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Evaluation of rhizospheric soil and nodules of native species of semi-arid/sub-humid forest ecosystem: coping with the growth barrier

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Key words: *Proline production, PGPR; Phosphorus Solubilization; Rhizospheric Soil, Rhizospheric Nodules.*

Abstract

The microorganisms (*Bacillus* and *Achromobacter* species) present in soil and root nodules play an important role in phosphorus solubilization and proline production. They are of utmost importance for growth enhancement in tree species especially which are surviving under stressed conditions.This study was carried out to solve the problem of stunted growth in native tree species which are striving in physiologically stressed conditions in semi-arid/sub humid forest ecosystem of Pakistan via application of plant growth promoting rhizobacteria (PGPRs). The rhizospheric soil and nodules under native tree species *viz a vizAcacia modesta, A. nilotica, Albizzialebbek*and*A.procera* were collected. A series of experiments were carried out to isolate Rhizobial strains from tree rhizospheric soil and nodules, through characterizing bacteria using molecular and biochemical techniques. Moreover the isolation was carried out through dilution plate techniques. Luria Bertani(LB)and Yeast ExtarctManitol(YEM) media were used. Strains were purified and preserved in glycerol stocks. The purified strains were also tested for proline and phosphorus solubilization. The study revealed that maximum phosphate solubilized by *Achromobacter muciolens*whileenhancedProline contents were found in *Achromobacter xylosoxidans*.This study identified that by application of PGPRs one can enhance the growth in tree species which are striving in stressed conditions.

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Introduction

Plant growth promoting rhizobacteria (PGPR) are the rhizosphere bacteria that can enhance plant growth by a wide variety of mechanisms like phosphate solubilization, siderophore production, biological nitrogen fixation, rhizosphere engineering, production of 1-aminocyclopropane-1 carboxylate deaminase (ACC), quorum sensing (QS), signal interference and inhibition of biofilm formation, phytohormone production, exhibiting antifungal activity, production of volatile organic compounds (VOCs), induction of systematic resistance, promoting beneficial plant-microbe symbiosis, interference with pathogen toxin production etc. (Bhattacharyaya and Jha, 2010). PGPR may influence growth, yield as well as supply of nutrients through different mechanisms. In leguminous crops, ability of nitrogen fixing can be enhanced by these. These bacteria can enhance different nutrient levels in soil, viz a viz. sulfur, copper, phosphorus and iron. These bacteria have the ability in the production of Phyto-hormones, develop other beneficial bacteria and provide protection from bacterial and fungal diseases (Saharan, 2011).

PGPR can be defined as the indispensable part of the rhizosphere biota that when grown in association with the host plants can stimulate the growth of the host. PGPR seemed as successful rhizobacteria in getting established in soil ecosystem due to their high adaptability in a wide variety of environments, faster growth rate and biochemical verstality to metabolize a wide range of natural and xenobiotic compounds.

The leguminous species *Albizzialebbek, A. procera, Acacia modesta, A. nilotica* strive naturally in the sub-tropical sub humid climate of the Pothwar plateau in Pakistan. Here these species shows stunted growth due to climatic factors as well as low fertility level of the soil.

Albizzialebbek

Albizialebbek is a deciduous, legume tree with perennial growth. It is a medium-sized tree. It reaches 3-15 m in plantations and up to 30 m in the open. Its dense, shade-producing crown can be as large as 30 m in diameter. Leaves are bipinnate with 3-11 pairs of bright green. Inflorescences are globular clusters of 15-40 white fragrant flowers. (Orwa *et al.*, 2009; Lowry *et al.*, 1992). The fruits are 10-30 cm long x 3-6 cm broad, reddish-brown pods that contain 5-15 flat rounded, free moving seeds. This tree needs a summer precipitation zone with annual rainfall of about 400-1000 mm. It favorssub-humid, slightly cold a, warm, subtropical and tropical climate with a temperature ranged from 4 to 40^{o}C.

Albizziaprocera

This tree species inherent in central as well as southern India, Burma and Bangladesh. It is planted in Punjab and KPK provinces of Pakistan. It is a deciduous, fast growing, having tree height of 12 to 30m. Diameters up to 1m have been observed. It performs well in low lying, humid savannas, whereas tolerates saline and sodic environments(Sheikh, M.I. 1993).

Acacia nilotica

Acacia nilotica is a medium sized, thorny, nearlyevergreentreethatcan reach up to 20-25 m height, but, on the other hand, may have shrubby growth inpoor growing conditions. It also influences on the environment through soil reclamation, soil enrichment, protection against fire and wind, and as a haven for biodiversity and ornament. However, it is considered a weed in some areas including Australia, the Galapagos Islands, and the Pacific islands.*Acacianilotica* is a useful fodder source, and sometimes a very important one, particularly in dry regions (Sheikh, M.I. 1993).

Acacia modesta

Pakistan, Afghanistan and India have naturally grown stands of production *Acacia modesta*. It exists below 1200 m in the Himalayan foothills in Pakistan. (Fagg *et al.*, 2005; Orwa *et al.*, 2009). These tree species are also found in Salt Range, Himalayas, Kirthar Range, Sulaiman Hills and Balochistan. It is also found in the plains. It is a very good forage and fodder for animals.

There is a habit of *Acacia modesta* to shed its leaves in winter.

These tree species are striving under the physiologically stressed conditions (low rainfall) in the semi-arid/sub humid forest ecosystem of Pakistan and resulting in stunted growth. It was hypothesized that by application of PGPRs the problem of stunted growth can be solved. Therefore this study was designed as novel approach with following objectives a) To isolaterhizobial strains from tree rhizospheric soil and nodules of native tree species b) To characterize the bacteria using molecular techniques c) To evaluate the Phosphorus solubilization and proline contents by isolated strains to determine their potential in enhancing growth.

Materials and mehtod

Study area

The study was carried out in Pothwar-plateau of the semi- arid/sub humid forest ecosystem of Pakistan that lies at 32° 10' to 34° 9' N and 71° 10' to 73° 55' E comprising an area of more than 1mha. The landscape is undulating. The rainfall ranges from 500 mm to 1000 mm per annum. During the summer months July and August (Monsoon) more than 70% of annual rainfall is received. The parent material of the soils is less, alluvial and colorful in nature. The soils are generally calcareous and alkaline in nature, having high pH and low organic matter (PARC, 1980).

Selection of sampling site and Collection of Forest Trees Nodules (Legumes) and Soil samples

Four legumes trees including; *Acacia modesta*(Linn.)Wall, *Acacia nilotica*(Linn) Delile, *Albizzialebbek* (L.)Benth and *Albizziaprocera* (Roxb) Benth nodules and rhizospheric soil was collected from four sites Pothwar Plateau. One sample from each district; Attock, Jhelum, Chakwal and Rawalpindi was collected. The tree species selected for nodules were in the sapling stage of development. The soil samples around rhizospheric soil were taken from 30 cm depth.

Isolation of bacteria from nodules

Roots of plants having nodules were removed from the root surface having cut on root 0.5 cm on each side of the nodule. The selected nodules were, pink colored and nodules were surface sterilized with soaking 95% ethanol for 1-2 min. followed by shaking in 10% chlorox for 2-3 min. subsequently nodules were washed successively 3-4 times in autoclaved distilled water. The sterilized nodules were grinded in autoclaved distilled water and the suspension was filtered/ centrifuged and the supernatant was spreaded on YEM medium and (Somasegaran*et al.*, 1984) picked with disinfected loop and the colonies were splashed on the medium plate for purification (Vincent, 1970).

Identification of bacteria by partial sequencing of 16SrRNA gene

The isolated strains were identified by nearly complete 16S rRNA gene sequences of the strains were obtained as described by Katsivels*et al.*, (1999) using universal forward and reverse primers: 9F(5-GAGTTTGATCCTGGCTCAG-3) and 1510R (5-GGCTACCTTGTTACGA-3). Reaction mixture prepared for full length will be initially denatured at 94°C for 2 minutes. followed by 30 cycles. Primer annealing was done at 55°C for 60 sec and primer extension at 72 °C for 10 min. in a thermo cycler (Ahmed *et al.*, 2007).

Sequence was carried out from Macrogen Korea.

P- Solubilization Index (SI)

The bacterial colonies containing tricalcium phosphate were inoculated in Pikovskaya's broth media (Pikovskaya, 1948) under sterile conditions and incubated at 28°C for 7, 13 and 18 days. The Solubilization index (SI) was calculated from the formula. (Premono*et al.*, 1996).

SI is measured from the formula;

Solubilization Index= Colony diameter + Halozone diameter / Colony diameter.

Inorganic Phosphorous Quantification

The p-solubilized by bacterial strains was quantified using Phosphorus molybdate blue color method adapted by Murphy and Riley (1962). For analysis the flask containing 100 ml autoclaved Pikovskaya's broth (pH adjusted to 7.00) supplemented with sucrose and tri-calcium phosphate as a source of energy and inorganic phosphate (0.3g/100 ml) was inoculated with 4-5 loops of p-solubilizing bacterial strain and kept on a rotary shaker for 12 days at 28 °C at a speed of 150 rpm. The culture suspension was thereafter centrifuged at 10,000 rpm for 15 min. and the supernatant was collected, filtered and pH was measured. The solubilized-p (P) by the strains was quantified at 882nm on the spectrophotometer and compared with standard solutions of P calibrated using standard phosphate curve.S.I is calculated as under:

E = ∑A/DC = 5.36402/12 = 0.447

Where No. of Standard absorbance (DC) = 12; Sum of Standard Absorbance (A) = 5.36402

Phosphorus Solubilization= C = A/Ed

It can be attain by dividing absorbance of your strain by 0.447; A = Absorbance of your strain; E = 0.447 (d = diameter of cuvit (which is usually 1).

Proline Contents of PGPRs

Proline contents were determined using method of Bates *et al*., 1973.

Results and discussion

Strains identified from nodules

Following strains were identified in the nodules of the native tree species (Table 1).

Table 1. Bacterial strains isolated from nodules.

Sr. No.	Strain ID	Forest Tree	Location
1	AM-1	*Acacia modesta*	33.0134° N, 73.3547° E
2	AM-2	*A.modesta*	33.7687° N, 72.3621° E
3	AM-3	*A.modesta*	33.8195° N, 72.6890° E
4	AL-1	*Albizzialebbek*	3.5984° N, 73.0441° E
5	AN-1	*Acacia nilotica*	3.5984° N, 73.0441° E
6	AP-1	*Albizziaprocera*	3.5984° N, 73.0441° E

Isolation and purification of bacteria from root Nodules

Isolation of PGPRs from selected tree species nodules and rhizospheric soil revealed following strains in all the native tree species (Table 2).

Table 2. Bacterial strains isolated from nodules.

Strain ID	Bacteria	Forest Tree	Location
1	*Bacillus stratosphericus*	*Acacia modesta*	33.0134° N, 73.3547° E
2	*Bacillus stratosphericus*	*Acacia modesta*	33.7687° N, 72.3621° E
12	*Bacillus stratosphericus*	*Acacia modesta*	33.8195° N, 72.6890° E
16	*Achromobacter xylosoxidans*	*Albizzialebbek*	3.5984° N, 73.0441° E
21	*Bacillus aerius*	*Acacia nilotica*	3.5984° N, 73.0441° E
25	*Achromobacter muciolens*	*Albizziaprocera*	3.5984° N, 73.0441° E

Phosphorus Solubilization (SI)

Maximum SI 7 was observed in *Achromobacterxylosoxidans*followed by *Bacillus aerius* 5.5 and *Bacillus stratosphericus* 3.6 (Table 3). The minimum SI was observed in strain *Achromobacter muciolens* 3.42.The maximum phosphorus solubilized index 5.43 was observed by *Achromobacter muciolens* (Fig. 1) and it differs significantly as compared to other three strains was followed by *Achromobacter muciolens, Bacillus aerius*and *Bacillus stratosphericus*1.63 and these three strains differ non significantly among each other.

Table 3. Phosphorus solubilization by bacterial strains.

Sr. No.	Strains	Results	Colony diameter (mM)	Halozone diameter (mM)	SI Index
1	*Achromobacter xylosoxidans*	+ve	5	30	7
2	*Achromobacter muciolens*	+ve	7	17	3.42
3	*Bacillus stratosphericus*	+ve	8	36	3.6
4	*Bacillus aerius*	+ve	8	36	5.5

Past studies pointed out thatPhosphorus is the major macronutrient for biological growth and development. Strains from bacterial genera Pseudomonas, Bacillus, Rhizobium, Achromobacter and Enterobacter along with Penicillium and Aspergillus fungi are the most powerful P solubilizers (Whitelaw, 2000). The is also indication of phosphorus solubilisation by Bacillus and Achromobacter species(Widada *et al.*, 2007).

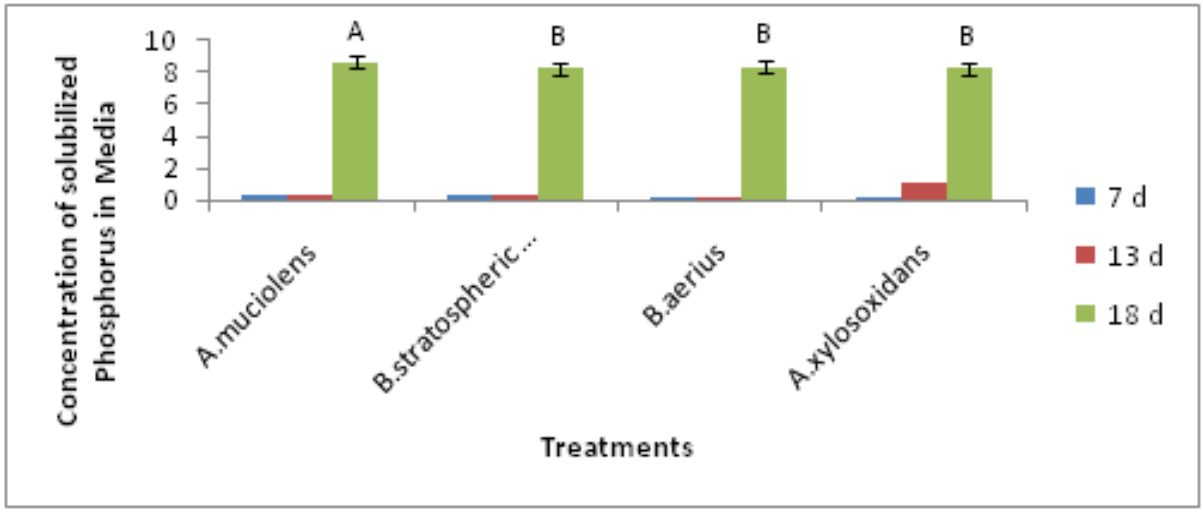

Fig. 1. Phosphate solubilization index (SI) by PGPR strains at 7th, 13th and 18thday.

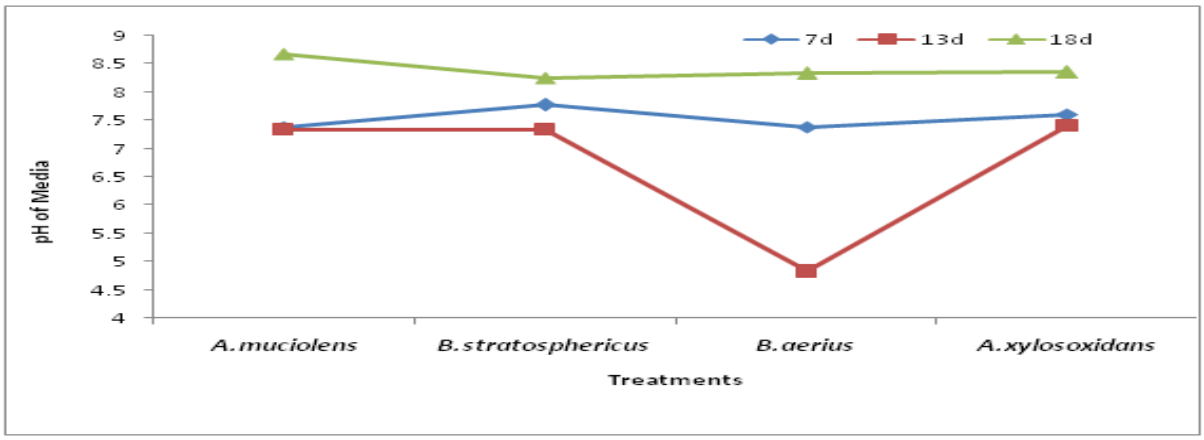

Fig. 2. The pH of PGPR strains at 7th, 13th and 18th day.

Inorganic Phosphorous Quantification

Maximumconcentration of solubilized phosphorus in media was by *Achromobacter xylosoxidans*5.1% (Fig 1-3) and it differs significantly as compared to other strains followed by *Achromobacter muciolens, Bacillus aerius*and *Bacillus stratosphericus*1.09% and these three strains differ non significantly among each other.

Proline Contents of PGPRs (ug/g)

Maximum proline produced by strain *Achromobacter xylosoxidans* 99.95% and *Bacillus stratosphericus* 99.95% (Fig. 4) followed by *Bacillus aerius*99.91 % and *Achromobacter muciolens* 99.90% as compared to control. Toluene was used as blank reading having OD 0.141. There was no significant difference between *Achromobacter xylosoxidans* and *Bacillus stratosphericus*. No significant difference observed between *Achromobacter muciolens* and *Bacillus aerius*.Proline is the enzyme that works during water stress conditions and it is conclueded from results that *Achromobacter xylosoxidans*and *Bacillusstratosphericus* has the maximum ability to work under water stress conditions (Bates *et al.*, 1973).

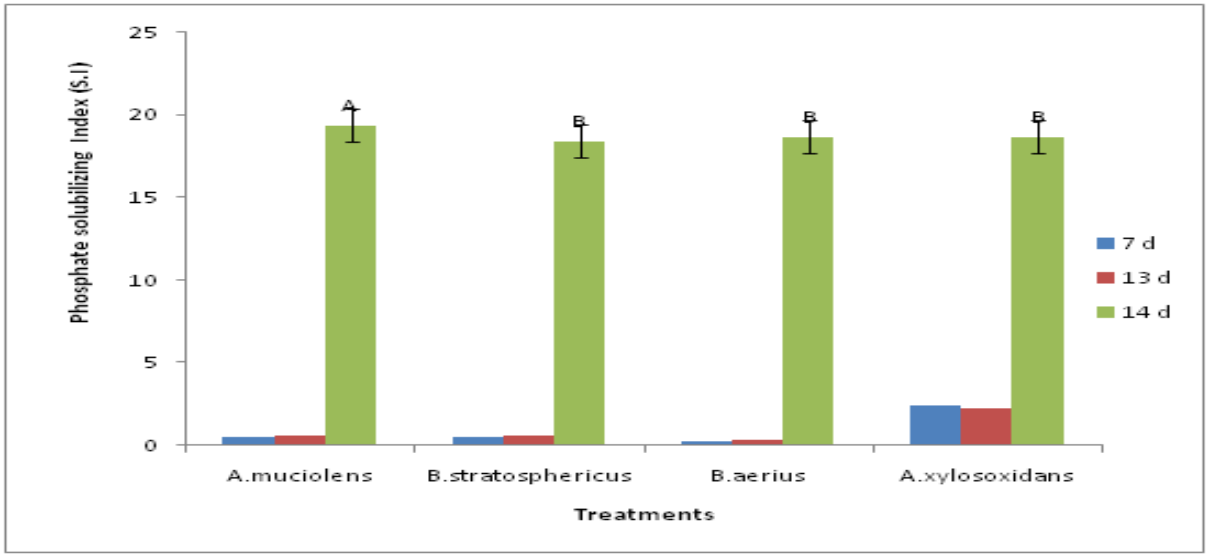

Fig. 3. Phosphorous solubilized (µg/ml) by PGPR strains at 7th, 13th and 18th day.

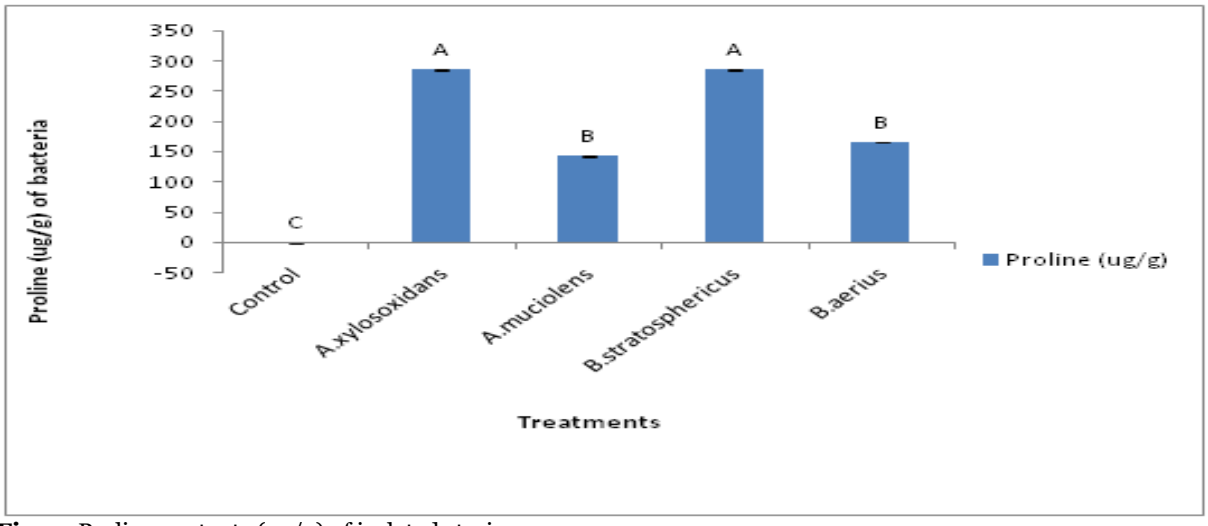

Fig. 4. Proline contents (ug/g) of isolated strains.

Molecular Identification or Characterization (16SrRNA)

Identification of strain AL isolated from nodules of *Albizzialebbek* and AP isolated from nodules of *Albizziaprocera,* AM strain isolated from nodules of *Acacia modesta*andAN strain isolated from nodules of *Acacia niloticaw*as carried out by 16S-rRNA sequence analysis. *Albizzialebbek*base spacing: 15.579729 has991 bases in 12041 scans. The comparison of the sequence showed 99% similarity

with the sequence of *Achromobacter xylosoxidans .Albizziaprocera*base spacing: 15.661685 having 997 bases in 12165 scans. Similarity with *Achromobacter muciolens.Acacia modesta*base spacing: 15.765433 having 798 bases in 9920 scans (The comparison of the sequence showed 99% similarity with the sequence of *Bacillus stratosphericus ..Acacianilotiica*base spacing: 15.513764 having 997 bases in 12150 scan. The comparison of the sequence showed 99% similarity with the sequence of *Bacillus aerius. The Phylogenetic tree of the strain isolated from Albizzialebbek , A.procera and Acacia modesta and A. nilotica has been presented in* (Figure 5 and 6) respectively.

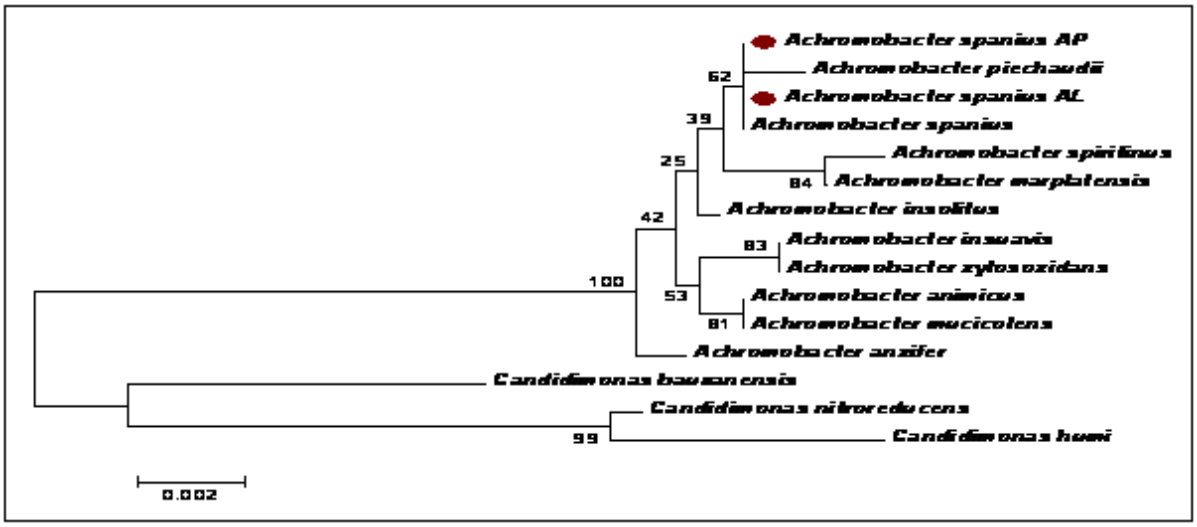

Fig. 5. Phylogenetic tree of the strain isolated from *Albizzialebbek* and *Albizziaprocera*.

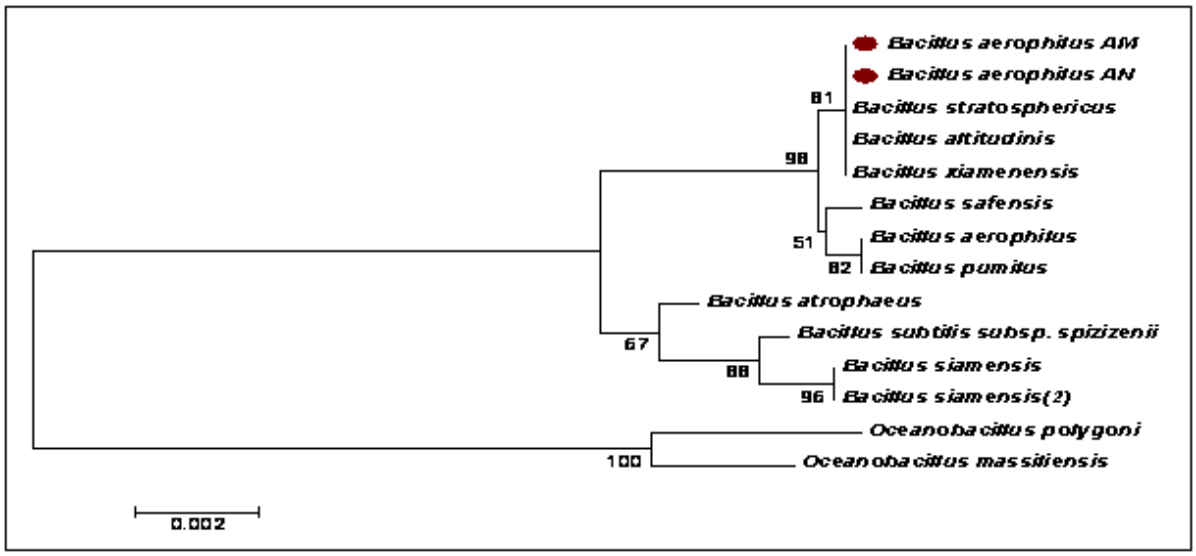

Fig. 6. Phylogenetic tree of the strain isolated from *Acacia modesta* and *Acacia nilotica*.

Conclusion

The study revealed that soil phosphorus is precipitated as orthophosphate and available to bacteria through their organic acid production and acid phosphatise secretion. Proline is the enzyme that works in water stress conditions thus the sustainable approach for managing nutrient deficiency and stress conditions in native trees.

The study highlights the novel techniques in enhancing the growth of tree species under stressed conditions.

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