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Isolation, characterization and identification of endophytic bacteria by 16S rRNA partial sequencing technique from roots and leaves of *Prosopis cineraria* plant

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ABSTRACT

Plant roots are potential hosts to a plethora of beneficial microorganisms including mycorrhizal fungi, rhizobia bacteria, antagonistic yeasts and endophytic fungi. Endophytic bacteria live symbiotically with the plant and in turn helping the plant in number of ways. The present investigations were undertaken to isolate and identify bacterial endophytes in root and leaf tissue of Prosopis cineraria plant growing at Pune, India. A total of two endophytic bacteria were isolated from the parts of the plant .On the basis of the morphological and biochemical characterization of the endophytes as well as 16S rRNA sequencing technique they are identified as Bacillus subtilis and Stenotrophomonas maltophilia. Both the isolates Bacillus subtilis & Stenotrophomonas maltophilia were present in root tissue while only one i.e. Stenotrophomonas maltophilia isolated from leaf tissue. Studies on the optimization of growth of the isolates were performed by varying pH & temperature conditions. The ability of bacterial isolates were tested for different enzyme activity and also screened for sensitivity of different antibiotics.

Keywords: *Bacillus subtilis, Stenotrophomonas maltophilia, Prosopis cineraria,* 16S rRNA partial sequencing technique, NCBI Gene Bank.

INTRODUCTION

Endophytes are group of microorganisms having the ability to enter inside the plant hosts colonizing the intercellular spaces and also the xylem vessels. They exist in a range of tissues types within a broad range of plants, colonizing the plant systemically with bacterial/fungal colonies and biofilms [10].They are ubiquitous, colonize most of the plants, and have been isolated from almost all plants examined till date. Endophytes are to live symbiotically within the plants. While growing inside the plant the endophytes shows no visible symptoms of infection and disease(Bacon and White, 2000). The close association of endophytes with internal tissues of host plant has increasingly gained them scientific and and commercial interest due to their potential to improve plant quality and growth (Carroll, 2001: Schulz et al 2000).They exhibit complex interactions with their hosts which involves mutualism and antagonism. Their association can be obligate or facultative. Plants strictly limit the growth of endophytes, and these endophytes use many mechanisms to gradually adapt to their living environments. In order to maintain stable symbiosis, endophytes produce several compounds that promote growth of plants and help them adapt better to the environment [17].Some of the endophytes are known to protect their host from being attacked by fungi, insect and mammals by producing secondary metabolites (Zhang, 2007). Among them, endophytic bacteria are thought to interact closely with their host plants, and therefore could be used as biological control agents in sustainable crop production potentially (Sturz and Nowak, 2000; Taechowisan et al., 2003; Zhang et al., 2008).

Endophytes are known to supply nutrients to plant by fixing atmospheric nitrogen and solubilizing ion (Marx 2004; Porras- soriaano et al 2009). This ultimately leads to increase in plant immune system as well as protects plant from infection by plant pathogens .studies have also shown role of endophytes in removal of soil contaminants (Barac et. al 2004; Doty et al 2009). Studies on endophytes their significance and role in plant metabolism is an important area to explore. Existing literature gives data on colonization pattern as well as their effect on plant growth.

Endophytic population is known to vary from plant to plant and also from species to species. Same species of plant may also show different endophytic population occurring at different regions. Hence temporal and climatic changes affect occurrence of entophytes (Nair & Padmavathy, 2014)

Prosopis cineraria (L) are a species of flowering tree of the Indian desert belonging to family Fabaceae. It is a deep rooted, nitrogen fixing, multipurpose tree endemic to the hot deserts of India [1].Almost all parts of this tree have lot of medicinal value. It has anti -inflammatory, anticonvulsant, antifungal, anticancer, antidiabetic, hypolipidemic, abortifacient, antioxidant, antimicrobial and wound healing properties. This is a preferred tree for agro-forestry and is a popular renewable source of fuel, fodder, timber and vegetables. The pods of this plant are also known to contain steroids, alkaloids, flavaons etc. The whole plant is used in indigenous system of medicine as a remedy for many diseases (Khandelwal et. al 2015). The data about the presence of endophyte in this plant is very less.

The present studies was therefore undertaken to investigate and identify endophytic bacteria present in the tissue of roots and leaves of *Prosopis cineraria* plant. The technique of 16s rRNA partial sequencing is also used for identification of the bacterial species. Studies were also performed on biochemical characterization of the endophytes and their ability to interact with different antibiotics.

MATERIALS AND METHODS

A. Collection of the sample:
Two months old plant of *Prosopis cineraria* were procured from a plant nursery near Aundh, Pune, and Maharashtra in the month of January. These plant were maintained under controlled environmental conditions in well insulated plastic pots in the green house of Department of Biotechnology, Modern College of Arts, Science & Commerce, Ganeshnkhind, Pune Maharashtra, India.

B.Isolation of endophytic bacteria from *Prosopis cineraria* roots and leave tissue
i. Surface sterilization
The root and leave sample of the *Prosopis* plant were taken and processed separately. Healthy and undamaged leave and root were collected from the plant grown under controlled environmental conditions. These were than washed under running water and dried followed by surface sterilization. Surface sterilization was done first washing in 1% savlon for 5 minutes. Subsequently these leaves and pieces of roots were treated with 0.1 % mercuric chloride for surface sterilization. Two sets of experiment with different timing for the sterilization were prepared to standardize the surface sterilization efficiency.

ii. Isolation of endophytic Bacteria
The samples of leaves and roots were cut into small pieces and macerated separately in phosphate buffer of pH 7.2 with a sterile pestle and mortar. Tissue extract were then prepared for tenfold dilution in sterile saline. Serial dilutions (10^{-5}, 10^{-6}, and 10^{-7})were prepared from this extract. For inoculations 0.1ml of the aliquot was used on Nutrient Agar medium. The inoculations were done in triplicates separately for both roots and leaf tissue extract. These plates were then incubated at 37^0 C. Observations were taken after 48 to 72 hrs .Bacterial colonies were differentiated on the basis of morphological colony characters. Bacterial isolates were picked from plates and purified by streaking techniques and incubated at 37^0C. The isolation process repeated till monocultures were obtained for further experimentations .The media used for isolation was Yeast Manitol Agar, Ashbeys and Picovasky's medium.

C. Characterization of endophytic bacteria
i) Morphological Characterization
a) Gram Staining Tests:
Gram staining was carried out by following standard staining procedures and techniques.

The microscopic examination was performed with the oil immersion objective of the bright field microscope.

b) Motility Test:
A loopful of the bacterial culture taken for microscopic examination of motility of the bacteria isolated for identification and characterization [10]. Slides were prepared by standard techniques.

ii) Physiological Characterization
a) Effect of pH on growth of isolates:
Optimization and standardization of growth of the isolates on range of pH was studied by inoculating the isolates on nutrient broth media (Hi-media) having pH range from 1 to 14. These were then incubated at 37 ^{0}C for 48 hours [2].The growth was determined by taking optical density at 520 nm.

b) Effect of temperature on growth of isolates:
Effect of temperature on growth of isolates was determined by inoculating the isolates in nutrient broth and incubated at different temperature that is at 4 ^{0}C, 28 ^{0}C, 37^{0}C, 50 ^{0}C for 48 hours[10]Optical density at 520 nm was measured.

iii) Biochemical Characterization
a)Catalase Test:
Catalase activity of the isolates was estimated by using H_2O_2 solution onto the microscopic slides containing the culture of the isolates separately. Observations were taken for immediate bubble formation (O_2 + water = bubbles).Release of O_2 bubbles indicated positive catalase activity [10]

b) Amylolytic activity:
Amylolytic activity was observed after inoculating the isolates in nutrient agar with 1% starch of pH 6.06. Culture plates were treated with iodine after incubation period. Clear zone was observed around colonies confirming the activity [10].

c) Urease activity
Isolates were grown on the medium containing urea agar. After incubation slant were observed for change in its colour from redish pink indicating positive urease activity

d)Antibiotic Sensitivity test
The isolates to be screened for their sensitivity for antibiotics were cultured on to the nutrient agar plate. Antibiotic Kanamycin of varying concentration (5µg/ml, 50µg/ml, and 100µg/ml) used for treating the culture by using well technique. The protocol was repeated for Ampicillin and Amoxycillin antibiotic with concentration – 5µg/ml, 50µg/ml, 100µg/ml on separate plates. Cultures inoculated for 24 hrs at 37^{0}C. The growth of the bacterial colonies was observed and zone of inhibition measured.

Observations and Results -
Two bacterial colonies were isolated from roots and one is isolated from leaf tissue extract. Two bacterial colonies isolate II& III were found to be of same species & were common in both root and leaf tissue.

Table 1: Morphological characterization of endophytes

Colony characters	Isolate I / root	Isolate II/ root	Isolate III/ leaf
Size	4mm	3mm	3.7mm
Shape	Circular	Circular	Circular
Colour	Orange	Yellowish	Yellowish
Margine	Regular	Regular	Regular
Opacity	Opaque	Opaque	Opaque
Elevation	Flat	Flat	Flat
Consistency	Smooth	Smooth	Smooth
Gram characters	Gram positive	Gram negative	Gram negative
Motility	Motile	Motile	Motile

Table 2: Screening of the isolates for enzyme activity

Organism	*Amylolytic activity*	*Urease activity*	*Catalase activity*
Bacillus subtilis	+	+	+
Stenotrophomonasmaltophilia	-	-	+

+ = Positive , - = Negative.

Table 3: Anti biotic sensitivity test

Name of Organism	Kenamycin µg/ml			Ampicillin µg/ml			Amoxycililn µg/ml		
	5	50	100	5	50	100	5	50	100
Stenotrophomonasmaltophilia	-	-	-	1mm	5mm diameter	8mm diameter	-	-	-
Bacillus subtilis	-	-	-	-	-	-	-	-	-

- = No Zone.

Sequencing and analyses of DNA

The identification of the endophyte isolate strain 1 and 2 by the 16S rRNA gene partial sequencing were performed at Genombio Technologies Pvt. Ltd., Pune, India using the universal primers. The partial16S rRNA gene of selective isolate of each tissue extract was sequenced and presented in FASTA format. Finally16S rRNA sequence of the endophyte isolate was compared with that of other bacterial sequence by way of BLAST (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). The result was compared with the sequence of GenBank based on partial 16S rRNA sequence to check the relationship and similarity with the endophytic isolate.

The sequence of strain 1 was found to be of 737 base pairs as follows:

GACGAACGCTGGCGGCGTGCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCTGATGTTA
GCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGG
GCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACA
GATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGA
CCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAG
GGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCG
TAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCA
GAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATT
ATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAG
GGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAAT
GCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGA

The sequence of strain 2 was found to be of 617 base pairs as follows:

GTCGTGGGGGATAACGTAGGGAAACTTACGCTAATACCGCATACGACTACGGGTGAAAGCAGGGGAT
CTTCGGACCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAA
GGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTC
CTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATACCGCGTGGG
TGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCTGGCTAATACCTGGTTG
GGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGT
GCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTCGTTTAAGTCCGTTGTGAAAGC
CCTGGGCTCAACCTGGGAACTGCAGTGGATACTGGGCGACTAGAGTGTGGTAGAGGGTAGCGGAATT
CCTGGTGTAGCAGTGAAATGCGTAGAGATCAGGAGGAACATCCATGGCGAAGGCAGCTACCTGGACC
AACACTGACAC

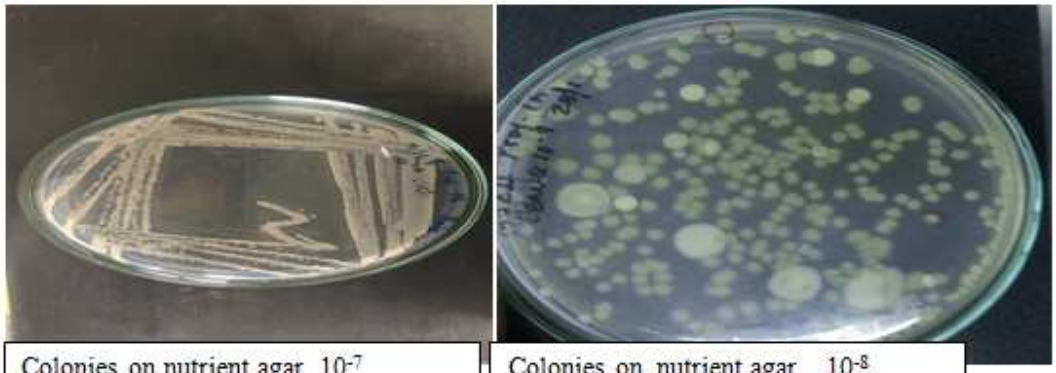


Fig 1: **Endophytes from roots and leaf tissue of *Prosopis cineraria on nutrient Agar medium***

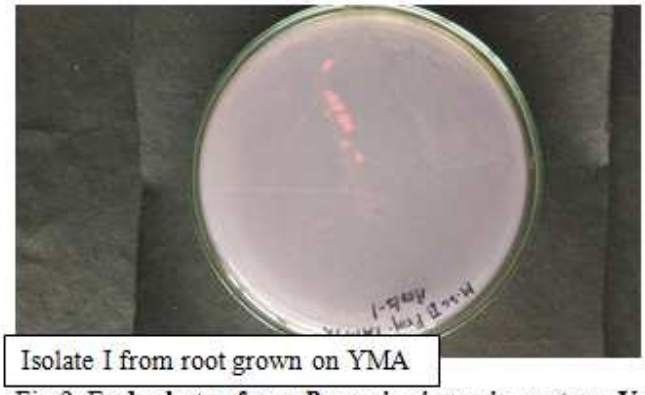


Fig 2: **Endophytes from *Prosopis cineraria* roots onYeast Mannitol Agar (YMA)**

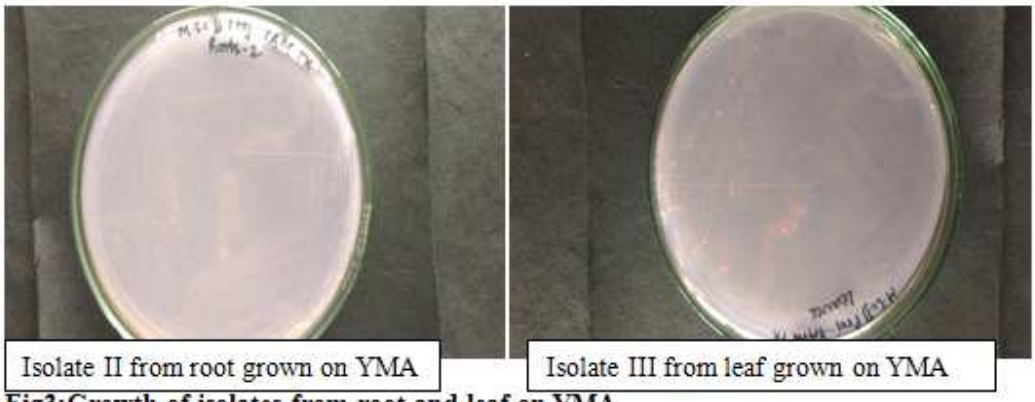


Fig3:Growth of isolates from root and leaf on YMA

IsolateI from rooton picovasky's medium

Fig 4: Growth **of endophytes from *Prosopis cineraria on Picovasky's medium***

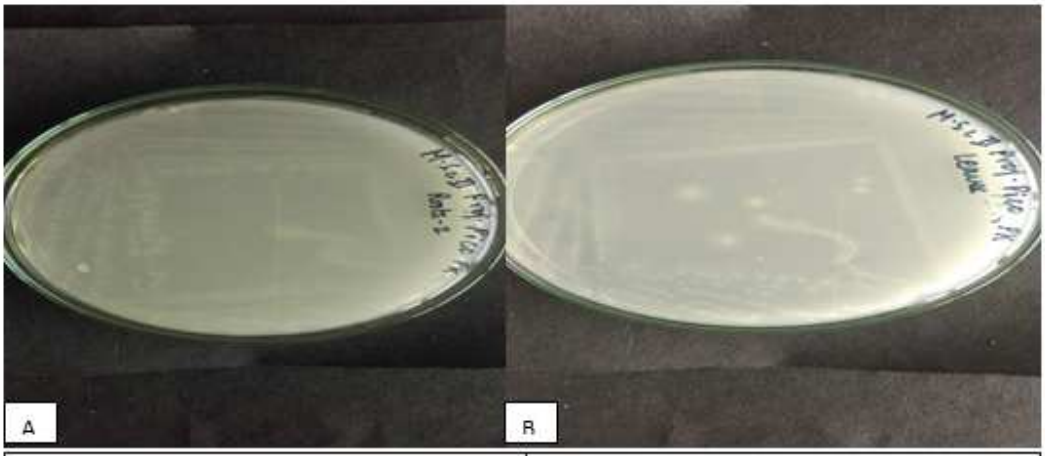
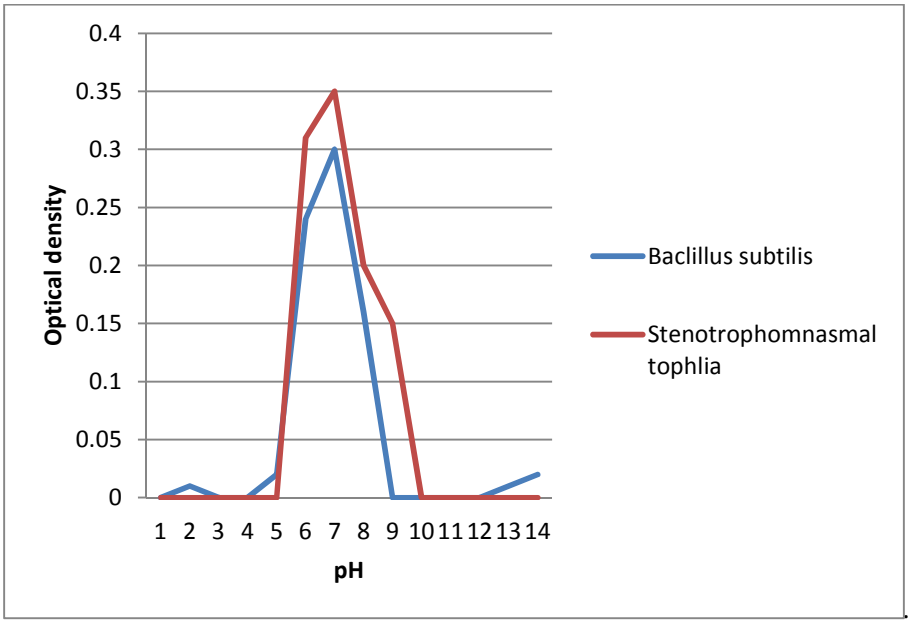
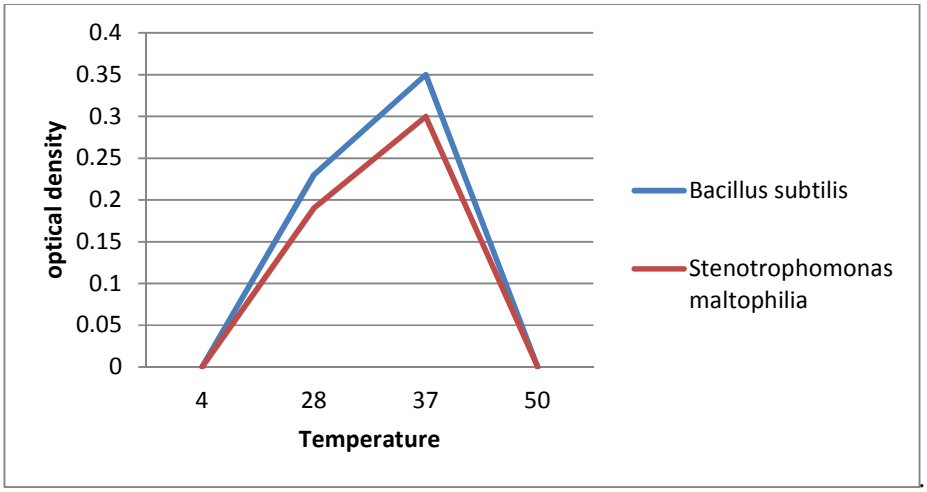


Fig 5: A. Isolate 2 of root on Picovasky medium | B. Isolate 2 of leaves on Picovasky medium

Graph 1. Effect of pH on growth of isolates
Media: Nutrient Broth incubation: temp37 ^{0}C time: 2 Days



Graph 2.Effect of temperature on growth of isolates
Media: Nutrient Broth incubation: temp37 ^{0}C time: 2 Days



RESULTS AND DISCUSSION

Prosopis cineraria Linn is used in traditional medicines since many years. However, new discoveries in this plant research leads to development and opening of many more areas to explore. The plant is now gaining importance to develop some more new search for the future development by understanding the gene level study. Therefore, considering its versatile medicinal uses, there is an ample scope for future research on *Prosopis cineraria* and hence further pharmacological investigations are warranted. Endophytic bacteria are reported to enhance the growth of plant and may also increase the supply of nutrients to plants in turn helping in building the defense mechanism of the plant.

The present investigations were undertaken to find out the presence of bacterial endophytes in root and leaf tissue of this plant. By using nutrient agar and selective medium (YMA and Picovasky'smedium) two endophytes were isolated from roots and one from leaves tissue of the plant. The characterization of these three isolates showed that one isolate from root was similar to the isolate from the leave tissue of the plant .Hence ultimately two endophyte were present in root and leaf tissue of the *Prosopis* plant and further characterization and sequencing were performed in these two isolates. Endophyte Isolate 1 & Isolate 2 were not able to grow on Ashbey's medium.

On the basis of the morphological and biochemical characterization of the endophytes as well as 16S rRNA sequencing technique they are identified as *Bacillus subtilis* and *Stenotrophomonas maltophilia.* The isolate I shows 99% identity to *Bacillus subtilis* strain UD 1022, complete genome. The isolate 2 shows 100% identity to *Stenotrophomonas maltophilia*s train psk2.

The endophyte *Stenotrophomonas maltophilia* was isolated from both roots and leaf tissue whereas *Bacillus subtilis* was present only in roots and absent in leaf tissue. This is the first report on the isolation and description of endophytic bacteria from roots and leaves of *Prosopis cineraria* plant found in Pune, Maharashtra.

The sequences are submitted to NCBI GENE BANK and they are under process. Submission Id of these sequences is 1806120 and 1806135 respectively.

During present studies the endophytic bacteria *Bacillus subtilis* isolated from roots showed high amylolytic, catalase and urease activity though only catalase activity observed in Stenotrophomonas *maltophilia*(Table2). Sensitivity of three different antibiotics at varying concentration was also tested for these two endophytes. *Bacillus subtilis* was observed to be resistant to all three antibiotics kanamycin, ampicillin &amoxicillinas no zone of inhibition was observed whereas *Stenotrophomonas maltophilia* showed sensitivity to ampicillin at higher concentration (Table3).

The optimum growth of both *Bacillu ssubtilis* and *Stenotrophomonas maltophilia* was observed to be at pH 7 and temperature 37^0C (Graph1&2). During present studies roots and leaf tissue were selected for isolation of endophytes and it was found that the population density and type of the endophyte was more in root than in leaves.

To conclude, two bacterial endophytes are isolated from roots and leaf tissue of *Prosopis cineraria* plant. Both the isolates *Bacillus subtilis* & *Stenotrophomonas maltophilia* were present in root tissue while only one i.e. *Stenotrophomonas maltophilia* from leaf tissue. Further research work is required to explore more about the role of these bacterial endophytes in the metabolism of the plant.

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REFERENCES

[1] KhandelwalPreeti , Sharma Ram Avatar , Agarwal Mala; *Journal of Plant Sciences* (**2015)** ; 3(1-1): 33-39
[2] Dhanya N. Nair and S. Padmavathy; Impact of Endophytic Microorganisms on Plants, Environment and Humans Hindawi Publishing Corporation Scientific World Journal, Article ID 250693, 11 pages Volume (**2014)**
[3] Rupert AnandYumlembam And S.G. Borkar ; *Phytopath*. 67 (4) : 353-357 (**2014**)
[4] Jema Haji and Abdu Mohammed *Ethiopia African Journal of Agricultural Research* Vol. 8(9), pp. 768-779, 18 March, (**2013)**
[5] Masroor Qadri, Sarojini Johri, Bhahwal A Shah, Anamika Khajuria, Tabasum Sidiq, Surrinder K LattooMalik Z Abdin and Syed Riyaz-Ul-Hassan; *SpringerOpen Journal* 2:8 (**2013)**
[6] M. S. Ambawade1, G.R. Pathade *International Journal of Science and Research* 6.14 (**2013)**
[7] Garcia A., Rhoden S.A., Bernardi-Wenzel J., Orlandelli R. C., Azevedo J.L., Pamphile J.A. *Journal of Applied Pharmaceutical Science* Vol. 2 (10), pp. 035-040, October, (**2012)**
[8] Hui Li, Xiaoxian Wang, Meizhe Han, Zhenzhen Zhao, Minqian Wang, Qin Tang, Changhong Liu, Brian Kemp, Yucheng Gu, Jinglei Shuang and YarongXue; *African Journal of Biotechnology* Vol. 11(1), pp. 231-242, 3 January, (**2012)**
[9] Zareen Khan, Grant Guelich, Ha Phan, Regina Redman, and Sharon Doty; *International Scholarly Research NetworkISRN Agronomy*, Volume (**2012)**
[10] Ruby E Jalgaonwala; *International Journal Of Pharma And Bio Sciences* ;Volume 2/Issue1/ Jan March (**2011)**
[11] R.E. Jalgaonwala and R.T. Mahajan; *Journal of Agricultural Technology* Vol. 7(6): 1733-1741 (**2011)**
[12] P.Chaudhry *Journal of Biodiversity and Ecological Sciences No.1, Issue4 Fall (***2011)**.
[13] Irene De Araújo Barros; Welingtonluiz Araújo; Joãolúcio Azevedo; *Brazilian Journal Of Microbiology* Issn 1517-8382 41: 956-965 (**2010)**
[14] SonaimuthuVisalakchi And JohnpaulMuthumary *International Journal Of Pharma And Bio Sciences* Vol.1/Issue-3/Jul-Sep**.(2010)**
[15] R E Jalgaonwala, B V Mohite and R T Mahajan *International Journal on Pharmaceutical and Biomedical Research (IJPBR)*Vol. 1(5), 136-141,(**2010**)
[16] Natalia Malfanova, Faina Kamilova, Shamil Validov,Andrey Shcherbakov, Vladimir Chebotar, Igor Tikhonovich and Ben Lugtenberg; *Microbial Biotechnology* (**2011)**4(4), 523–532
[17] Ulrich K., Ulrich A., and Ewald D; *FEMS Microbial Ecol*, 63:169-180, (**2008**).
[18] Arite Wolf, Antje Fritze, Martin Hagemann and Gabriele Berg; *International Journal of Systematic and Evolutionary Microbiology* (**2002**), 52, 1937–1944
[19] Ajit Varma, Savita Verma, Sudha, Nirmal Sahay, Britta Bu¨Tehorn, and Philipp Franken *Endophyte Applied And Environmental Microbiology,;*P. 2741–2744 June (**1999)**