

## Zinc Solubilizing Fluorescent Pseudomonads as Biofertilizer for Tomato (*Solanum lycopersicum* L.) under Controlled Conditions

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### ABSTRACT

Zinc solubilization by Plant Growth Promoting Rhizobacteria (PGPR) is relatively a newer strategy and can be a potential alternative for zinc-based chemical fertilizers to alleviate zinc deficiencies. The following study characterized the zinc-solubilization potential of fluorescent pseudomonads to observe their role in growth promotion of tomato. Nine strains of *Pseudomonas* spp. were screened to solubilize ZnO, ZnSO<sub>4</sub> and ZnCO<sub>3</sub> on tris-minimal agar plates. *P. chlororaphis* strain RP-4 solubilized ZnCO<sub>3</sub> whereas, *P. aurantiaca* strains GS-4 and Type strain (NCIB 10068T) solubilized ZnCO<sub>3</sub> and ZnO, only. Moreover, cotton isolate *P. aurantiaca* ARS-38, solubilized ZnO, however, rest of the strains could not solubilize ZnCO<sub>3</sub> or ZnO. All bacterial strains were analyzed for their growth enhancement potential, at pot-scale plant experiments and effect was observed on growth parameters. Plant experiments were set-up in RCBD, conducted in triplicate and harvested after four weeks. Maximum fresh weights of shoots and roots were noted for the plants inoculated with *P. aurantiaca* PB-St2 (non Zn-solubilizer), however, maximum dry root weights were recorded for the Zn-solubilizer strain RP-4 (*P. chlororaphis*) inoculated plants. Likewise, substantial increase in root and shoot lengths and area were observed for the Zn-solubilizer strains GS-4, and ARS-38 (*P. aurantiaca*) inoculated plants in comparison of un-inoculated controls. These findings indicate the potential of Zn-biofertilizer strains of *Pseudomonas* spp. in increasing bioavailability of zinc to plants for sustainable agriculture.

**Key words:** Biofertilizers, *Pseudomonas aurantiaca*, *Pseudomonas chlororaphis*, PGPB

### INTRODUCTION

Rhizosphere plant-microbe interactions are the key factors in plant growth, soil fertility and overall productivity. PGPR constitute heterogeneous groups of microorganisms adhered to rhizosphere and on the root surfaces that can significantly stimulate plant growth, impart phytopathogenic protection, and abiotic stresses [1,2]. PGPB utilize diverse mechanisms such as biological nitrogen-fixation, inorganic mineral solubilization and phytohormone production to increase availability of nutrients to plants [3,4]. Among the dominant PGP bacterial genera, fluorescent pseudomonads are well established bioinoculants for successfully colonizing their hosts. Pseudomonads are ubiquitous in agricultural soils and have demonstrated positive effects on seed germination and plant growth. Especially, extensive research is underway globally on the fluorescent pseudomonads (FLPs), which have been significant contributors in soil health and exhibit varied metabolic functions [5]. Positive impacts of other pseudomonads were reported by researchers on crops including chick pea, sugar cane, potato, radish and sugar beet [6]. When used as fertilizers for these crops, fluorescent pseudomonads employed mechanisms like production of phytohormones and insoluble mineral solubilization that influenced root growth and shoot growth, tissue differentiation and responses to the associated abiotic factors for this purpose [7]. Moreover, *Pseudomonas* spp. were reported for promoting zinc and phosphorus bioavailability to plants which are globally widespread micronutrient deficiencies [8].

Apart from above-mentioned growth-promoting effects by diverse mechanisms, various *Pseudomonas* species have been highlighted as significant biocontrol agents because of the production of antagonizing metabolites that include HCN, pyrrolnitrin, pyoluteorin, phenazines, 2,4-diacetylphloroglucinol, siderophores, and pyoverdines [9]. These

compounds were evaluated for the successful suppression of many fungal and bacterial diseases such as tobacco black root rot, root rot of pea and wheat, sugarcane red rot, and sugar beet damping-off [10].

This study evaluated the biofertilizer potential of zinc-solubilizing fluorescent pseudomonads in growth promotion of tomato which is one of the major crops in Pakistan. Nutritional requirements of tomato crops are very high and chemical fertilizers are extensively used to provide nutrients to the growing crop. Chemical fertilizers and pesticides are expensive and cause serious environmental challenge, and also ineffective against certain fungal and bacterial pathogens. Keeping in view the mentioned facts, nine identified strains of *Pseudomonas* spp. were characterized for their zinc-solubilization ability and their effect on tomato growth through pot experiments.

## MATERIALS AND METHODS

### Bacterial isolates

Nine identified *Pseudomonas* strains were used in this study (Table 1). *P. aurantiaca* strains GS-1, GS-3, and GS-4 were isolated from cactus, ARS-38 (*P. aurantiaca*) from cotton, RP-4 (*P. chlororaphis*) from halophyte para grass, whereas, PB-St2 (*P. aurantiaca*), OKSt (*P. putida*), and PBCSt (*P. fluorescens*) were the isolates of sugarcane [10,11]. *Pseudomonas* isolates were revived from glycerol stocks, streaked on King's B agar plates [g/L: protease peptone, 20.0; K<sub>2</sub>HPO<sub>4</sub>, 1.5; MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.5; agar, 20.0, distilled water, 1000 mL] purchased from HiMedia [12], incubated at 28 ± 2 ° C for 48 h and observed for culture purity.

**Table 1.** List of bacterial strains, their molecular identification and zinc solubilization potential

Sl. No.	Bacterial Strains	Identification	Accession No.	Host	Tris-minimal		Reference
					ZnO	ZnCO <sub>3</sub>	
1	RP-4	<i>P. chlororaphis</i>	KT888010	Para grass	-	+	[10]
2	GS-1		KT888006	Cactus	-	-	
3	GS-3		LN898138		-	-	
4	GS-4		KT888007		+	+	
5	ARS-38	<i>P. aurantiaca</i>	KJ094432	Cotton	+	-	
6	Type (NCIB 10068T)		DQ682655	Unknown	+	+	[14]
7	PB-St2	<i>P. putida</i>	EU761590	Sugarcane	-	-	[11]
8	OKSt		CP000712		-	-	
9	PBCSt	<i>P. fluorescens</i>	EU439419		-	-	

### Assessment of zinc solubilization potential of *Pseudomonas* spp.

Tris-miniaml agar [g/L: NaCl, 4.68; KCl, 1.49; NH<sub>4</sub>Cl, 1.07; Na<sub>2</sub>SO<sub>4</sub>, 0.43; D-glucose, 10.0; tris-HCl, 6.06; MgCl<sub>2</sub>·2H<sub>2</sub>O, 0.20; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.30; agar, 20.0] was used for detection of zinc solubilization ability of fluorescent pseudomonads [13]. Tris-minimal agar was prepared from scratch ingredients purchased from Roth, Germany, according to the method described by Sharma et al., [14]. Plates individually supplemented with 0.1% zinc sources including ZnO, ZnCO<sub>3</sub>, and ZnSO<sub>4</sub> were spot-inoculated in triplicates and incubated at 28 ± 2 oC for 14 days in dark. Halo-zones around bacterial colonies were observed for positive test results.

### Colony Forming Unit (CFU) count of bacterial strains

For the calculation of CFU, all bacterial strains were individually inoculated in 10 mL King's B broth and incubated at 28 ± 2 ° C for 24 hours in orbital shaking incubator. Optical Density (OD) of each strain was determined at 600 nm and adjusted at 1.0 with sterile King's B broth. Serial dilutions for each bacterial culture were prepared individually in sterile saline (0.85%) and 100 µL of each dilution were spread on King's B agar plates (Somasegaran and Hoben, 1994). Plates were incubated overnight at 28 ± 2 ° C and CFU were calculated.

### Seed sterilization and germination

Tomato variety Pomodoro Marmande (Pride Seeds) was used for this study. Seeds were bought from local importers and packaging was aseptically opened. Tomato seeds were surface sterilized using 0.01N NaClO solution (Chlorex) for 8-10 minutes, and washed with autoclaved distilled H<sub>2</sub>O (dH<sub>2</sub>O). Sterilized seeds were air-dried in laminar air-flow

cabinet, transferred to water-agar plates (1%) and incubated at  $28 \pm 2^\circ \text{C}$  for germination.

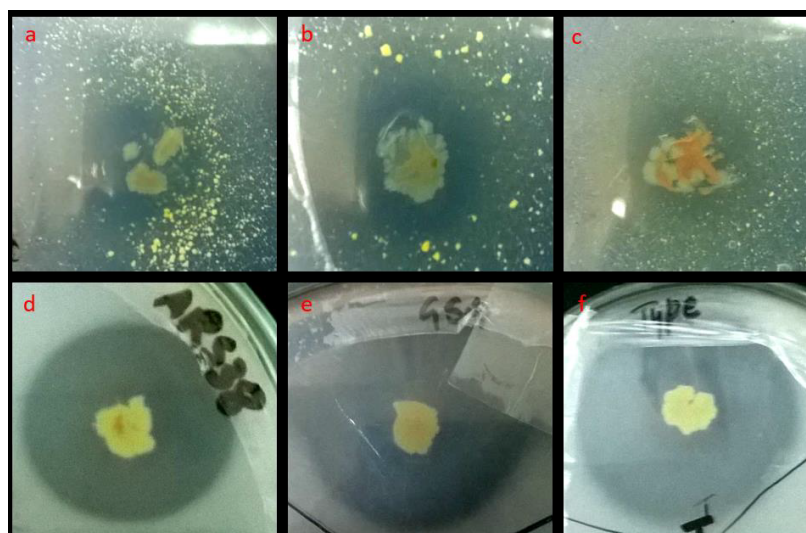
#### **Pot experiments with *Pseudomonas* spp. as bioinoculants of tomato**

Pot experiment was performed in climate-control room to evaluate the effectiveness of *Pseudomonas* spp. as biofertilizers for tomato. Germinated seedlings were transferred to disposable plastic cups, each containing 250 g of autoclaved sand, 1%  $\text{ZnCO}_3$ , and 50 mL of Hoagland's solution (Macronutrients (1M):  $\text{KNO}_3$ , 5 mL;  $\text{Ca}(\text{NO}_3)_2$ , 5 mL;  $\text{K}_2\text{HPO}_4$ , 5 mL;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 5 mL; FeNa EDTA, 6.55 g/L, micronutrients (g/L),  $\text{MnSO}_4$ , 1.81;  $\text{ZnSO}_4$ , 0.22;  $\text{H}_3\text{BO}_3$ , 2.86;  $\text{Na}_2\text{MoO}_4$ , 0.25;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.80; add 2 mL of FeNaEDTA and 1 mL micronutrient solution in 1000 mL  $\text{dH}_2\text{O}$  along with 5 mL of each macronutrient; Hoagland and Arnon, 1950) [15]. All plants were inoculated with 1 mL ( $1 \times 10^7$  cells/mL) of each bacterial culture, individually and six replicates of each treatment were maintained. Plants were kept in a climate control room at relative humidity of 60% with 12 h photoperiod (200  $\mu\text{M}$  m-2s-1 at pot heights with fluorescent lights,  $15^\circ/20^\circ \text{C}$ ). Experiment was set up in completely randomized design and repeated in triplicate. Pots were daily watered with sterile water and plants were harvested after six weeks. Roots and shoots were separated and washed to remove adhered sand, following the measurement of root and shoot lengths of each plant. Shoot lengths and area were measured using ImageJ software [16]. Roots and shoots were dried in hot-air oven for 72 h at  $68 \pm 2^\circ \text{C}$  to record the dry weights. The effectiveness of *Pseudomonas* spp. as bioinoculants was based on the analysis of variance using IBM SPSS Statistics 23.0, and means were compared with application of the Tukey test at  $\alpha 0.05$ .

## RESULTS

#### **Zinc solubilization of *Pseudomonas* spp.**

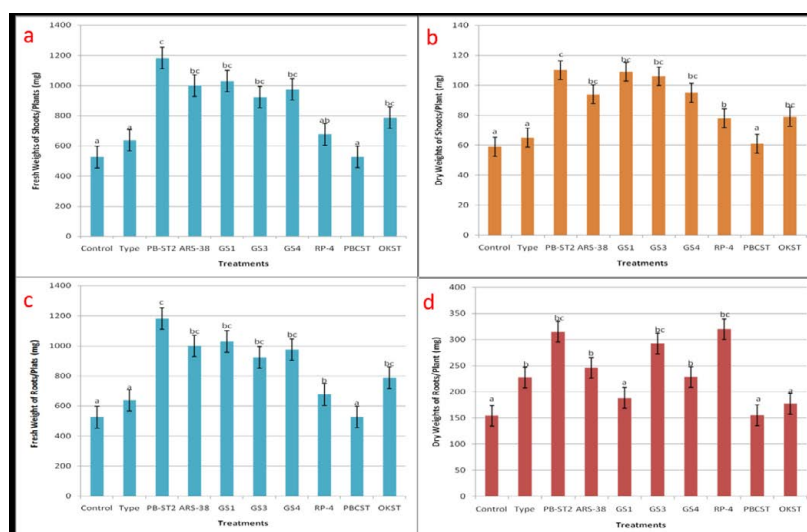
Among all *Pseudomonas* spp., *P. aurantiaca* Type strain (NCIB 10068T) and GS-4 could solubilize both, ZnO and  $\text{ZnCO}_3$ , ARS-38 (*P. aurantiaca*) solubilized ZnO whereas RP-4 (*P. chlororaphis*) was positive for  $\text{ZnCO}_3$  solubilization (Table 1). None of the pseudomonads solubilized  $\text{ZnSO}_4$  in petri-plate assay (Figure 1).



**Figure 1.** Zinc solubilization potential of *P. aurantiaca* (a, f) type-strain, (b, e) GS-4, *P. chlororaphis* (c) RP-4, and *P. aurantiaca* (d) ARS-38 on tris-minimal agar plates

#### ***Pseudomonas* spp. strains enhanced growth of tomato plants**

Plant experiment results manifested considerably increased shoot and root lengths, area, and, biomass. Shoots of the tomato plants inoculated with PB-St2 (*P. aurantiaca*) showed maximum fresh weights (1181 mg) as compared to all inoculated treatments and un-inoculated control. Plants inoculated with *P. aurantiaca* GS-1 also significantly increased shoots fresh weights (1026 mg) as compared to other treatments (Figure 2a). Considerable difference in dry weights of shoots was also observed in comparison to un-inoculated control (Figure 2b). *P. aurantiaca* PB-St2 inoculated plants exhibited the highest dry shoot weight (110 mg) followed by *P. aurantiaca* GS-1 (106 mg).

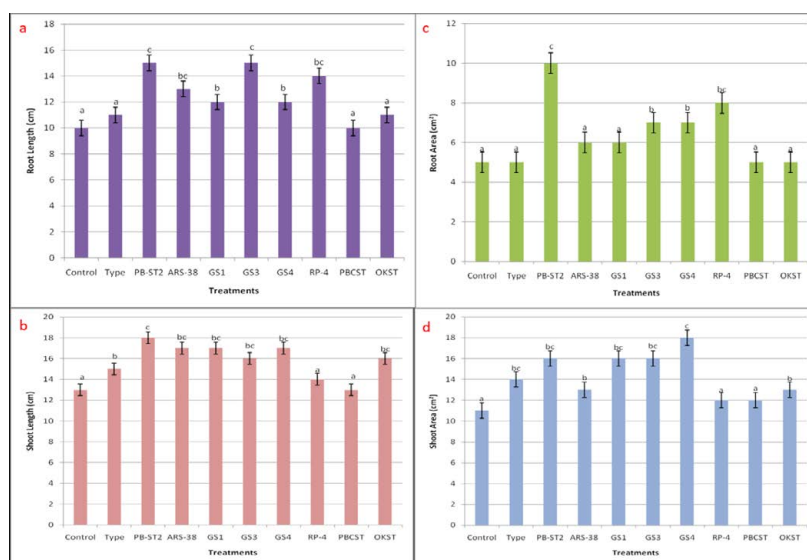


**Figure 2.** Effect of fluorescent pseudomonads on the (a) fresh weight of shoots, (b) fresh weight of roots, (c) dry weight of shoots, and (d) dry weight of roots of tomato plants. Bacterial treatments are indicated on x-axis.

Highest fresh weights of roots were also shown by the plants inoculated with PB-St2 (*P. aurantiaca*) followed by *P. aurantiaca* GS-4, i.e., 836 mg and 678 mg, respectively (Figure 2c). However, maximum dry weight of roots were observed for *P. chlororaphis* RP-4 inoculated plants (320 mg), closely followed by *P. aurantiaca* PB-St2 (310 mg) treated plants (Figure 2d).

*P. aurantiaca* PB-St2 and GS-3, and *P. chlororaphis* RP-4 inoculated plants demonstrated significant increase in root lengths (Figure 3a). PB-St2 inoculated plants showed root lengths of 15 cm, whereas plants inoculated with GS-3 and GS-4, each showed the root length of 13 cm. All *Pseudomonas spp.* strains increased shoot lengths of the tomato plants, however, maximum shoot lengths were observed for *P. aurantiaca* PB-St2 (18 cm) inoculated plants followed by *P. aurantiaca* ARS-38, GS-1, and GS-4, all with average shoot lengths of 17 cm (Figure 3b).

Notable increase in root area was also observed for the tomato plants inoculated with *P. aurantiaca* PB-St2 (10 cm<sup>2</sup>), closely followed by *P. chlororaphis* RP-4 inoculated plants (8 cm<sup>2</sup>) as compared to uninoculated control (Figure 3c). Maximum shoot area was observed for the plants inoculated with *P. aurantiaca* GS-4 (18 cm<sup>2</sup>), closely followed by *P. aurantiaca* PB-ST2, GS1 and GS3, each with the area of 16 cm<sup>2</sup> (Figure 3d).



**Figure 3.** Effect of fluorescent pseudomonads on the (a) root lengths, (b) shoot lengths, (c) root area, and (d) shoot area of tomato plants. Bacterial treatments are indicated on x-axis. **Figure 1.** Zinc solubilization potential of *P. aurantiaca* (a, f) type-strain, (b, e) GS-4, *P. chlororaphis* (c) RP-4, and *P. aurantiaca* (d) ARS-38 on tris-minimal agar plates

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## DISCUSSION

Zinc is among the vital micronutrients necessary for the normal development and growth of plant tissues and humans and its deficiency is associated with significant crop losses globally. Even-though many countries have zinc-deficient soils, zinc fertilizers are under-utilised in developing countries including Pakistan, and are not cost-effective [17]. This deficiency hampers the growth and yield in many economically important crops in the country including wheat, rice, sugarcane, and tomato and application of expensive zinc-fertilizers was shown to enhance the yield [18,19]. However, application of synthetic chemicals to enhance crop yield threatens environment, public health and also challenges farmer's livelihood hence, growers are abandoning chemical fertilizers and returning to organic farming.

This study focused to characterize zinc-solubilizing fluorescent pseudomonads to be applied as zinc-based biofertilizers for tomato. Based on biofertilizer and biocontrol properties of these *Pseudomonas spp.* such as production of indole-3-acetic acid (IAA), hydrolytic enzymes, hydrogen cyanide (HCN), and siderophores, solubilization of insoluble phosphorus and potassium, and suppression of phytopathogens [10], these strains were selected for evaluating their potential for tomato growth promotion. Fluorescent pseudomonads have been widely reported to colonize wheat and sugarcane rhizosphere and stimulating plant growth [20]. For instance, IAA producing *P. fluorescens* has shown considerable increase in onion and wheat yield [21]. Likewise, biocontrol properties of this genus are well documented [22,23]. Some of the studies suggest the potential of zinc solubilizing *Pseudomonas* in chickpea, wheat, [24,25]. However, there are only few reports available for the zinc-solubilizing pseudomonads in growth promotion of tomato. In this study, tomato plants inoculated with ZnCO<sub>3</sub> solubilizing strain RP-4 (*P. chlororaphis*) considerably increased root lengths and areas, and dry shoots and roots weights of when put together with uninoculated controls. Likewise, *P. aurantiaca* strain GS-4 solubilized both ZnCO<sub>3</sub> and ZnO and plants inoculated with GS-3 indicated highest shoot areas when noticed in comparison to uninoculated control plants and inoculated plant with non-zinc solubilizing strains. GS-4 inoculated tomato plants also showed increased root areas and shoots dry weights. *P. aurantiaca* strain ARS-38 solubilized ZnO only on tris-minimal agar plates and upon inoculation; it showed significant increase of dry weights of roots and shoots. Additionally, ARS-38 inoculated plants demonstrated notable increase in root, shoot lengths and shoot areas. *P. aurantiaca* type strain (NCIB 10068T) used as control in this study, however did not contribute in tomato growth promotion despite the fact that it solubilized both ZnCO<sub>3</sub> and ZnO. The reason associated with the maximum growth promotion of tomato by *P. aurantiaca* strain PB-St2 (non-zinc solubilizer) followed by *P. chlororaphis* strain RP-4 (zinc-solubilizer) can be their high production of phytohormone IAA [10]. Moreover, most of the inoculated plants indicated increase in roots and shoots biomass, lengths, and areas when compared to uninoculated controls. These findings show consistence with previously reported literature where zinc-solubilizing pseudomonads showed encouraging results upon inoculation and increased zinc content in wheat grains, shoots, and roots [26,27]. Based on these facts, it can be predicted that inoculating tomato plants with these strains would have increased zinc content of the fruit to alleviate zinc deficiency.

## CONCLUSION

Zinc solubilization by PGPR is comparatively a newer strategy and very few PGPR have been tested for this activity so far. This study indicates the potential of *Pseudomonas spp.* to be used as zinc-biofertilizers to overcome zinc deficiency in countries like Pakistan where zinc fertilizers are expensive and are usually not preferred.

## CONFLICT OF INTEREST

Authors declare no competing interests.

## AUTHORS' CONTRIBUTIONS

SM is the project leader and conceived the study and designed experiments. KT carried out experiments and IS performed statistical analyses and wrote the manuscript.

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