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Assessment for plant growth promoting activities of *Azotobacter vinelandii* AV7 from rhizospheric soil of tomato

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Abstract

The strain isolated from rhizospheric soil of tomato was revealed as *Azotobacter vinelandii* by biochemical characteristics and 16S rRNA sequencing. *A. vinelandii* strain AV7 was evaluated for some plant growth promoting activities. It produced significant amount of indole acetic acid (IAA), the highest concentrations were 22.95 µg/ml (with 0.2% tryptophan) and 72.8 µg/ml (with 0.5% tryptophan). It was found that the highest amount of phosphate (218 µg/ml) was solubilized on 5 days incubation. The strain also produced 24% of siderophore unit. For abiotic stress tolerance, the isolate can tolerate temperature up to 50 °C. The highest vigor index was observed in the seeds inoculated with the isolate AV7 in germination test. In greenhouse experiment, tomato plants treated with the strain AV7 showed significantly higher in fresh and dry weights of plant than the plants used as control.

1. Introduction

Plants need sunlight, proper temperature, moisture, air, and nutrients mineral nutrients form their optimal growth. Abiotic stresses, various adverse environmental factors such as drought, high salt, heavy metals, high temperature, pH which are the limiting factors for plant growth and development [1-3].

Microorganisms which can stably colonize the rhizosphere of various plant species are called plant growth promoting bacteria. They can improve soil structure, promote plant growth and enhance plant mineral-nutrition absorption [4]. Plant growth promoting bacteria (PGPB) enhance plant tolerance to abiotic stresses by inducing physical and chemical changes in plants and it can be termed as induced systemic tolerance (IST)[5]. Many PGPB including *Pseudomonas*, *Bacillus, A. chroococcum, A. benjerinkii, A. vinelandii, A. paspali* can grow and survive at extreme environmental condition such as higher salt concentration, extreme pH value and maximum temperature [6, 7]. Since last two decades the diversity and morphological characteristics of these species are well investigated because of their plant growth promoting activities for sustainable agriculture. They can also enhance crop productivity and nutrient content and suppress the growth of pathogens [8].

In this present study, the soil samples were collected from agricultural soil of Chaungoo region exposed to higher temperature above 40 °C. The goal of our work was to search heat tolerant bacterial isolate with PGP activities from agricultural fields which can be used to develop environmentally friendly biofertilizer for agricultural sector.

Material and Methods

2.1. Sampling and isolation

Soil samples were collected from rhizospheric soil of tomato in Sagaing province. 1 g soil sample was mixed with 10 ml sterile normal saline solution and shaken well by vortex and allowed to stand for 30 min. And then they were kept at 50 °C in incubator for 5 hours to get the strains resistance to high temperature. For isolation of *Azotobacter* sp., glucose nitrogen free mineral medium GNFMM (g/L): 1.0 K_2HPO_4, 1.0 $CaCl_2$, 0.5 NaCl, 0.25 $MgSO_4 \cdot 7H_2O$, 0.01 $FeSO_4 \cdot 7H_2O$, 0.01 $Na_2MoO_4 \cdot 2H_2O$, 0.01 $MnSO_4 \cdot 5H_2O$ and 7.0 glucose was used. Soil suspensions were spread plated on GNFM medium and incubated at 37°C for about 2-3 days.

2.2. Identification of isolated bacteria by biochemical characteristics and 16S rRNA sequencing

Colonial and microscopic morphologies of AV7 were interpreted by Bergey's Manual of Determinative Bacteriology. Gram's staining, gelatin liquefaction, starch hydrolysis, catalase production, methyl red and voges-proskauer tests were carried out. For molecular identification, 16S rRNA gene from the isolated strain was amplified using pair of universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′ TACGGCTACCTTGTTACGAC-3′). For phylogenetic analysis, the 16S rRNA gene sequences were aligned with the Clustal W and the tree was constructed with the maximum likelihood method based on the Tamura-Nei model [9] integrated in the MEGA X software [10]. The phylogenetic tree was tested with 1,000 bootstrap replicates.

2.3. Qualitative detection of siderophore production

Qualitative siderophore production was detected using chrome azurol S (CAS) agar [11]. The strain AV7 was streaked on CAS plates and observed for development of orange halo zone around the colonies after 72 hr incubation at 37°C. A change in color from blue to orange was considered as a positive reaction for siderophore production [12] .

2.4. Quantitative spectrophotometric assay for siderophore production

The strain AV7 was grown in GNFM medium at 37 °C for 5 days under shaking condition (150 rpm). Broth culture was centrifuged at 10,000 rpm for 10 minutes. 0.5 ml of the culture supernatant was then mixed with 0.5 ml CAS solution. The color obtained was determined using the UV-Vis spectrophotometer at 630 nm after 20 minutes of incubation at room temperature. Siderophore produced by strain was expressed in percent siderophore unit (psu) which was calculated according to the formula by Payne [13].

2.5. Estimation of indole acetic acid production

To determine the amount of indole acetic acid (IAA) produced by the isolate, a colorimetric technique was performed with the Salkowski's method [14]. The isolate AV7 was grown in liquid GNFMM and incubated at 37 °C with 150 rpm shaking for 7 days. On 7^{th} days of incubation, the culture broth was centrifuged at 12,000 rpm for 20 minutes. 1ml of supernatant was taken and mixed with 2ml of Salkowski's reagent (2% 0.5 M $FeCl_3$ in 35% $HCLO_4$ solution) and then added one drop of orthophosphoric acid. The mixture was incubated at room temperature for 20-30 minutes. The absorbance of developed pink color was recorded at 530 nm and the amount of IAA produced was calculated by a standard IAA curve.

2.6. Phosphate solubilizing activity

The strain AV7 was screened for phosphate solubilizing activity. The colony of the isolated strain was transferred to Pikovskaya's medium; 10 g glucose, 0.5 g yeast extract, 0.5 g $(NH_4)_2SO_4$, 0.2 g of NaCl, 0.2 g of KCl, 0.1 g $MgSO_4$, trace $MnSO_4$, trace $FeSO_4.7H_2O$ gL^{-1} supplemented with 0.5% $Ca_3(PO_4)_2$. The plates were then incubated at 37 °C and the clear zones were examined after 7 days of incubation and data were recorded. After confirming phosphate solubilizing ability on solid medium, the quantitative determination was carried out according to the blue color method [15].

2.7. Screening of zinc solubilization

The strain AV7 was inoculated on mineral salts medium by Saravanan et al [16] containing dextrose: 10.0; $(NH_4)_2SO_4$: 1.0; KCl: 0.2; K_2HPO_4: 0.1; $MgSO_4$: 0.2; pH: 7.0 and insoluble Zn compound (0.5% ZnO) Agar: 15.0 gL^{-1} and autoclaved at 121 °C for 20 min. The strain was spot-inoculated onto the medium and incubated at 37 °C for 5 days. The clear zones around the colony were recorded.

2.8. Resistance to heat stress

In order to determine the tolerance of high temperature, 1 ml seed culture of the strain AV7 was transferred to 10 ml sterilized liquid GNFMM and incubated at 37°C for 19 hours. The liquid culture was then incubated again at three different high temperature; 40 °C, 45 °C and 50 °C for 5 hours. Incubation at these alternate temperatures was done for 5 days. The growth after heat stress was checked by streaking 100 µl of broth culture on the medium and then the results were recorded daily.

2.9. Effect of isolate AV7 on plant growth parameters of tomato

For seed germination test, 1.5×10^8cfu/ml inoculum size of AV7 was prepared. Tomato seeds (*Solanum lycopersicum* L.) were sterilized by soaking them first in 70% ethanol for 5 min and then in 0.2% sodium hypochlorite for 5 min and rinsed three times with sterilized distilled water. The sterilized seeds were immersed for 2 h in cell suspension prepared earlier and the seeds treated with GNFMM and distilled water were used as control. All treated seeds were then transferred to plates containing wetted filter papers (10 seeds per plate) and incubated at 25 °C. After 5 days, the germination rates, shoot and root lengths of seedlings were measured. Three replicates were done for this experiment. The vigor index was calculated as follows [17]:

Vigor index = percent germination × seedling length (shoot length + root length)

Sterilized tomato seeds were sown in the trays filled with the sterilized soil. After two weeks, uniform sized seedlings were selected and planted in plastic bags filled with the sterilized soil. Three treatments; water, liquid GNFMM and strain AV7, were conducted for this experiment, water and liquid GNFMM were used as controls. The seedlings were treated with 20 ml of each treatment every week. After 30 days, the plants were harvested, and then root length, shoot length, fresh weight and dry weight were measured.

2.10. Statistical analysis

Data were analyzed by analysis of variance (ANOVA) followed by the least significant difference (LSD) tests using the Minitab v 19.0 statistical software package and all hypotheses were tested at the 95% confidence level. All experiments were done in triplicate.

3. Results and discussion

3.1. Isolation and identification of isolated strain

The isolated strain obtained from the rhizospheric soil of tomato was characterized morphologically, biochemically and subjected to 16S rRNA gene sequence analysis. The colonial morphology of the selected isolate was creamy white in early days of incubation and after 5 days of incubation, they produced brown color pigment. Gram reaction of that isolated strain was negative and cells are usually oval, but may take various forms from rods to spheres. Some biochemical characteristics of the selected isolate was shown in Table 1. A phylogenetic tree based on neighbor-joining method for the strain AV7 along with the closest relatives of the genus *Azotobacter* species was shown in Figure 1. According to the biochemical characteristics and 16S rRNA gene sequence analysis, the isolated strain was characterized as *Azotobacter vinelandii* and designated as AV7 for this study.

Table 1: Biochemical characteristics of isolated strain AV7

Test	Result
Gram stain	-
Methyl red	-
Voges–Proskauer	+
Catalase	+
Citrate utilization	+
Gelatinase	+
Starch hydrolysis	+

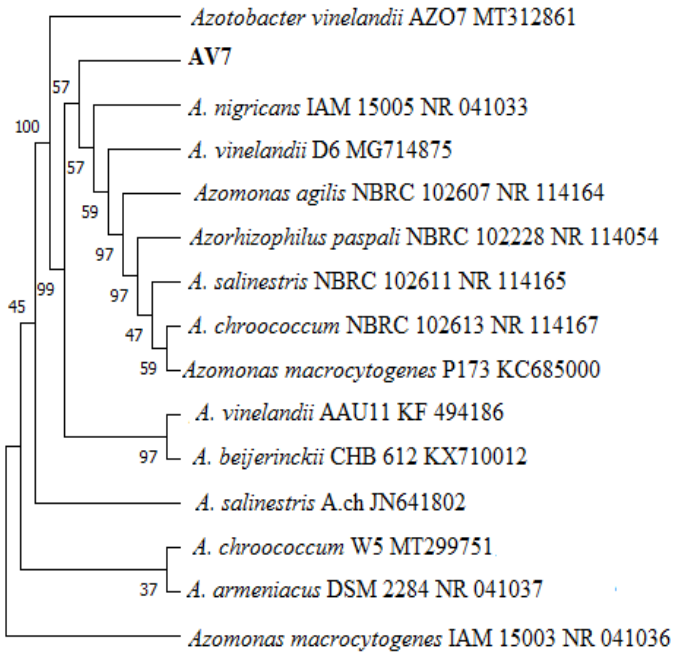

Figure1: Neighbor-joining tree based on 16S rRNA gene sequences, showing the relationships between strain AV7 and other related *Azotobacter* species.

3.2. Determination of siderophore production

Microbial siderophores provide Fe to plants to enhance their growth when the bioavailability of Fe is low in the soil [18]. Production of siderophore has significant role in the biological control mechanism as competitors for Fe in order to reduce the Fe availability for the phytopathogens [19, 20]. Positive for

siderophore production activity was determined according to the formation of orange colored zone around the bacterial colonies. The isolated strain AV7 showed the orange halo zone after 3 days incubation. When siderophore production was estimated quantitatively, the strain AV7 showed 24% of siderophore production unit at 5 days incubation period.

3.3. Indole acetic acid production of strain AV7

Indole acetic acid was produced by microorganisms which was isolated from rhizospheric region of various crop due to rich supply of substrates. IAA helps the production of longer roots and increase number of root hairs and lateral roots which are involved in nutrient uptake [21].

IAA stimulates cell elongation by modifying certain conditions like, increase in osmotic contents of the cell, increase in permeability of water into cell, decrease in wall pressure, an increase in cell wall synthesis and inducing specific RXA and protein synthesis (Zhao, 2010).

In our present study, the impact of two different concentrations (0.2% and 0.5%) of tryptophan on IAA secretion by the isolated strain AV7 was investigated at 3, 5 and 7 days incubation periods. Significantly different IAA concentrations were produced by the strain on two different tryptophan concentrations. The highest IAA concentrations were observed at 5 days incubation for both tryptophan concentrations by the strain. The results were compared in (Figure 2).

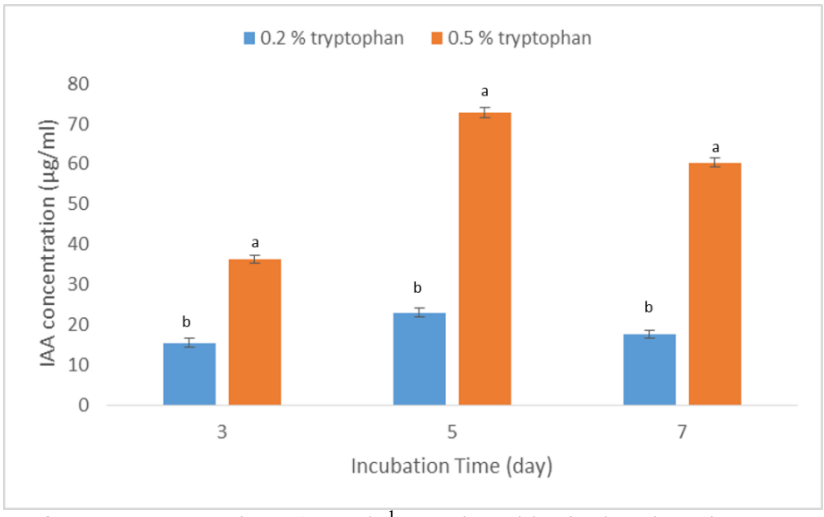

Figure 2: Comparison of IAA concentrations (µg ml $^{-1}$) produced by isolated strain AV7 on two different tryptophan concentrations

3.4. Phosphate solubilizing activity of the isolate AV7

Phosphorus (P) is the second most essential macro nutrients for plant growth and development that is absorbed only in soluble forms of phosphate ion. However, it is mostly immobilized in the forms of organic and inorganic compounds such that only a small fraction of P is available for plant growth [22, 23]. Phosphate solubilizing microorganisms enhance plant growth through solubilization of insoluble phosphate. Phosphate solubilizing PGPR included in the genera *Arthrobacter*, *Bacillus*, *Beijerinckia, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Mycobacterium, Pseudomonas*, *Rhizobium*, *Rhodococcus* and *Serratia,* and have attracted the attention of agriculturists as soil inoculums to improve plant growth and yield [24]. The strain AV7 was evaluated for phosphate solubilizing activity qualitatively and quantitatively. In plate screening, the strain showed 33 mm in diameter of solubilization zone and in quantitative analysis, the strain AV7 can solubilize significant amount of phosphate; the highest concentration was 218.28 µg/ml at 5 days incubation period followed by 169.65 µg/ml at 7 days incubation and 159.1 µg/ml at 3 days incubation. It was shown in Figure 3.

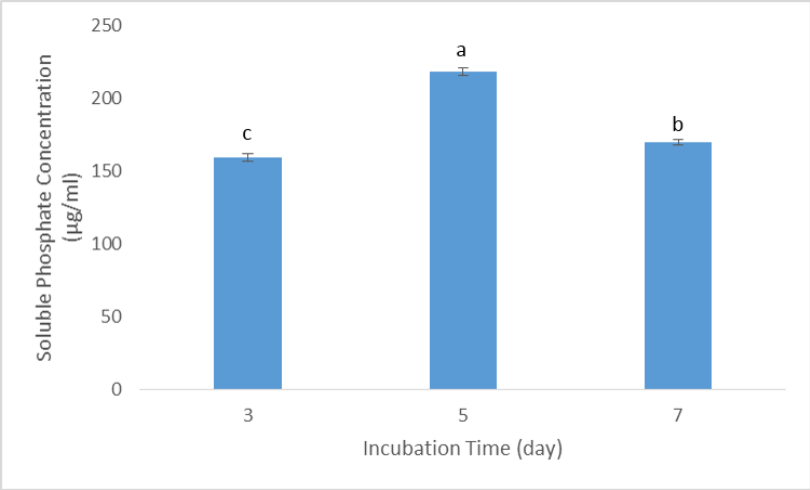

Figure 3: Phosphate concentrations (µg ml $^{-1}$) solubilized by strain AV7

3.5. Zinc solubilizing activity

Zinc (Zn) is one of the essential micronutrients required for optimum plant growth. Zinc deficiency in plants leads to reduced membrane integrity and synthesis of carbohydrates, auxins, nucleotides, cytochromes, and chlorophyll and develops susceptibility to heat stress [25]. In this study, the strain AV7 showed 24 mm in diameter zone of solubilization.

3.6. Tolerance of heat stress by the strain AV7

Plant growth-promoting bacteria found in association with plants grown under chronically stressful conditions, including high salinity, may have adapted to the stress conditions, and could provide a significant benefit to the plants. *Pseudomonas*, *Bacillus* and *Azotobacter* can grow and survive at extreme environmental conditions such as higher salt concentration, pH value and in dry soil with extreme temperature [26]. In this present study, the isolate AV7 was able to grow well at temperature up to 50 °C. The results were shown in Table 2. This result indicates that the strain AV7 may be suitable for agricultural soil in high temperature area.

Table 2: Heat stress tolerance of strain AV7

Isolated strain	**Temperature**		
	40 °C	45 °C	50 °C
AV7	+++	+++	+++

+++ = well growth

3.7. Effect of isolated strain AV7 on growth of tomato seedlings

Although the germination percent of the tomato seeds was not different in all treatments, the shoot length and root length of tomato plants treated with the strain AV7 were significantly different from that of the controls. The results were shown in Table 3 and (Figure 4).

Based on initial studies for PGP activities, the strain AV7 was tested in pots for growth promotion of tomato plants. In greenhouse experiment, the greater accumulation of fresh and dry weights was occurred in bacterially treated plants compared with the controls. Mechanisms employed for plant growth may include enhancement of solubilization of phosphate, production of IAA and siderophore. These results

indicated that this rhizospheric strain AV7 therefore has the potential to promote plant growth. The significant different results were shown in Table 4 and (Figure 5).

Table 3: *In vitro* seed germination test

Treatment	Inoculum size	Germination percent	Shoot length	Root length	Vigor index
Water	0	100	1.7 ± 0.22b	3.9 ± 0.21b	560
GNFMM	0	100	1.8 ± 0.23b	4.1 ± 0.27b	590
strain AV7	1.5 x 10^8	100	2.4 ± 0.25a	5.5 ± 0.50a	790

Values with different letters within a column are significantly different according to LSD test at P ≤ 0.05.

Table 4: Effect of strain AV7 on the growth of tomato plants in greenhouse experiment

Treatment	Shoot length (cm)	Root length (cm)	Fresh weight (g)	Dry weight (g)
Water	21.47 ± 1.23b	4.87 ± 0.57b	2.67 ± 0.42b	0.25 ± 0.03b
GNFMM	22.13 ± 1.15b	5.07 ± 0.50b	2.97 ± 0.50b	0.28 ± 0.02b
AV7	27.47 ± 1.11a	6.50 ± 0.44a	4.87 ± 0.25a	0.57 ± 0.02a

Values with different letters within a column are significantly different according to LSD test at ≤ 0.05.

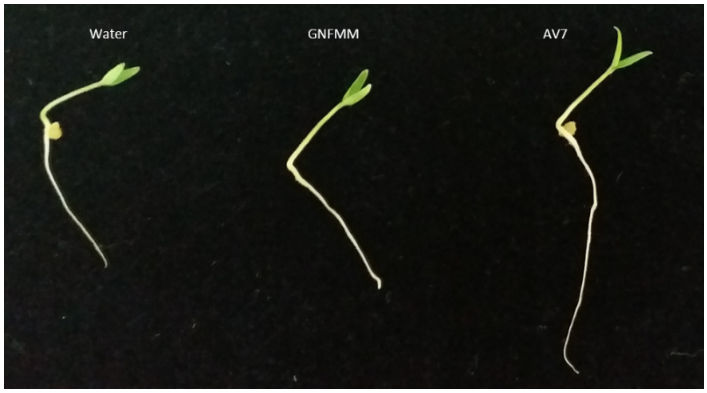

Figure 4: *In vitro* seed germination for isolated strain AV7

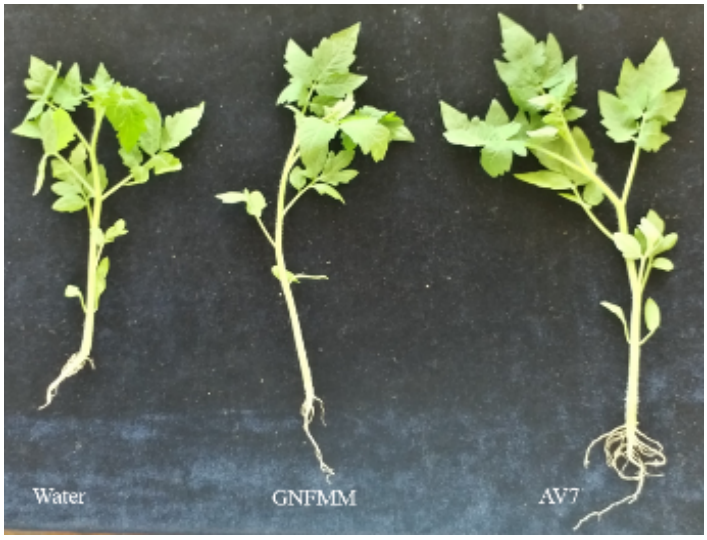

Figure 5: Different growth parameters of tomato plants (*Solanum lycopersicum* L.) inoculated with AV7, GNFMM and water

Conclusion

This rhizospheric bacteria enhance plant growth through PGP activities such as the ability of auxin production, phosphate solubilization, siderophore production and zinc solubilization as well as by suppressing fungal pathogens. Collectively, this study demonstrated that heat tolerant strain AV7 has potent plant growth promoting activities under both laboratory and greenhouse conditions.

References

1. T.A. Crane, C. Roncoli, G. Hoogenboom, Adaptation to climate change and climate variability: The importance of understanding agriculture as performance, *NJAS-Wageningen Journal of Life Sciences,* 57(3-4) (2011) 179-185.
2. P. Ahmad, A. Hashem, E.F. Abd-Allah, A. Alqarawi, R. John, D. Egamberdieva, S. Gucel, Role of *Trichoderma harzianum* in mitigating NaCl stress in Indian mustard (*Brassica juncea* L) through antioxidative defense system, *Frontiers in plant science*, 6 (2015) 868.
3. Q.Y. Jiang, F. Zhuo, S.H. Long, H.D. Zhao, D.J. Yang, Z.H. Ye, S.S. Li, Y.X. Jing, Can arbuscular mycorrhizal fungi reduce Cd uptake and alleviate Cd toxicity of *Lonicera japonica* grown in Cd-added soils?, *Scientific reports*, 6(1) (2016) 1-9.
4. Q.S. Wu, R.X. Xia, Y.N. Zou, Improved soil structure and citrus growth after inoculation with three arbuscular mycorrhizal fungi under drought stress, *European journal of soil biology,* 44(1) (2008) 122-128.
5. J. Yang, J.W. Kloepper, C.M. Ryu, Rhizosphere bacteria help plants tolerate abiotic stress, *Trends in plant science,* 14 (1) (2009) 1-4.
6. A. Srivastava, A.K.Mishra, Regulation of nitrogen metabolism in salt tolerant and salt sensitive Frankia strains, (2014).
7. S. Shaikh, P. Patel, S. Patel, S. Nikam, T. Rane, R. Sayyed, Production of biocontrol traits by banana field fluorescent Pseudomonads and comparison with chemical fungicide, (2014).
8. D.J. Jiménez, J.S. Montaña, M.M. Martínez, Characterization of free nitrogen fixing bacteria of the genus *Azotobacter* in organic vegetable-grown Colombian soils, *Braz. J. Microbiol.,* 42 (3) (2011) 846-858.
9. S. Kumar, K. Tamura, M. Nei, MEGA: molecular evolutionary genetics analysis software for microcomputers, *Bioinformatics,* 10 (1994) 189-191.
10. S. Kumar, G. Stecher, M. Li, C. Knyaz, K. Tamura, MEGA X: molecular evolutionary genetics analysis across computing platforms, *Molecular biology and evolution*, 35 (2018) 1547-1549.
11. B.C. Louden, D. Haarmann, A.M. Lynne, Use of blue agar CAS assay for siderophore detection, *Journal of microbiology & biology education,*12 (1) (2011) 51.
12. B. Schwyn, J.B. Neilands, Universal chemical assay for the detection and determination of siderophores, *Anal. Biochem.,*160 (1) (1987) 47-56.
13. S.M. Payne, Iron acquisition in microbial pathogenesis, *Trends. Microbiol.,* 1 (2) (1993) 66-69.
14. A. Ehmann, The Van Urk-Salkowski reagent—a sensitive and specific chromogenic reagent for silica gel thin-layer chromatographic detection and identification of indole derivatives, *J. Chromatogr.,* 132 (2) 91977) 267-276.
15. S.R. Olsen, Estimation of available phosphorus in soils by extraction with sodium bicarbonate, *US Dept. of Agriculture*, (1954).

16. V. Saravanan, P. Kalaiarasan, M. Madhaiyan, M. Thangaraju, Solubilization of insoluble zinc compounds by *Gluconacetobacter diazotrophicus* and the detrimental action of zinc ion (Zn2+) and zinc chelates on root knot nematode *Meloidogyne incognita*. *Lett. Appl. Microbiol.,* 44(3) 92007) 235-241.
17. A. Vashisth, S. Nagarajan, Effect on germination and early growth characteristics in sunflower (*Helianthus annuus*) seeds exposed to static magnetic field, *J. Plant. Physiol.,* 167 (2) (2010) 149-156.
18. D.E. Crowley, Microbial siderophores in the plant rhizosphere, Iron nutrition in plants and rhizospheric microorganisms, *Springer*, (2006) 169-198.
19. A. Beneduzi, A. Ambrosini, L.M. Passaglia, Plant growth-promoting rhizobacteria (PGPR): their potential as antagonists and biocontrol agents, *Gent. Mol. Biol.,* 35 (4) (2012) 1044-1051.
20. J.W. Kloepper, J. Leong, M. Teintze, M. N. Schroth, Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria, *Nature*, 286(5776) (1980) 885-886.
21. C. Datta, P.S. Basu, Indole acetic acid production by a *Rhizobium* species from root nodules of a leguminous shrub, *Cajanus cajan*, *Microbiol. Res.,* 155 (2)(2000) 123-127.
22. A. Deubel, W. Merbach, Influence of microorganisms on phosphorus bioavailability in soils, Microorganisms in soils: roles in genesis and functions, *Springer*, (2005) 177-191.
23. M. Turan M, N. Ataoğlu, F. Şahın, Evaluation of the capacity of phosphate solubilizing bacteria and fungi on different forms of phosphorus in liquid culture, *J. Sustain. Agr.,* 28(3) (2006) 99-108.
24. P.N. Bhattacharyya, D.K. Jha, Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World. J. Microbiol. Biotechnol.,* 28(4) (2012) 1327-1350.
25. B. Singh, S.K.A. Natesan, B.K. Singh, K. Usha, Improving zinc efficiency of cereals under zinc deficiency, *Curr. Sci.,* 88(1) (2005) 36-44.
26. G. Chennappa, M.K. Naik, C.R. Adkar-Purushothama, Y.S. Amaresh, M. Y. Sreenivasa, PGP potential, abiotic stress tolerance and antifungal activity of *Azotobacter* strains isolated from paddy soils, *Indian. J. Exp. Biol.,* 54(5) (2016) 322-331.