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Detection of abiotic stress tolerant *Azotobacter* species for enhancing plant growth promoting activities

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Abstract

Detection of Azotobacter species AC9 was done for tolerance of high temperature and analysis of phosphate solubilizing activity, zinc solubilizing activity, hormone production activity and plant growth promoting traits on tomato seedlings. For heat stress tolerance assessment of the isolated strain, our experiment was designed to be suitable for the climate of our country. In this study, Azotobacter chroococcum was isolated from agricultural soil in Kyaukse province and identified by some biochemical characteristics and 16S rRNA sequencing. The isolated strain can tolerate high temperature up to 50˚C. In phosphate solubilizing activity, the strain AC9 showed 183 µg/ml and 166 µg/ml at 5 days incubation period under normal and heat stress conditions respectively. AC9 also expressed highest IAA producing activity at 5 days incubation period for both normal and heat stress conditions. The highest IAA concentrations were 23.72 µg ml^{-1} (with 0.2% tryptophan), 55.55 µg ml^{-1} (with 0.5% tryptophan) and 17.73 µg ml^{-1} (with 0.2% tryptophan), 45.06 µg ml^{-1} (with 0.5% tryptophan) respectively. Under greenhouse condition, tomato plants inoculated with AC9 grew to a significantly greater extent than control plants.

Keywords: Plant growth promoting activity, Indole acetic acid (IAA), Azotobacter chroococcum, phosphate solubilizing activity, Heat stress, 16S rRNA.

INTRODUCTION

Excessive use of chemical fertilizer can cause deleterious effects on soil microorganism, the fertility status of soil and also pollutes environment [1, 2]. The application of these fertilizers on a long-term basis often leads to reduction in pH and exchangeable bases thus making them unavailable to crops and the productivity of crop declines. To obviate this problem and obtain higher plant yields, farmers have become increasingly dependent on chemical sources of nitrogen and phosphorus. Besides being costly, the production of chemical fertilizers depletes nonrenewable resources, the oil and natural gas used to produce these fertilizers, and poses human and environmental hazards [2]. In modern cultivation process indiscriminate use of fertilizers, particularly the nitrogenous and phosphorus, has also led to substantial pollution of soil, air and water.

The current agricultural policy emphasized to have major changes to sustainable production system. Researchers have been interesting for research in the environment of microorganisms around plant root called rhizosphere. The rhizosphere microorganisms have a major influence on plants today and become important tool to guard the health of plants in ecofriendly manner [3].

The rhizobacteria that can be found in the rhizosphere, at root surfaces and in association with roots are heterogeneous bacteria [4]. The benefits of these bacteria such as producing plant growth hormones, asymbiotic nitrogen fixation, solubilization of mineral phosphate, potassium and zinc and biocontrol activity against plant pathogen have been studied on numerous crops, including tomato (*Lycopersicon esculentum* Mill.) grown under green house in organic media [5] or field conditions [6].

Climate changes can also cause vulnerable effect in agricultural sector. Abiotic and biotic stresses has become major cause for yield declines in various crops in many parts of South East Asia due to increasing water stress, reduction in number of rainy days and increased temperature. Besides salt stress, high temperature, droughts, elevated CO_2, extreme rainfall events, more floods, cold waves, heat waves, and cyclones are the other important natural disasters that cause serious economic losses, are likely to because of global warming [7]. Abiotic stress can also restrict plant growth and yield improvement because of the

limited uptake of water and nutrients [8]. Nevertheless, 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 [9]. Plant growth promoting bacteria could play a significant role in stress management, and also provide excellent models for understanding stress tolerance mechanisms that can be subsequently engineered into crop plants [7]. Plant growth promoting bacteria most involved are: *Agrobacterium*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Caulobacter*, *Chromobacterium*, *Erwinia*, *Flavobacterium*, *Micrococcous*, *Pseudomonas* and *Serratia* [10].

This present study was conducted in Kyaukse province, Mandalay, Myanmar where crop production is very important. Here we evaluate and characterize heat stress tolerance and plant growth promoting activities of *Azotobacter chroococcum* for tomato plants.

MATERIALS AND METHODS

Sampling and isolation

The present investigation was under taken with an objective to select heat tolerant plant growth promoting *Azotobacter* species. The soil samples were collected from different sites of Kyaukse province. 1g of soil samples was added to 10 ml of 0.9% NaCl solution and then kept at 50° C in incubator for 5 hours. After this, 0.1ml supernatant was surface spread over glucose nitrogen free minerals agar 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 at 37°C for 3 days. The colonies formed on these plates were then checked colonial and microscopic morphology for *Azotobacter* species.

Morphological characterization and strain identification

The selected bacterial isolate was examined for morphological features of the colony like the colour, shape, size, surface, pigment production and gram staining. One isolate out of 10 was selected and tested by some biochemical characteristics for strain characterization. For molecular identification, genomic DNA extraction and PCR amplification of the 16S rRNA gene of the isolate was performed. The 16S rRNA gene sequence of the selected isolate was aligned with that of related species using clustal W and phylogenetic analysis was performed by software package MEGA X [11].

Resistance to heat stress

The strain AC9 was assessed for heat tolerance at different temperature 37°C, 40°C, 45°C and 50°C according to our experimental design. The strain was first inoculated in 5 ml GNFMM broth for 24 hours at 37°C with 150 rpm/min shaking. And then 1 ml of culture broth was transferred to 10 ml sterilized liquid GNFMM and incubated at 37°C for 19 hours. After that, they were further incubated at respective above-mentioned temperatures for 5 hours. To ascertain growth after heat stress, 100 µl of broth culture from each tube were spread on GNFMM plates and incubated at 37°C for 48–72 hours and then checked for growth.

Monitoring IAA production

The strain AC9 was inoculated into 10 ml of GNFMM supplemented with 0.2 % and 0.5 % tryptophan and incubated at 37°C with 150 rpm/min shaking for normal quantification and to investigate IAA production after heat stress, bacterial culture was incubated at 37°C for 19 hours and then continued incubation at 50°C for 5 hours alternatively for 7 days.

Broth cultures were centrifuged at 12,000 rpm for 10 minutes. To one ml supernatant of the culture, 2 ml of Salkowski's reagent [12]and one drop of orthophosphoric acid were added and incubated for 25 minutes at room temperature. Thereafter, the absorbance of the developed pink colour was measured at 530 nm and the values of IAA were expressed as microgram per ml over uninoculated control.

Determination of phosphate solubilization

To examine phosphate solubilization capability, the strain AC9 was spotted on the center of modified Pikovskaya's (PVK) agar [13] medium plate containing insoluble phosphate. PVK medium composed of 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 $FeSO_4.7H_2O$ was supplemented with 0.5% $Ca_3(PO_4)_2$ (TCP) and 15 g agar (in solid medium) per liter at pH: 7.2. The inoculated plates were incubated at 37°C. The diameter of zone of clearance (halo) surrounding the bacterial colony as well as the diameter of colony were measured after 3, 5- and 7-days incubation. Phosphate solubilizing index (PSI) was calculated using the formula: (colony diameter + halozone diameter) - colony diameter.

The isolate AC9 was also assayed for phosphatase activity quantitatively according to the blue color method [14]. Fresh seed culture of AC9 was inoculated in the PVK broth (20 ml) at 37℃ for 24 hours with continuous shaking at 150 rpm min^{-1}. 1 ml of broth culture was then transferred to the flask containing sterilized liquid PVK medium with 0.5 % $Ca_3(PO_4)_2$ and incubated for 7 days with continuous shaking at 37℃ and the sterilized uninoculated medium served as a control. 10 ml of each culture and control were taken and centrifuged at 12,000 rpm for 10 min. The supernatant was used in determining the amount of phosphate released into the medium.

For the assessment of phosphate solubilizing activity of the isolate under abiotic heat stress condition, it was inoculated in sterilized liquid PVK medium and then incubated at alternate temperature described earlier with continuous shaking at 150 rpm min^{-1}. After 7 days incubation, phosphate solubilizing activity was determined. The amount of soluble phosphate was calculated using the standard curve prepared with KH_2PO_4.The experiment was done as triplicate.

Screening of zinc solubilization

The isolate AC9 was inoculated into liquid mineral salts medium ($g \cdot lit^{-1}$) specified by Saravanan et al [15] 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 g and autoclaved at 121˚C for 20 min. Actively growing culture of the strain was spot-inoculated onto the agar and plates were incubated at 37℃ for 48-72 h. The clear zones around colony were recorded.

Estimation of siderophore production

The isolate AC9 was tested for the production of siderophore on chrome azurole-S-agar (CAS) plates by incubation at 37°C for 5 days [16].

Quantitative estimation of siderophore production was conducted by the method Hu and Xu 2011 [17]. Siderophore produced by strain was measured in percent siderophore unit (psu) which was calculated according to the formula by Payne [18].

Vigor index and plant growth condition under green house

In vitro seed germination test was performed using a paper towel method by [19]. The strain AC9 was incubated in sterilized liquid GNFMM at 37˚ C and inoculum size (1.5×10^8cfu/ml) was prepared. Tomato seeds were surfaced sterilized with 70% ethanol for 5 min followed by 0.2% sodium hypochlorite for 5 min and rinsed four times with SDW. Sterilized seeds were soaked in the cell suspension prepared earlier and kept 150 rpm shaking condition for 2h. Seeds treated with water were used as control. The seeds were then transferred to sterile plates containing wetted filter papers at the rate of 10 seeds per plate. Each treatment was replicated three times. Seeds were germinated in a growth chamber at 28˚ C. After 5 days, the number of germinated seeds was counted, and root and shoot lengths of individual seedling were measured. Vigor index was determined with the following formula: Seedling length (i.e. mean root length + mean shoot length) x germination %] [20].

Tomato seeds were sown in trays with the sterilized soil. After 1-week, uniform sized seedlings were selected and planted in plastic bags filled

with the sterilized soil. The seedlings were treated with 20 ml of strain AC9, liquid GNFM medium and water as controls every week. The seedlings were maintained under greenhouse at a day/night temperature of 25 °C. After 30 days, the plants were harvested, and then root length, shoot length, fresh weight and dry weight were measured for each treatment.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA), and all hypotheses were tested at the 95% confidence level.

RESULTS

Characterization of the isolated strain

Some biochemical tests were done for selected bacterial isolate and the results were shown in Table 1. 16S rRNA sequence analysis of the selected strain indicated that the strain was 99.93% identical to *Azotobacter chroococcum* Fig.1. The strain that was selected for further study was therefore designated as AC9. Phylogenetic distances were calculated with the Kimura two-parameter model [21] and tree topologies were inferred using the neighbour-joining method and bootstrap analysis was performed with 1000 resampling [22].

Heat stress tolerance of strain AC9

In order to analyze the heat stress tolerance of AC9, the strain was incubated at different temperatures 37°C, 40°C, 45°C and 50°C on GNFMM plates. The strain was observed able to grow up to temperature 50°C. The results were shown in Table 2.

Indole acetic acid (IAA) production

The impact of two different temperatures (37°C and 50°C) on IAA secretion by heat tolerant AC9 in GNFMM broth supplemented with 0.2% and 0.5% tryptophan under normal and heat stress conditions was investigated at 3, 5, 7 days incubation. The strain produced IAA under normal condition and heat stress condition. The results were compared in Fig. 2.

Phosphate solubilizing activity

Screening of phosphate solubilizing activity of AC9 was conducted on PVK media supplemented with 0.5% $Ca_3(PO_4)_2$. The formation of halo zone around the colony indicates the solubilization of phosphate and the results of zone diameter were shown in Table 3. Quantitative analysis of phosphate solubilizing activity was also investigated by Olsen P method and the results were shown in Fig. 3.

Zinc solubilizing activity

When zinc solubilizing activity of the isolated strain AC9 was evaluated on mineral salts medium, the isolated strain could effectively solubilize insoluble Zn compound used. The results were shown in Fig.4.

Production of siderophore by strain AC9

The siderophore production of strain AC9 was determined by the formation of orange-coloured zone around the bacterial colonies. Quantitative estimation of siderophore production for AC9 was also done and 24% of siderophore production unit was found at 5^{th} day incubation period.

Seed vigor index

Tomato seeds were treated with the strain AC9 and after five days, the germination percentage, root length and shoot length of seedlings were recorded. *In vitro* seed vigor index results were shown in Table 4.

Influence of selected strain on the growth of tomato plants

In pot trial experiment under greenhouse condition, plants inoculated with AC9 grew to a significantly greater extent than un-inoculated control plants. The greater accumulation of fresh and dry weights were occurred in bacterially treated plants. The significant different results were shown in Table 5.

DISCUSSION

The goal of today's world is yield improvement and enhanced quality of the crop as well as fertility of soil to get in an ecofriendly manner. Hence, the research has to be emphasized on the new concept of rhizo engineering based on favorably activities of microbes, which create a unique setting for the interaction between plant and microbes [23]. Besides plant growth promoting mechanisms, microorganisms can also impart some degree of tolerance to plants towards abiotic stresses like drought, chilling injury, salinity, metal toxicity and high temperature [24]. In this present work, plant growth promoting bacterium AC9 was isolated and identified as *A. chroococcum* by 16S rRNA sequencing. The phylogenetic analysis further confirmed that the strain is phylogenetically related to other *Azotobacter* species obtained from NCBI Gen Bank database. To determine the heat tolerance of the strain AC9, our experiment was designed to be suitable for the climate of our country. For the whole day, the highest (extreme) temperature is about 5 hours and then the temperature drops in the rest 19 hours. So bacterial culture was incubated at high temperature for 5 hours and at normal temperature for 19 hours alternatively. When temperature tolerance of the isolated strain was examined, it can tolerate high temperature up to 50˚C.

Phosphorus plays an important role in virtually all major metabolic processes in plant including photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis and respiration. It is abundantly available in soils in both organic and inorganic forms [25]. Plant growth promoting rhizobacteria present in the soil employ different strategies to make use of unavailable forms of phosphorus and in turn also help in making phosphorus available for plants to absorb. The ability of *Azotobacter* species to dissolve inorganic and organic phosphate compounds was recently described by [26]. In this study, there was no significant difference in phosphate solubilization of the isolated strain AC9 under normal and heat stress conditions.

Indole acetic acid (IAA) is the most common natural auxin found in plants and effects on root formation of plants. Most of rhizobacteria can produce indole acetic acid (IAA), enhance the host's uptake of minerals and nutrients from the soil [27]. The effect of different temperatures on production of IAA by *Azotobacter* sp. was also studied by Mahrouk et al. [28]. Regarding production of IAA, strain produced indole acetic acid with different ranges to the first treatment (0.2% tryptophan) and to the second treatment (0.5% tryptophan). Although there was significant difference in IAA production under normal and heat stress conditions, the strain AC9 can produce significant amount of IAA under heat stress condition.

Solubilization of zinc can be accomplished by excretion of metabolites such as organic acids, proton extrusion, or production of chelating agents [29]. Qualitative study of zinc solubilization exhibited that the strain could effectively solubilize the insoluble Zn compound (ZnO) used.

The production of iron chelating siderophores is another important function of strains *Azotobacter* species with a dual effect of promoting plant growth by increasing nutrient availability to the plant and at the same time reducing iron availability for soil-borne plant pathogens [30]. The siderophore production was documented in our isolated strain AC9.

Similar observation was also recorded by Dey et al. [31] that *Azotobacter* which can improve seed germination to a considerable extent, has significant role in plant growth promotion including production of growth regulators, protection from root pathogens, and modification of nutrient uptake by the plant. One month after planting of tomato plants, the shoot and root dry weights of seedlings with AC9 inoculation were significantly higher than those of the control plants.

The overall results of the present study have shown that soil dwelling bacteria (PGPR) form an essential constituents of soil ecosystems and play a crucial role in growth and enhancement of plant by diverse mechanisms.

Table 1: Biochemical characteristics of *A. chroococcum* strain AC9

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

Table 2: Heat stress tolerance of strain AC9

Isolated strain	Temperature			
	37˚C	40˚C	45˚C	50˚C
AC9	+++	+++	+++	+++

+++ Well growth

Table 3: Zone diameter of phosphate solubilization

Isolated strain	Zone diameter in mm		
	3 days	5 days	7 days
AC9	11.7 ± 0.06	15 ± 0.1	20.7 ± 0.15

Table 4. *In vitro* PGPR effect on tomato seed germination

Treatment	Inoculum size	Germination percent	Shoot length (cm)	Root length (cm)	Vigor index
Water(control)	0	100	1.7 ± 0.42	4.8 ± 0.7	650
Strain AC9	1.5 x 10^8	100	2.3 ± 0.32	6.2 ± 0.57	850

Table 5: Effect of strain AC9 on the growth of tomato plants under greenhouse

Treatment	Shoot length (cm)	Root length (cm)	Fresh weight (g)	Dry weight (g)
Water (control)	21.5 ± 1.33	4.6 ± 1.1	2.6 ± 0.66	0.25 ± 0.07
GNF	24.1 ± 1.12	5.4 ± 0.9	3.95 ± 0.52	0.39 ± 0.06
AC9	29 ± 0.9	6.2 ± 0.64	4.83 ± 0.23	0.56 ± 0.05

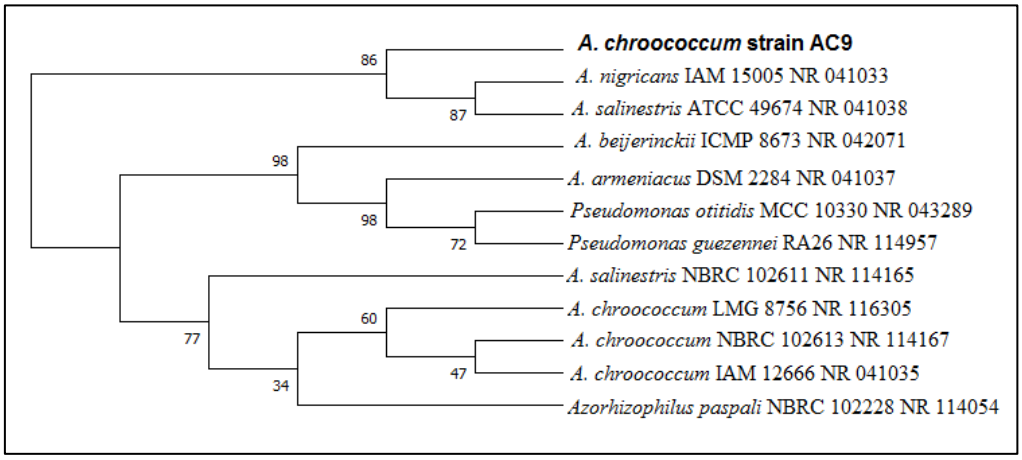

Figure 1: Neighbour-joining tree based on 16S rRNA gene sequence, showing the relationships between the strain *Azotobacter chroococcum* AC9 and other related strains of *Azotobacter* species

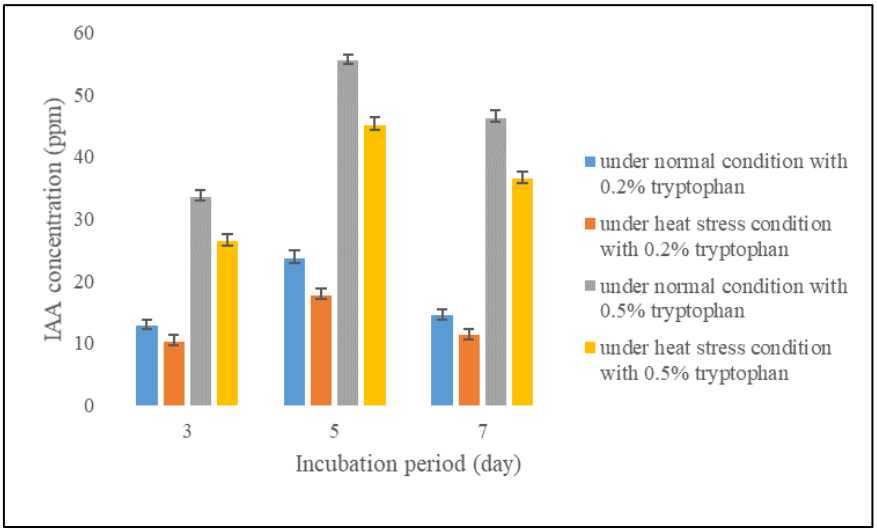

Figure 2: Production of indole acetic acid by *Azotobacter chroococcum* strain AC9

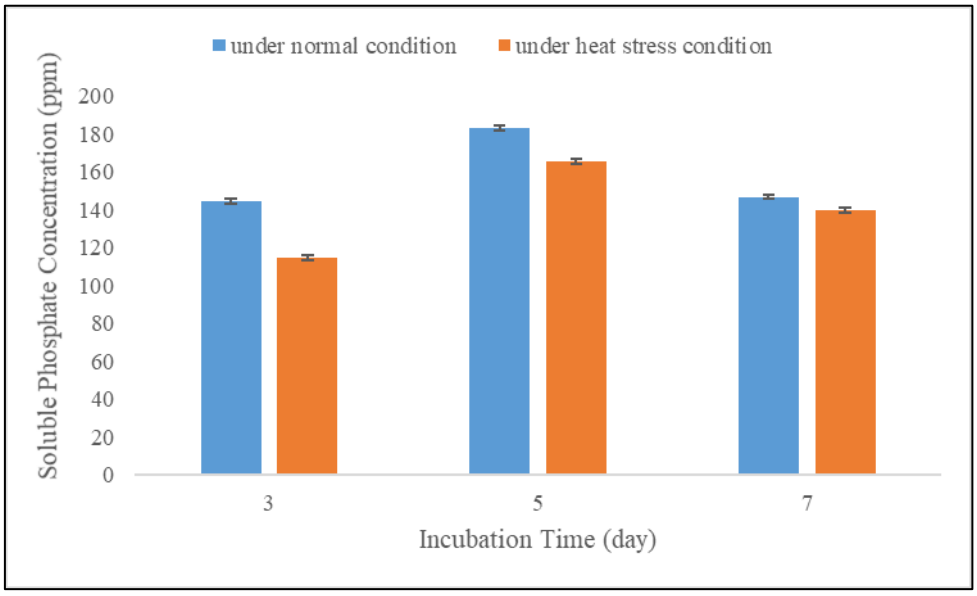

Figure 3: Phosphate solubilizing activity of the isolated strain AC9

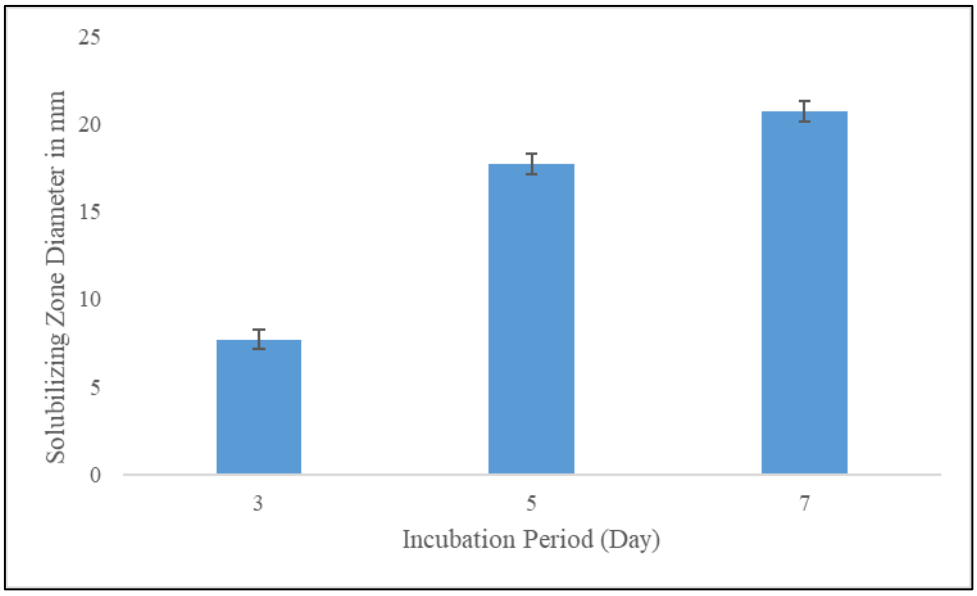

Figure 4: Zinc solubilizing ability of strain AC9 with insoluble zinc substrate on mineral salts medium

CONCLUSION

Agricultural and ecological basis, stress tolerant PGPRs are studied to be able to enhance crop production and maintain environmental quality under sustainability. Results obtained in this study show that *Azotobacter chroococcum* strain AC9 has the abilities to tolerate high temperature and to promote growth of plant. Further work need to be evaluated to develop the significantly better mutant strain constructed by genetic engineering and testing of improvement in existing PGPR activities.

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