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Impact of *Pseudomonas Plecoglossicida* AVP1, Plant Growth Promoting Rhizobacteria on Growth and Physiological Attributes in Chilli

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Plant growth-promoting rhizobacteria (PGPR) are known for their significant role in agriculture systems. But little is known about their effect on high-energy reactions of photosynthesis and transpiration. *Pseudomonas plecoglossicida* AVP1, isolated from chilli (*Capsicum annum L*), was used to evaluate its efficacy on chilli. *P. plecoglossicida* AVP1 strain showed significantly high levels of phosphate solubilisation (845ppm/ml), IAA (66±0.49 µg/ml) and Ammonia production (55 ± 0.67µg/ml) and maximum acid phosphatase activity (0.176 IU/ml) at pH 3.0 and pH 3.4 after the optimization of growth conditions. The strain AVPI also increased the fresh weight (109.82%), root length (16%), and shoot length (56.44%) at 4 and 8 weeks of growth compared to untreated control. Physiological attributes were analysed using the ADC Bio-scientific Le pro-system (33292). The 8-weeks-old untreated seedlings showed 0.55 µmol/m^2/s of Transpiration (**E**), 230.5 ppm of Internal carbon(**Ci**), 0.027 µmol/m^2/s of stomatal conductance(**gs**) and 2.01 µmol /m^2/s and seedlings treated with AVP1 strain increased the **gs** and **A** by 100% and 93.73%. In contrast, **E** and **Ci** production was not significant among treatments. Our results suggest that *P. plecoglossicida* AVP1 stimulates the stomatal conductance (**gs**), a regulator of gas exchange of CO2 and water, and allows the plant to increase CO2 uptake and assimilation, thereby subsequently enhanced the photosynthesis (**A**). We hypothesized that these attributes increased the plant growth of chilli.

Keywords: *Pseudomonas plecoglossicida;* phosphate solubilisation; stomatal conductance; photosynthetic rate; *Capsicum annum L*

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1. Introduction

High-energy metabolisms (carbon and nitrogen metabolism) influence crop production and crop improvement (Richard, 2000). Environmental stresses like biotic stress, water stress, and high irradiance cause severe photo-oxidative damage to the photosynthetic apparatus and dysfunction of the stomata regulatory mechanism.

Under stress environments, plants have developed many protective mechanisms such as triggering oxidative stress enzymes against reactive oxygen species (ROS) by lowering the stomata conductance (**gs**), internal carbon (**Ci**), and photosynthetic rate (**A**). On the contrary, plant growth-promoting rhizobacteria act as bio stimulators and significantly enhanced crop growth by providing nitrogen, solubilization of phosphorous, and producing biologically active compounds such as IAA,

siderophore, etc. (Kensuke 2012), However, the contribution and stimulatory effect of PGPR on plant physiological attributes and their *denovo* mechanism are not understood enough.

As the stomata act as portals for two essential plant processes like photosynthesis and respiration, the CO_2 uptake and water evaporation are regulated by crop plants in response to environmental and biochemical stimuli through stomatal movement. Under well-watered growth conditions, stomatal conductance increases stomatal conductance (gS), regulates gas exchange (CO_2 and H_2O), and increases CO_2 uptake, thereby enhance photosynthesis (Kensuke 2012). The relationship between stomatal conductance Photosynthesis and internal carbon concentration is critical, as several environmental factors control the stomatal movement mechanism.

An increase in plant growth by applying PGPR bacteria is r early (Holguin and Glick, 2001). According to earlier reports, the production of IAA, GA, etc., of PGPR to increase root length growth and root length results in greater root surface area, enabling the plant to access more nutrients from the soil. A wide range of research work done by several researchers revealed that PGPR such as *P. fluorescens* NJ101 (Bano and Musarrat, 2004), *P. aeruginosa* (Jha *et al.,* 2009), and *Bacillus* sp. (Ahmad *et al.,* 2008) as good phosphate solubilizers and enhance the production of IAA as well as ammonia production, thereby improving the growth of chickpea plant. The most efficient inoculation like *Pseudomonas putida and Pseudomonas aer*uginosa, for enhancement of shoot and root length and the dry matter, followed by *Bacillus subtilis, Paenibacillus polymyxa,* and *Bacillus bosonophillus* over control in several crop plants. Thus, a study aimed to unravel the Phyto-stimulatory effect of phosphate solubilizing PGPR on physiological attributes influencing plant growth and production.

2. Materials and Methods

Pseudomonas plecoglossicida AVP1 **(KF527824.),** a phosphate-solubilizing rhizobacterium (PSRB) isolated from chilli (*Capsicum annum L*), was used as a test organism. Seeds of *Capsicum annum L var CSH5* collected from Agriculture station, LAM, Guntur district, Andhra Pradesh, India.

2.1 Plant Growth Promoting Traits:

As per standard protocols, Phosphate (PO^{4-}) solubilisation, IAA, and ammonia production of *Pseudomonas plecoglossicida* AVP1 screened qualitatively (Gaur, 1990; Brick *et al.,* 1991; Cappuccino and Sharma, 1992).

2.2 Quantitative analysis of plant growth-promoting (PGP) traits

The method referred by Nautiyal and Jackson, 2001 is used to estimate inorganic phosphate solubilisation. The National Botanical Research Institute's Phosphate (NBRIP) broth containing 0.5% Tricalcium phosphate (TCP) was used for the growth of bacterial isolate. 500µl of bacterial broth was added to 50 ml of medium and incubated at 30±0.1 °C at 180 rpm for five days in Incubator Shaker and the uninoculated medium as control. The culture is centrifuged at 10,000 rpm for 10 min. The Vanado-molybdate yellow colour method was used to estimate inorganic phosphate present in supernatant using Barton's reagent. 0.5 ml of the supernatant was added to 2.5 ml Barton's reagent, and volume was made to 50 ml with de-ionized water. After 10 min of incubation, the absorbance was read at 430 nm in UV-Visible Spectrophotometer and calculated the total soluble phosphorous (ppm/ml) from the regression equation of the standard curve.

Acid phosphatase activity was estimated quantitatively (Gugi *et al.,* 1991). Cells were grown overnight in Citrate salt medium (pH7.0) and centrifuged at 8,000 rpm for 8min. 100µl of cell suspension was added to the enzyme assay mixture containing 100 µl of 500mM citrate buffer and 50 µl of 0.12mM PNPP at six different pH ranging from pH3.0, pH3.4, pH3.8, pH4.0, pH4.4, and pH4.8 separately and incubated at 30^0 C for 30 minutes and centrifuged the mixture at 8,000 rpm for 8min. After centrifugation equal volume of 0.5M NaOH was added to the supernatant and measured the OD at 420nm. Enzyme activity expressed as µmol of Para-nitro phenol formed.

Indole acetic acid production determined using the method of Pattern and Glick (2002). Bacteria inoculum was added to nutrient broth supplemented with L-tryptophan (1µg/ml) for 72hrs and centrifuged at 10,000g for 10minutes. 1ml 0f culture filtrate was allowed to react with 2ml of Salkowski reagent (1ml of 0.5M Fecl3 in 50ml of 35% perchloric acid) at 28 ± 2^0 C for 30minutes. The development of pink colour in the reaction mixture confirmed IAA and was estimated at 530nm using a UV-Visible spectrophotometer. A standard graph plotted with different concentrations of IAA ranging from 10µg/ml to 100µg/ml for calibrations.

Ammonia production was estimated using peptone water (Baligh, 1996). The freshly grown culture was inoculated in 10ml peptone water and incubated for 48-72 hours at 36 ± 2^0 C. After incubation, centrifuged the culture at 10,000g for 10 minutes, collected the supernatant, and added 0.5ml of Nessler's reagent. The development of brown to yellow colour was a positive test for ammonia production. A standard graph was plotted with different concentrations of ammonium sulphate from 10µg/ml to 100µg/ml for calibrations.

2.3 Optimization studies

Growth optimization of rhizobacteria isolates was analysed, at different temperatures (25^0 C, 37^0 C and 50^0 C), pH ranging from pH3, pH5, pH7, pH9 and pH11, four different carbon sources (sucrose, maltose, lactose and dextrose) and five different concentrations NaCl (0.3%, 0.5%, 0.7%, 0.9% and 1%) separately. After 48rhs of incubation at room temperature, the O.D values were recorded at 600nm (Bhutto *et al.,* 2010).

2.4 Greenhouse studies of Rhizobacteria

The bacterial cell suspension was prepared by harvesting the cells grown in the optimized medium at 28^0 C$\pm1^0$ C for 48 hours and then centrifugation at 6000rpm for 15 minutes. The inoculum was re-suspended in sterile distilled water, and the concentration adjusted to OD 1.0 (Thompson, 1996). Seeds of chilli were treated with the 48hrs old culture (10^8 CFU/ml) for 30minutes and shade-dried at 28^0 C for 1hr. Sowed treated seeds in pots containing coco peat and grow under a greenhouse. Root length, Shoot length, No. of leaves, and fresh weight of the seedlings was measured. We recorded observations at two weeks intervals using ten seedlings from each replication (Sudhir *et al.,* 2014).

2.5 Gas exchange experiments

8-week old seedlings used for gas exchange experiments to measure Photosynthesis (**A**), Leaf conductance (**gs**), Leaf temperature (**T**), intracellular carbon dioxide (**Ci**), and Transpiration (**E**) using ADC Bio scientific LC pro-SD system Serial No.33292. 8-week old plants grown on soil in pots under GH conditions were used for the analysis in the present study. The middle portion of the 7^{th} (or) 8^{th} leaf was used for the measurements between 10.00 Hr and 12.00 Hr (Itoh *et al.,* 2005).

Calculated the photosynthetic activity and internal carbon dioxide as described (Carmmerer and Farquhar, 1981).

2.6 Statistical Analysis

All the triplicate experiments were performed and expressed the results as the Mean, Analysed Means and Standard Errors (SE) using XLSTAT software package.

3. Results and Discussion

The ability of PGPR to solubilize insoluble phosphorus minerals has attributed to their capacity to reduce pH by the excretion of organic acids such as gluconate, citrate, lactate, and succinate (Gyaneshwar *et al.*, (1999) & Mullen 2005). In various studies, PGPR present in different soils solubilize inorganic phosphate and making it available to the plants (Sharma *et al.*, 2012). The phosphate-solubilizing efficiency of *P. plecoglossicida* AVP1 in Pikovskaya's broth indicated that the strain efficiently solubilized inorganic phosphate in the medium containing 0.5% tri-calcium phosphate (Table 1). *P. plecoglossicida* AVP1 was produced 845 (ppm/ml) of soluble phosphate, 66 (µg/ml) of IAA, and 55µg/mlof Ammonia under optimized condition (pH7, 37^0C and 0.7% NaCl and lactose as carbon source as shown in **Fig1.**

Table1: Quantitative Analysis of Plant growth promoting traits of *P. plecoglossicida* AVP1

Rhizobacteria	Po_4^- (ppm/ml)	IAA (µg/ml)	Ammonia (µg/ml)
AVP1	845	66	55

pH declined to 3.4 and 3.0 from the initial pH of 4.8 and correlation was observed between maximum drop of pH value and elevated levels of acid phosphatase activity of AVP1 (Fig2A). Acid phosphatase activity was reported maximum with Re Xylose and Galactose followed by mannose (Fig2B) and Cysteine and methionine followed by Glycine (Fig2C). Our findings are in support with earlier studies. Research done by Park *et al.*, (2009) also reported the maximum drop of pH recorded at pH 4.0 by *P. fluorescens* RAF15**.** Researchers reported producing organic acids (gluconic acid, 2-ketogluconic acid, lactic acid, acetic acid, oxalic acid, citric acid, etc.) from insoluble phosphates (Rodriguez and Fraga 1999). In a study done by Vasslev *et al.*, (2006) and Ma *et al.*, (2009) indicated that the acidification of culture supernatants resulted in the production of organic acid which seemed to be the primary mechanism for phosphate stabilization.

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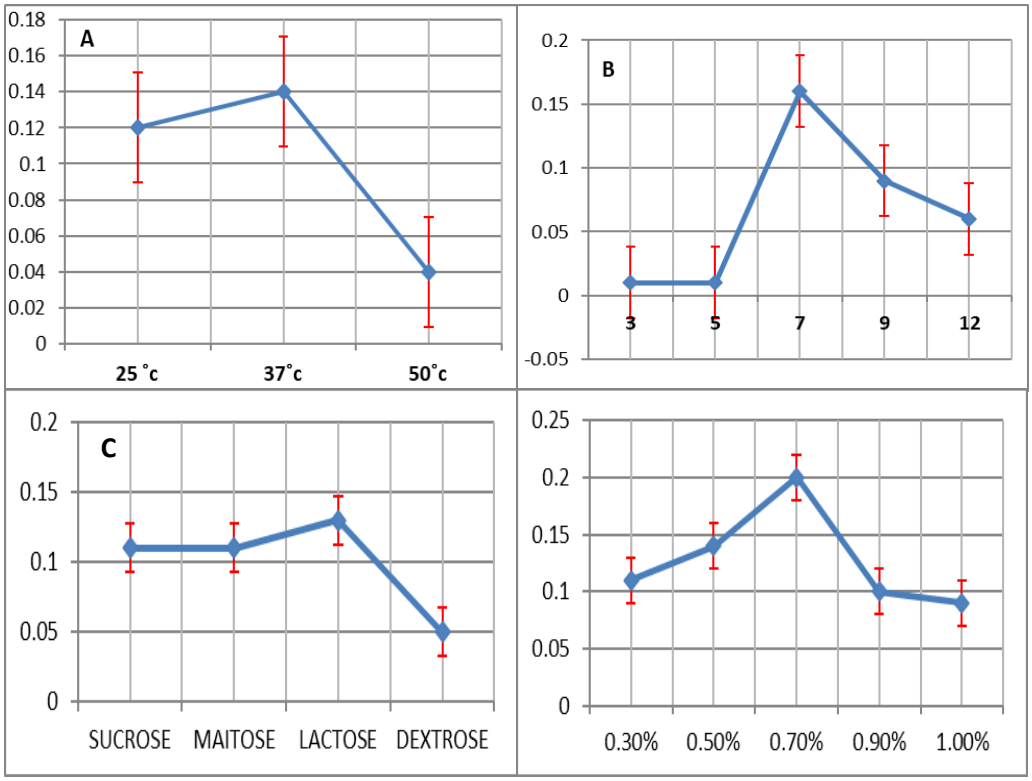
Fig.1: Growth optimization of *P. plecoglossicida* AVP1
A) Temperature B) pH C) 1% of Carbon source and D) % of Salinity.



From the analysis of **Fig 3**, it is clear that the root length and shoot height of chilli plants were highly affected by AVP1 bio-stimulation (Fig 4) and notices that AVP1 gave the highest shoot and root ratio 3:1 in 4th week and 5:1 in 8 weeks in treated chilli plants, while in control root growth was noticed high after four weeks balance has maintained 2:1. Our results evidenced that phosphate solubilizers trigger the greater surface area of the shoot,

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Fig.2 Optimization of Acid phosphatase activity (IU/ml) of *P. plecoglossicida* AVP1 A) Ph. B) carbon source and C) Amino acids

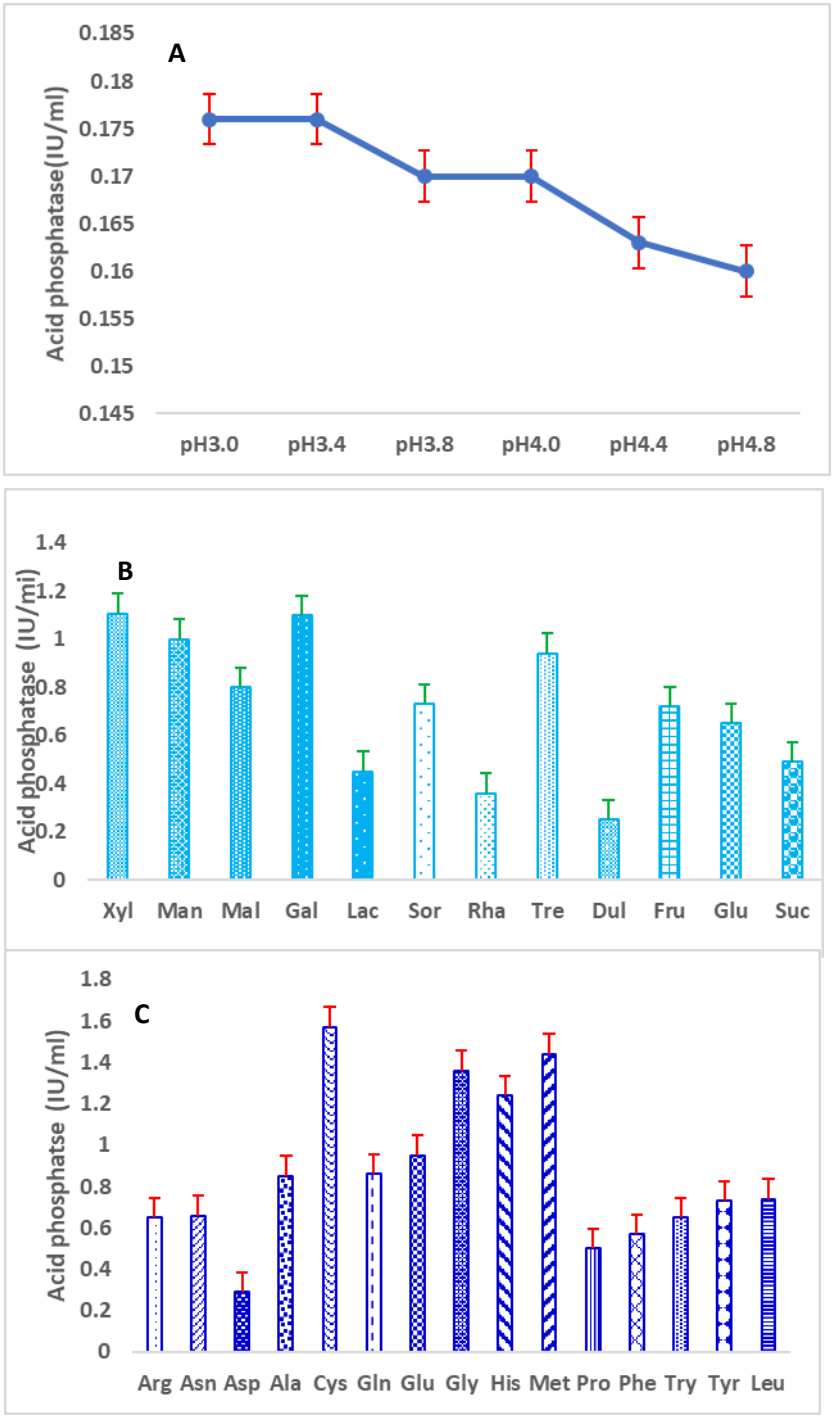

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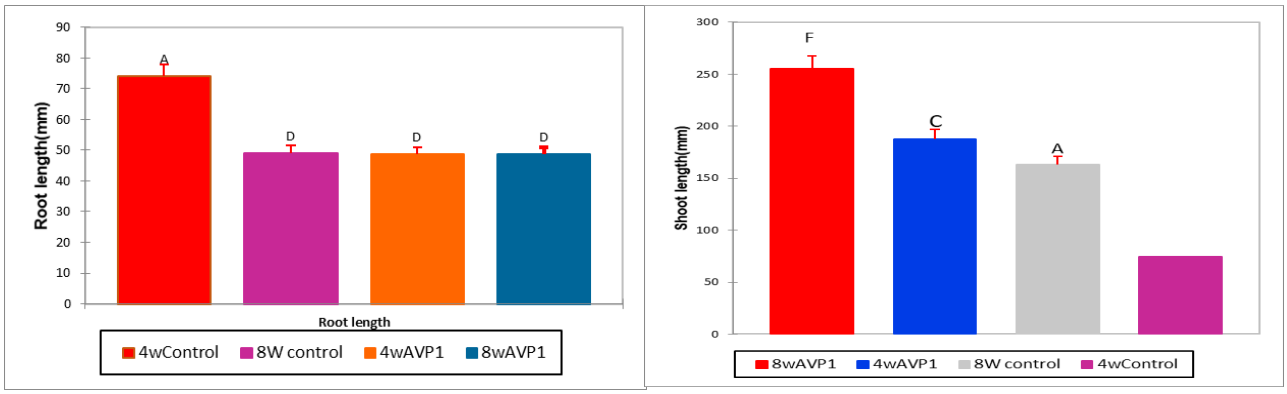
Fig3: Effect of *P. plecoglossicida* AVP1 inoculation on growth attributes of 4- and 8-weeks old Chilli plants.

(A) Root length and (B) Shoot height. Values are mean of ±S.E.M (n=15), *P<0.05vs.Control.

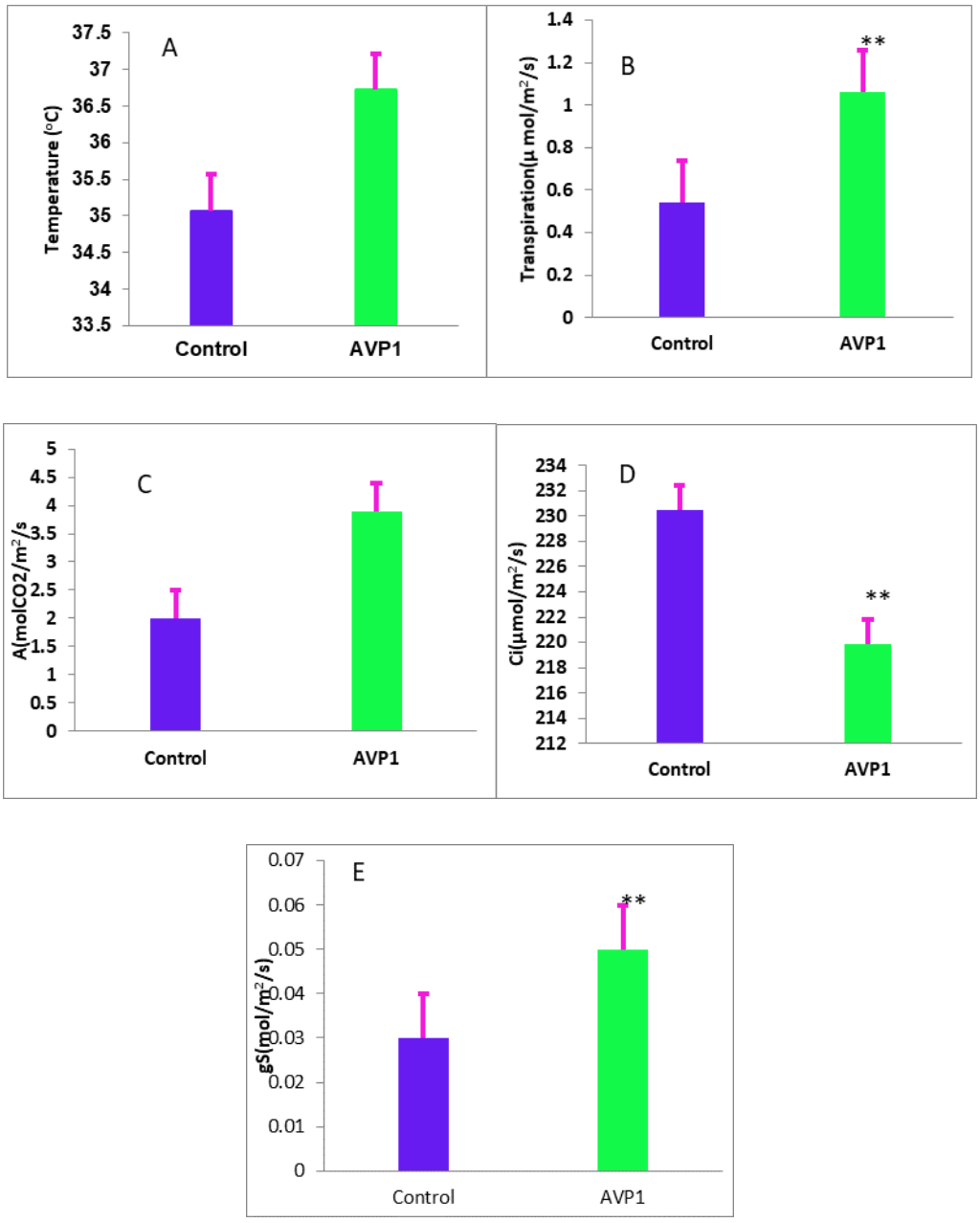


, which enables the plant to promote productivity. Holguin and Glick, (2001) reported that the production of Phytohormones implicated in the PGPR enhances root growth. An increase in the root growth results in greater root surface area, enabling the plant to access more nutrients from the soil. Several researchers revealed that *P. fluorescens* NJ101 (Bano and Musarrat, 2004), *P. aeruginosa* (Jha *et al.*, 2009), and Bacillus sp. (Ahmad *et al.*, 2008), as good phosphate solubilizers enhanced the production of IAA and ammonia production in chickpea and improved the growth of chickpea plant. The study reported that the most efficient inoculations for the enhancement of shoot and root length and dry matter over control in several crop plants were *P. putida and P.* aer*uginosa* followed by *B. subtilis*, *P. polymyxa*, and *B. bosonophillus.*

Data analysed in Fig 3 showed that the root length and shoot height of chilli plants were highly affected by AVP1 bio-stimulation (Fig3). Also noticed thatAVP1 gave the highest shoot and root ratio of 3:1 in 4th week and 5:1 in 8 weeks in treated chilli plants, while in control root growth was noticed high after four weeks and shoot root ratio maintained 2:1. Our results evidenced that phosphate solubilizers trigger greater l surface area of the shoot, which enables the plant to promote productivity. Application of PGPR increases plant growth revealed that the production of growth hormones implicated in the PGPR is believed to increase root growth. Root length enables the plant to access more nutrients from the soil due to greater root surface area. Several researchers revealed that species of *Pseudomonas* and *Bacillus* (Bano and Musarrat, 2004, Jha *et al.*, 2009, Ahmad *et al.*, 2008) were reported as good phosphate solubilizers and can enhance the production of IAA and ammonia production, thereby improving the growth of chickpea plant. *P. putida and P. aer*uginosa observed as the most efficient inoculations for enhancement of shoot and root length and the dry matter, followed by *B. subtilis*, *Paenibacillus polymyxa*, and B. bosonophillus over control in several crop plants.

Fig.4: Effect of *Pseudomonas plecoglossicida* AVP1 inoculation on Physiological attributes of 8 weeks old Chilli seedlings.

(A) Temperature of leaf (B) transpiration (c) Internal carbon concentration (D) stomatal conductance and (E) photosynthetic rate. Values are mean of ±S.E.M(n=15),*P<0.05vs.Control



A striking correlation was observed between photosynthetic capacity and stomatal conductance under various stress conditions (Ishihara and Saito, 1987; Hirasawa *et al.*, 1988). However, the relative significance of stomatal conductance in limiting the supply of stomatal CO_2 for metabolism through stomata (stomatal limitation) and in altering metabolism to reduce the potential rate of photosynthesis (non- stomatal limitation) is still unclear. Higher O_2 environments speed up carbon fixation and modulate carbon and nitrogen balance via changes in the levels of structural and non-structural carbohydrates and proteins (Allen *et al.*, 1988).

In the present research, we attempted to analyse the stimulatory impact of AVP1 on physiological attributes to show how PGPR, an external biotic factor works as a biostimulator instead of a stress inducer. We analysed physiological attributes in 8 weeks old chilli plants (Fig5). Control plant showed 0.55µmol/m^2/s of transpiration (E), 230.5ppm of internal carbon dioxide (Ci), 0.027 µmol/m^2/s of stomatal conductance (gs) and 2.01 µmol/m^2/s of photosynthetic rate (**A**). While AVP1 treated plants showed significantly highest values (%increase) of **E** (96.29%), **gS** (100%), and **A** (93.73%) with a significant decrease of Ci by 46.3%. stimulation of AVPI on physiological and growth attributes of chilli plant was graphically represented in the Fig 5.

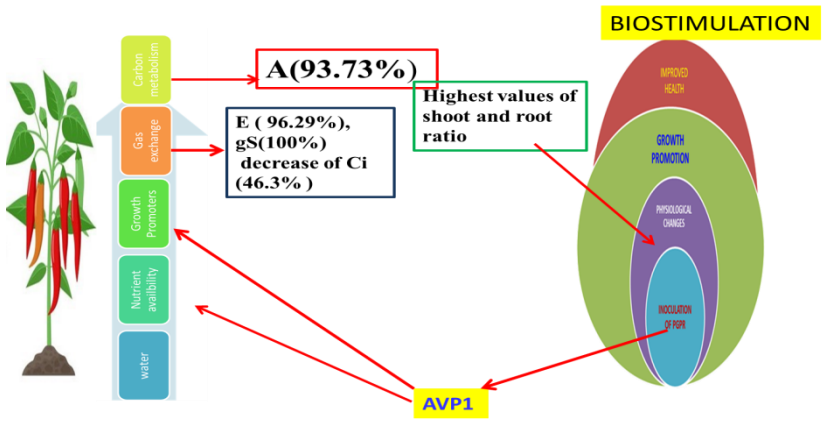


Fig 5. Graphical representation of phytostimulatory effect of *Pseudomonas plecoglossicida* AVP1 inoculation on Physiological and plant growth attributes of Chilli.

Earlier reports revealed stomatal closure in response to alteration in CO2 is the limiting factor for photosynthesis under stress conditions. In rice plants an artificial increase in stomatal conductance via genetic engineering is the primary determinant of photosynthetic capacity (Kensuke Kusumi, 2012). Similarly, in our findings, we observe that application of AVP1 triggered stomatal conductance and biochemical ability of the photosynthetic rate, which helps the plant tolerate drought and a potential increase in biomass. Earlier literature proved that water conditions optimized for photosynthesis to avoid drought stress. Higher stomatal conductance may enhance carbon CO_2 diffusion into chloroplasts. The Photosynthetic activity of plants through CO_2 response curves was assumed in the present study. Estimation of the relative contribution of transpiration versus biochemical limitation was done by analysing response curves between A and Ci (Van caemmeres and Farquhar 1981).

4. Conclusion

Production of Plant growth-promoting traits implicated in the *P. plecoglossicida* AVP1 enhances root growth. An increase in the root growth results in greater root surface area, enabling the plant to access more nutrients from the soil. We conclude that the application of *P. plecoglossicida* AVP1 like phosphate solubilizing PGPR stimulates the stomatal conductance and biochemical ability of the photosynthetic rate and transpiration/Significant reduction of Ci clearly emphasized that PGPR would be a potential bio stimulant and enable the plant to the possible increase in biomass.

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