after certain time the seed germination percentage was calculated as compared to uninoculated set. The calculation of seed germination percentage, PGPR's effects on root length and shoot length, are presented in Figure 2, Figure 3, Figure 4, respectively

Table1. Standard biochemical characterization of
Pseudomonas fluorescens

Characteristics	*Pseudomonas fluorescens*
1. Oxidase 2. Growth at 41^0 C 3. Pyoverdin (fluorescein) 4. Pyocyanin 5. Gelatinase	Positive Negative Positive Negative Positive

Table2. Identification of isolated siderophore producing bacteria

Characteristics	Bacterial Morphology
1. Colony size 2. Surface 3. Margin 4. Elevation 5. Optical features –pigment production 6. Microscopic examination 7. Gram staining 8. Motility 9. Endospore formation 10. Morphology	Pinpoint Shiny Smooth Raised Yellowish green Rod Negative Polar flagella Negative Rod shaped

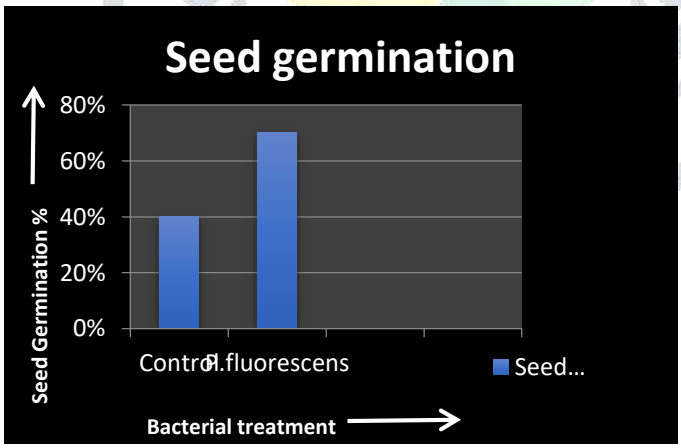

Figure 2. Effect of isolated PGPR on seed germination of spinach

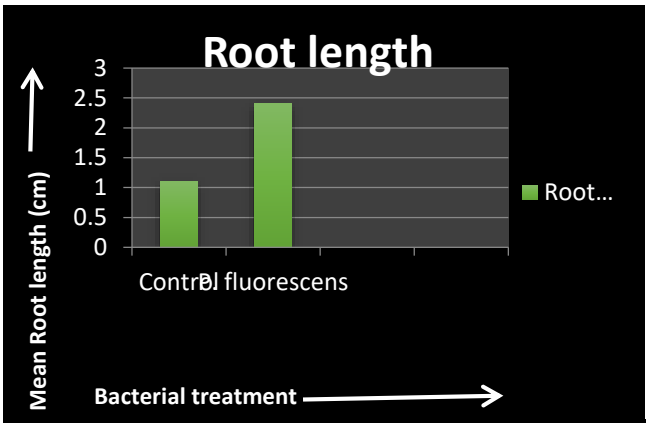

Figure 3. Effect of isolated PGPR on root length (cm.) of spinach seedlings

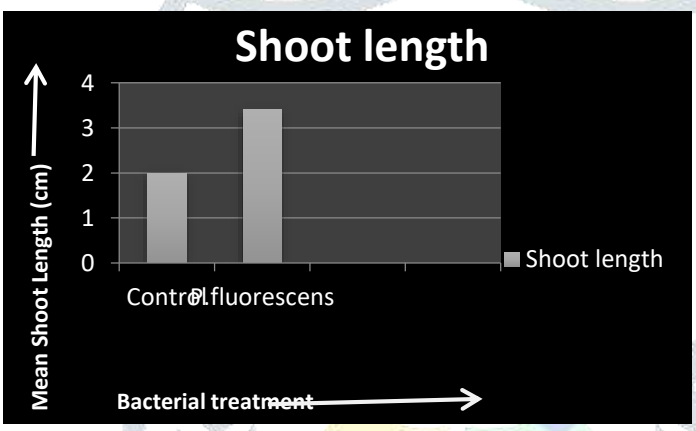

Figure 4. Effect of isolated PGPR on shoot length (cm.) of spinach seedlings

(A) **(B)** **(C)**

Figure 5. (A)Fluorescens of siderophore, (B) & (C) Effect on germination and growth of spinach in trays

(A) Control **(B) P.fluorescens**

Figure 6. Effect on germination and growth of spinach in pot trials

V. DISCUSSION

On the basis of screening for intense siderophore producers from spinach roots surface, *Pseudomonas fluorescens* was identified as potential siderophore producers in iron free Succinate medium. Results obtained after confirmation of siderophore produced shows that siderophore produced is of pyoverdine type. The result of this experiment denoted that the isolated PGPR strains showed profound positive effect on enhancing seed germination of spinach. The PGPR isolates exhibited their individuality based on various characterizations such as, morphological, staining and biochemical properties that is depicted in table 1.

In a subsequent study the isolated PGPR strains were applied as inoculants and they showed remarkably improved growth of spinach seedling in respect to root length (cm.) and shoot length (cm.) over the uninoculated plant (i.e. without PGPR treatment).

The PGPR is one of the most effective soil microorganisms that can enhance plant performance. Therefore, in this experiment also the isolated PGPR strains are found to enhance the seed germination and growth. The result further indicated that the isolated PGPR having plant growth promoting activity can be used as alternative source of biofertilizers in future. Although further study needs for to reach any firm conclusion.

VI. CONCLUSION

From the above study it can be clearly indicated that if the isolated PGPR strains were applied either alone or in combinations they can enhance plant germination, growth and development as well. Without any application of any chemical fertilizers the spinach plant growth can be increased by the using of these PGPR strains. So, there is a scope of using these PGPR strains as good biofertilizers in future.

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