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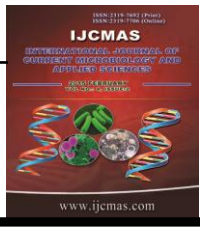

Original Research Article

Isolation and Identification of a novel Endophyte from a plant *Amaranthus spinosus*

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Keywords

Exiguobacterium profundum strain N4, *Amaranthus spinosus, Penincillum, Aspergillus, Cladosporium spps.*

A B S T R A C T

A total number of 536 bacterial and fungal endophytes were isolated from root, stem and leaves of the plant *Amaranthus spinosus*. Roots supported more number of bacterial endophytes than either stem or leaves whereas stem supported more number of fungal endophytes than either roots or leaves. The plant harbored more of gram negative compared to gram positive bacterial endophytes. The fungal endophytes isolated from root, stem and leaves of the plant *Amaranthus spinosus* revealed the presence of *Penincillum, Aspergillus Cladosporium, Phoma, Bipolaris* and *Fusarium* spps. All the isolated fungal endophytes belonged to the class hypomycetes. Dominant fungal endophyte was Cladosporium spp. which was found in all the plant parts studied. Roots of the plant possessed maximum nitrogen fixers followed by stem and leaves. A novel bacterial endophyte *Exiguobacterium profundum strain N4* was isolated from root of the selected plant. 62.75% of the bacterial endophytes isolated from the plant *Amaranthus spinosus* were able to fix nitrogen whereas 45.10% were able to soluibilize phosphorous. The phosphate solubilization efficiency was found to be highest for the fungal genera Aspergillus.

Introduction

An ever increasing human population has resulted in rapid industrialization and urbanization in India as well as all over the world. As a result the man is confronted with two major problems: constant depletion of natural resources and pollution of the environment. Textile dying and printing industry is among one of those industries which has greatly flourished in order to satisfy the demands of ever increasing human population. In order to make textiles more appealing, these industries make immense use of synthetic dyes.

Synthetic dyes constitute a class of xenobiotic compounds. These dyes, like xenobiotic compounds are capable of bioaccumulating and biomagnifications along the food chains. Although synthetic dyes have wide application in textile industry but a major portion of the dye applied is lost in waste water in the form of colored effluents. These untreated effluents are characterized by high salt content, COD, color and extreme pH (Tak et al. 2002). The effluents so released from textile dying and printing industries pose risk not only to the

aquatic life but also leads to contamination of the adjacent water bodies and soil.

In the present study Sanganer town was selected as the study area due to the concentration of large number of small and large scale textile dying and printing units. These industries make immense use of synthetic dyes. The effluents so released are highly colored visualizing the presence of these toxic dyes. The effluents from these industries are discharged untreated in nearby water bodies or even in adjoining soil due to poor law enforcement. Consequently, creating an environment which is unfavorable for the growth and development of flora and fauna. Therefore, the plants which are abundant in such unique environmental niches of Sanganer region must be possessing some novel endophytes for their survival under such harsh environmental conditions and thereby unusually out numbering rest of the plants in that area. Bacteria degrading recalcitrant compounds are more abundant among endophytic populations than in the rhizosphere of plants in contaminated sites (Siciliano et al. 2001; Rosenblueth and Martinez 2006) which could mean that endophytes have a role in metabolizing these substances. Hence, it is of utmost importance to study the kind of endophytes harbored by these plants.

During the present study, Amaranthus spinosus was found flourishing immensely in the contaminated area of Sanganer. Earlier scientists have reported the growing potential of the plants belonging to Amaranthaceae family at the sites contaminated with the toxic and hazardous pollutants. These plants have also been found to be good hyperaccumulators of heavy metals and thereby are ideal for phytoremidiation. There are a few literature available on *Amaranthus spinosus*, where scientists have reported their medicinal uses (Olajide et al. 2004; Hilou et al. 2006), for the presence of symbiotic fungi (Zhang et. al. 2011; Santos et al. 2013) and for phytoremediation purposes (Osu and Okoro, 2012; Chinmayee et al. 2012). Therefore, an attempt has been made in the present study for the isolation and identification of bacterial and fungal endophytes in the selected plant species that enable the selected plant to survive under harsh environmental conditions where the survival for the rest of the plant species becomes quite difficult.

Materials and Methods

Collection of plant samples

For the present study healthy fresh flowering plants of the selected plant species were carefully selected, removed and were collected in previously sterilized zip lock poly bags. The plants collected were identified with the help of published regional flora and by comparing the voucher specimens with identified herbarium collections in the herbarium of Department of Botany, University of Rajasthan, Jaipur. The identified plant was then deposited in the Botany Department of the University. The collected plant sample Amaranthus spinosus was allotted RUBL No. as 20592.

Isolation of Bacterial Endophytes

The bacterial endophytes were isolated from root, stem and leaves of the healthy flowering plant Amaranthus spinosus. The isolation was done from the plant immediately after collection. The plant samples were washed under running tap water for 10-15 mins. to remove adhering soil particles, air-dried and roots, stem and leaves were separated out. The separated plant roots, stem and leaves were weighed

up to one gram on a weighing balance. The weighed samples were soaked in distilled water and drained. The samples were then surface-sterilized by dipping in 70% ethanol for 1 minute, stem and leaves with 4 % sodium hypochlorite for 5 minutes and roots with 2% sodium hypochlorite for 10 minutes and then treated with 70% ethanol for 30 secs. followed by rinsing five times in sterilized distilled water. The surface sterilized samples were blot-dried using sterile filter paper. The samples were then macerated in one ml of distilled water in pestle and mortar. For each macerated sample, that is root, stem and leaves sample serial dilutions were made, up to 10^{-5} dilutions. One hundred micro liters from each dilution of the respective sample was then poured in their respective petri plates so labeled from 10^{-1} to 10^{-5} containing nutrient agar medium and potato dextrose agar medium separately and then spread with spreader for the isolation of the bacterial and fungal endophytes. The plating was done in triplicate for each dilution. The plates were then incubated at 37° C for 72 - 96 hours for isolation of bacterial endophytes whereas for the fungal endophytes plates were incubated at 28°C for two weeks. Sterility check was performed by imprinting the surface sterilized plant root, stem and leaves samples in nutrient agar medium and the potato dextrose agar medium respectively. The isolated bacterial endophytes were maintained as pure cultures on nutrient agar media whereas the fungal endophytes were maintained on potato dextrose agar medium.

Population size of bacterial and fungal endophytes

Colony forming units (cfu) of bacterial and fungal endophytes were calculated only after appropriate incubation at 37°C for four days and 28°C for fourteen days respectively. The colony count was expressed in cfu/g, all counts were performed in triplicate. Colony forming units (cfu) of bacterial and fungal isolates in roots, stem and leaves of the selected plant was calculated according to the following formula.

$$\text{Cfu/g} = \frac{\text{number of colonies}}{\text{dilution factor} * \text{dilution plated}}$$

Morphological Characterization of bacterial endophytes

The different morphological traits which were analyzed during the present study includes: colony type, margin of the colony, its elevation, color of colony, its surface and the opacity of colony.

Biochemical Characterization of bacterial endophytes from plant Amaranthus spinosus

The biochemical traits that had been analyzed during the present investigation includes: Gram's reaction, carbohydrate fermentation test, indole test, citrate test, catalase test, MR-VP, hydrogen sulphide production test, gelatin test, starch, capsular staining and motility test.

Morphological Characterization of fungal endophytes

The isolated fungal endophytes have been identified on the basis of different morphological features like colony characterization, growth of fungi, colour of colony (front and reverse), size, shape of conidiophores and conidia. The microscopic identification of fungal endophytes was carried out by lacto phenol cotton blue staining method.

Molecular characterization of a novel bacterial endophyte

The molecular characterization of culture AMR-1 was carried out by Xcelris Labs Ltd.

Ahmedabad using 16SrDNA. The DNA was isolated from the culture AMR-1. Its quality was evaluated on 1.2% Agarose Gel, a single band of high-molecular weight DNA was observed. Fragment of 16SrDNA gene was amplified by PCR from the above isolated DNA. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose Gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 27F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 1416bp 16SrDNA gene was generated from forward and reverse sequence data using aligner software. The 16SrDNA gene sequence was used to carry out BLAST with the nrdatabase of NCBI genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 5 (Tamura et al. 2007). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987).

Screening of the endophytes for PGP traits

The PGP traits of the isolated bacterial and fungal endophytes were evaluated based on their nitrogen fixing and phosphate solubilizing abilities.

Nitrogen fixation by bacterial endophytes

Nitrogen fixing ability of the bacterial endophytes were detected by inoculating the isolated pure enodphytic bacterial test cultures on the petri plates containing Jensen's media at 37°C for 5 days.

Phosphate solubilization by bacterial and fungal endophytes

Phosphate solubilizing ability of the bacterial and fungal endophytes were detected by spot inoculating pure isolated endophytic bacterial and fungal cultures separately on Pikovaskayas medium (Pedraza 2008) and incubated at 370C for three days and 28°C for seven days respectively along with the control plates. The uninoculated plates served as control. All the inoculations were done in triplicate. Phosphate solubilizations by the endophytic bacterial and fungal cultures were tested by their ability to solubilize inorganic phosphate. Pikovskayas agar medium containing calcium phosphate as the inorganic form of phosphate was used in assay. The phosphate solubilization efficiency (PSE) (Nguyen et al. 1992) was determined by:

$$PSE = \frac{\text{Diameter of clearing zone} \times 100}{\text{Diameter of entire colony}}$$

Results and Discussion

Isolation of Bacterial Endophytes

Total number of bacterial and fungal endophytes which were isolated from root, stem and leaves of the plant Amaranthus spinosus was 536. A total number of 394 bacterial endophytes and 142 fungal endophytes were isolated from the plant Amaranthus spinosus.

Population size of bacterial and fungal endophytes

The results clearly showed that roots supported more number of bacterial endophytes than supported either by stem or leaves, whereas stem supported more number of fungal endophytes than by roots

or leaves. The roots of the plant *Amaranthus spinosus* had the maximum colonization frequency $9.2*10^3$ followed by stem $7.3*10^3$ and leaves $3.4*10^3$ for bacterial endophytes whereas for the fungal endophytes, stem of the plant had the maximum colonization frequency $6.9*10^3$ followed by leaves $4.1*10^3$ and roots $3.2*10^3$. Out of 536 bacterial and fungal endophytes only 51 bacterial endophytes and 10 fungal endophytes had the ability to grow under laboratory conditions.

Morphological Characterization of bacterial endophytes

The results showed that out of 51 bacterial endophytes isolated from roots, stem and leaves of the plant Amaranthus spinosus, 88.24% of the bacteria had round colony shape, 82.35% had entire margins and 60.78% had convex elevation. The color of the colony which was most pronounced among the bacteria isolated from the roots of the plant was white or cream and of those isolated from the stem and leaves of the plant were found to be yellow. The morphological characteristics observed for Exiguobacterium profundum strain N4 were: Gram-positive, rod-shaped, motile and circular with entire margins.

Biochemical Characterization of bacterial endophytes from plant Amaranthus spinosus

Roots

Out of 16 endophytic bacteria isolated from roots of the plant Amaranthus spinosus, 81.25% were found to be Gram negative. In most of them capsule was absent, they were rod shaped, all were positive for citrate and catalase, while most of them were positive for VP, gelatin, motility and starch and only a few of them could ferment three sugars and most of them were MR and indole negative. The biochemical characteristics observed for Exiguobacterium profundum strain N4 is shown in Table-1.

Stem

Out of 20 endophytic bacteria isolated from stem of the plant Amaranthus spinosus, 55% were found to be Gram positive, were rod shaped, capsule was absent and most were citrate, VP, and starch positive and only a few of them could ferment three sugars i.e. lactose, sucrose and glucose and only few of them were catalase positive, were negative for gelatin, MR and indole and motility.

Leaves

Out of 15 endophytic bacteria isolated from leaves of the plant Amaranthus spinosus, 60% were found to be Gram positive, all were rod shaped, capsule was absent and only a few were citrate and catalase positive and most of them were negative for VP, gelatin, starch were also found to be negative for MR, indole and motility.

The endophytic bacteria isolated from roots, stem and leaves of the plant Amaranthus spinosus were mostly rod shaped, capsule was absent and 54.90% were found to be gram negative whereas 45.10% were found to be gram positive. Catalase, citrate and VP positive except for those found in leaves which were MR-VP negative. They were positive for starch except for those in leaves. They did not produce H_2S except for those found in roots. They were indole, MR, gelatin and motility negative, while those found in roots were motile.

Morphological Characterization of fungal endophytes

Roots

The fungal endophytes isolated from healthy roots of the plant Amaranthus spinosus

revealed the presence of Penincillum, Aspergillus and Cladosporium spps. The isolated fungal endophytes belonged to the class hypomycetes.

Stem

The fungal endophytes isolated from healthy stem of the plant Amaranthus spinosus revealed the presence of Phoma, Aspergillus, Cladosporium and Bipolaris spps. The isolated fungal endophytes belonged to the class hypomycetes.

Leaves

The fungal endophytes isolated from healthy leaves of the plant Amaranthus spinosus revealed the presence of Penincillum, Fusarium and Cladosporium spps. The isolated fungal endophytes belonged to the class hypomycetes.

The fungal endophytes isolated from roots, stem and leaves of the plant Amaranthus spinosus revealed the presence of Penincillum, Aspergillus Cladosporium, Phoma, Bipolaris and Fusarium spps Figure 1, 2,3,4,5,6. All the isolated fungal endophytes belonged to the class hypomycetes. Dominant fungal species was Cladosporium spp. which was found in all the plant parts studied.

Molecular characterization of a novel bacterial endophyte

The culture AMR-1 was identified as Exiguobacterium profundum strain N4 (GenBank Accession Number: KF928335.1) Figure-7 based on nucleotide homology and phylogenetic analysis.

Screening of the endophytes for PGP traits

62.75% of the bacterial endophytes isolated from the plant Amaranthus spinosus were able to fix nitrogen. Roots of the plant possessed maximum nitrogen fixers followed by stem and leaves, whereas 45.10% were able to soluibilize phosphorous. Leaves of the plant possessed maximum phosphate solubilizers followed by roots and stem. The phosphate solubilization efficiency was found to be highest for the fungal genera Aspergillus.

Nitrogen fixation by bacterial endophytes

The growth stimulation by the microorganisms can be a consequence of nitrogen fixation (Hurek et al. 2002; Iniguez et al. 2004; Sevilla et al. 2001). Out of 16 bacterial endophytes isolated from roots of the plant Amaranthus spinosus 87.5% were able to fix nitrogen whereas out of 20 bacterial endophytes isolated from the stem 52.17% were able to fix nitrogen and out of 15 bacterial endophytes isolated from leaves 46.67% were able to fix nitrogen. In total 62.75% of the bacterial endophytes isolated from the plant Amaranthus spinosus were able to fix nitrogen. Roots of the plant possessed maximum nitrogen fixers followed by stem and leaves.

Phosphate solubilization by bacterial and fungal endophytes

The bacterial endophytes isolated from roots, stem and leaves of the plant Amaranthus spinosus showed positive test for phosphate solubilization. Out of 16 bacterial endophytes isolated from roots of the plant Amaranthus spinosus 43.75% were able to solubilize phosphorous whereas out of 20 bacterial endophytes isolated from stem 34.78% were able to solubilize phosphorous and out of 15 bacterial endophytes isolated from leaves 53.33% were able to solubilize phosphorous. In total 45.10% of the bacterial endophytes isolated from the plant Amaranthus spinosus were

able to soluibilize phosphorous. Leaves of the plant possessed maximum phosphate solubilizers followed by roots and stem.

Solubilization Efficiency of isolated fungal endophytes

All the fungal isolates of the plant Amaranthus spiunosus showed positive test for phosphate solubilization as shown in Table-2. The phosphate solubilization efficiency was found to be highest for the fungal genera Aspergillus isolated from stem of the plant.

The results of the present study clearly revealed that the plant Amaranthus spiunosus harbored large endophytic bacterial and fungal communities. Almost all vascular plant species examined to date have been found to harbor endophytic microorganisms (Firakova et al. 2007). This obviously indicated that the plants provided an environment favorable for the growth of endophytic bacteria and fungi. This was possible only when the selected plant was also benefitted by their presence, thereby revealing that a symbiotic or mutualistic relationship existed between them. The roots of the plant Amaranthus spinosus had the maximum colonization frequency which has also been reported by Rosenblueth and Martínez-Romero, 2004, followed by stem and leaves for bacterial endophytes whereas for the fungal endophytes stem of the plant had the maximum colonization frequency which has also been reported by Sun et al. 2011, followed by roots and leaves. This might indicate to be the site of entry for endophytes or a better place which was more favorable for their growth with plenty of food substrates available to them.

The biochemical characteristics showed that the plant harbored 54.90% of gram negative bacteria and 45.10% of gram positive bacteria. Earlier also scientists have reported a predominance of Gram negative bacteria in the tissues of various plants (Elbeltagy et al. 2000). The bacterial endophytes inhabiting roots being motile moved to different plant parts with the help of their flagella and they were also peculiar, for their being H_2S positive.

The fungal endophytes isolated from roots, stem and leaves of the plant Amaranthus spinosus revealed the presence of Penincillum, Aspergillus Cladosporium, Phoma, Bipolaris and Fusarium spps. Earlier also scientists have reported dominance of certain fungal endophytes isolated from specific tissues which suggest that some fungal endophytes have an affinity for different tissue types, and this might be due to their capacity for surviving within specific substrates (Rodrigues 1994). The present study also showed that the dominant fungal endophyte was Cladosporium spp. which was found in all the parts of the plant studied.

The culture AMR-1 was identified as Exiguobacterium profundum strain N4 (GenBank Accession Number: KF928335.1) based on nucleotide homology and phylogenetic analysis. Earlier scientists have reported the ability of this bacterial genus in bioremediation of toxic textile azo dyes (Dhanve et al. 2008; Tan et al. 2009). There have also been reports on the isolation of the same from deep-sea hydrothermal vents (Crapart et al. 2007) and soil (Periasamy et al. 2013).

Endophytes exert beneficial effects upon plants. They are capable of increasing crop yields, remove contaminants, inhibit pathogens, and fix nitrogen (Rosenblueth and Martínez 2006) (Hurek et al. 2002; Iniguez et al. 2004; Sevilla et al. 2001) and solubilize phosphorous.

Table.1 The biochemical characteristics of the bacterial endophyte Exiguobacterium profundum strain N4

Culture No.	Gram's reaction		Sugar Fermentation					Indole	Citrate	Catalase	MR	VP	Gelatin	Starch	Motility	Capsular Staining
	R_X	Shape	Dextrose	Sucrose	Lactose	Gas	H_2S									
AMR 1	G +	Rod	+	-	+	No	No	-	+	++	-	+	+	+	+	-

Table.2 Solubilization Efficiency of isolated fungal endophytes

Fungal endophytes	**Size of colony (cm)**	**Size of halozone (cm)**	**Solubilization Efficiency (E)**
FAMR 1 Asper	3.5	0.70	20
FAMR 2 Clado	2.8	0.46	16.43
FAMR 3 Penin	3.1	0.51	16.45
FAMS 1Phoma	3.2	0.42	13.13
FAMS 2Asper	3.4	0.65	19.12
FAMS 3Clado	2.9	0.35	12.07
FAMS 4 Bipola	2.4	0.22	9.17
FAML 1Fusa	2.1	0.23	10.95
FAML 2Penin	2.7	0.43	15.93
FAML 3Clado	2.4	0.28	11.67

Figure.1 Depicting conidiophores bearing sterigmata and chains of conidia of ***Aspergillus*** **spp.** isolated in the present study

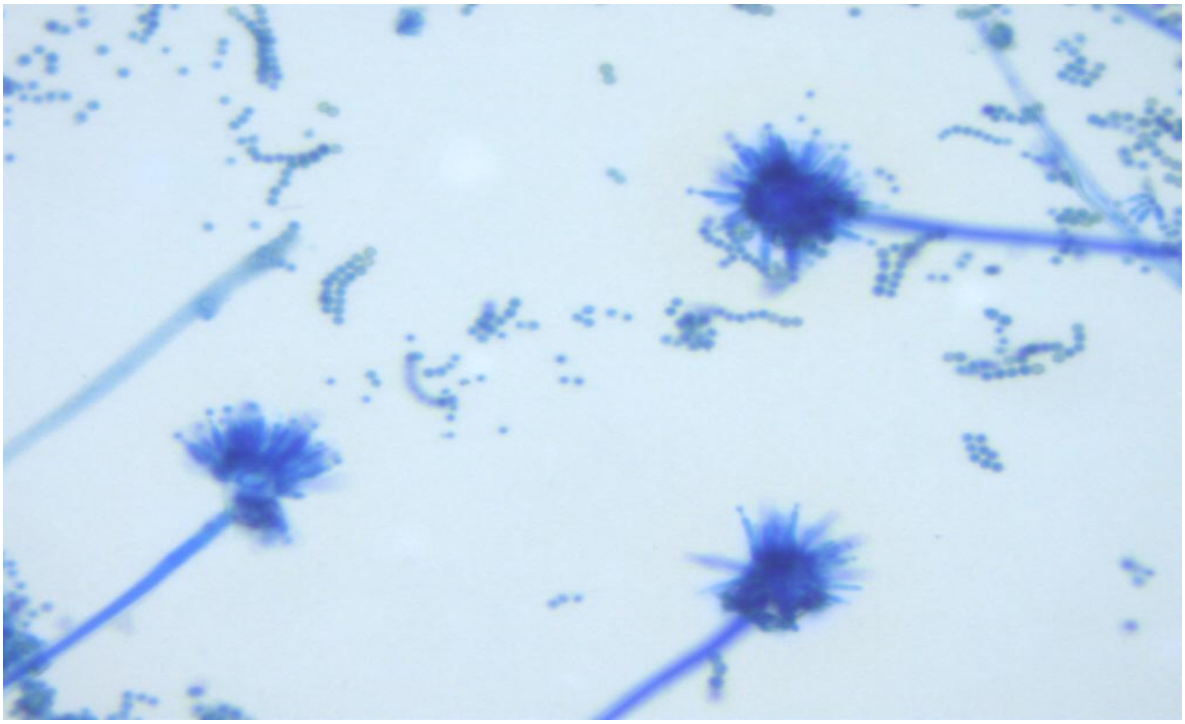


Figure.2 Depicting conidia of ***Cladosporium*** **spp.** isolated in the present study

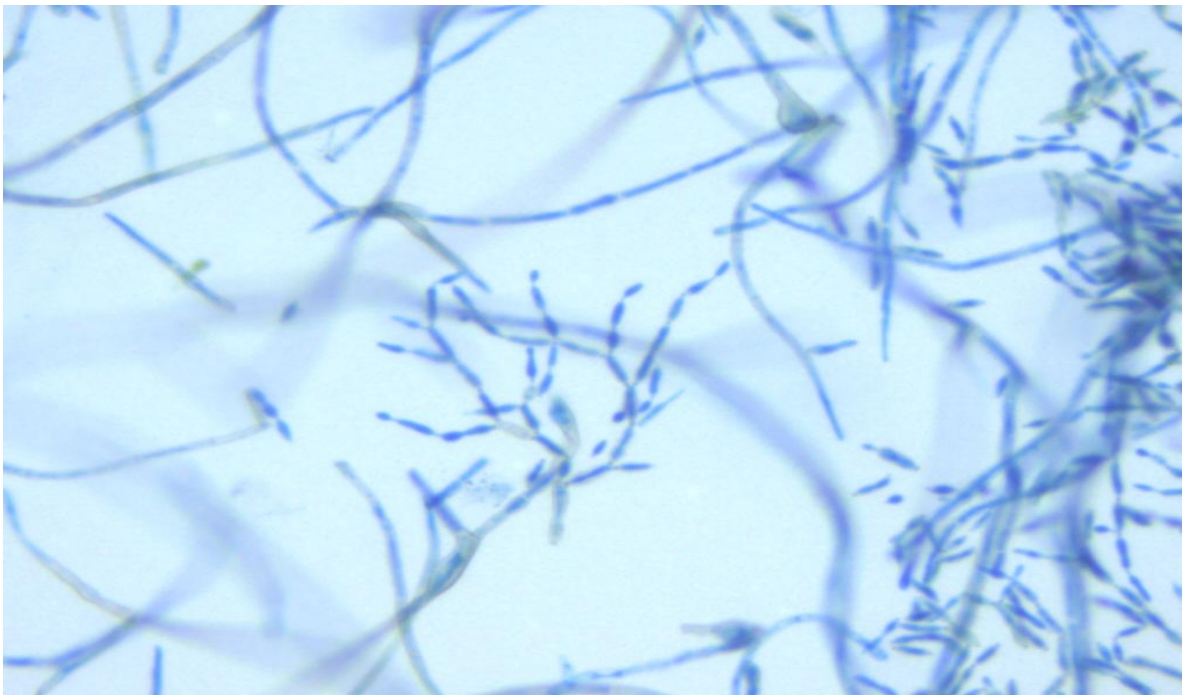


Figure.3 Depicting conidiophores bearing metulae and chains of conidia of ***Penincillum*** **spp.** isolated in the present study

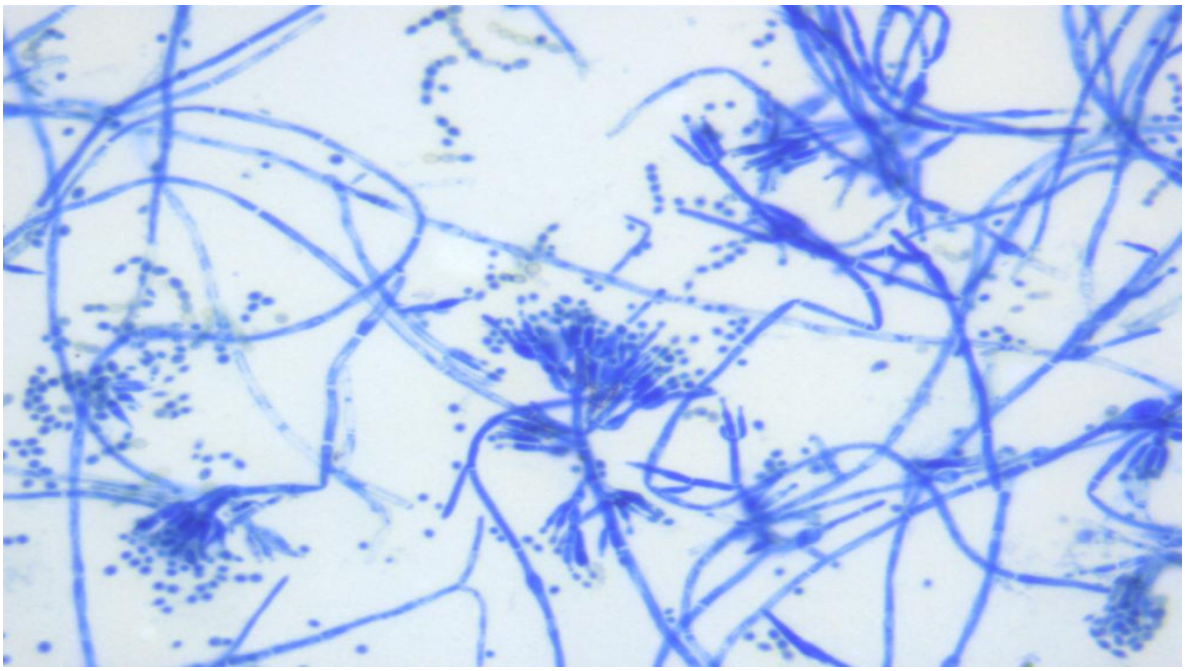


Figure.4 Depicting pycnidium of ***Phoma*** **spp.** isolated in the present study

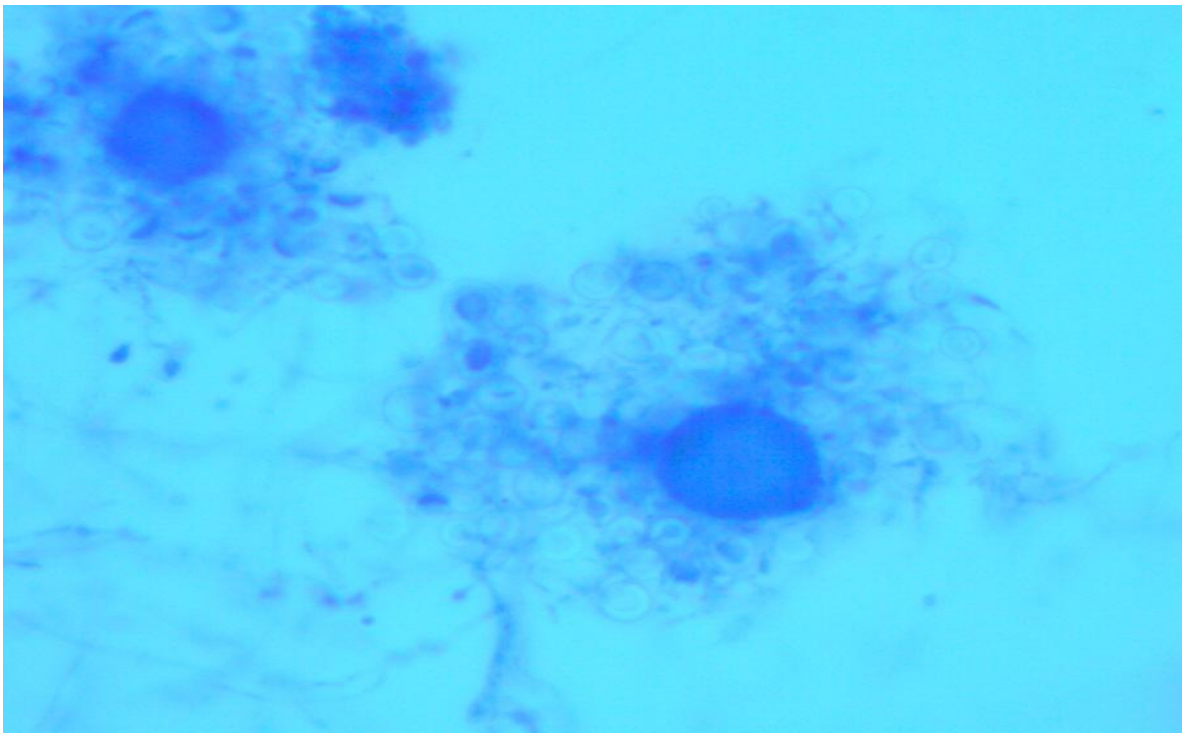


Figure.5 Depicting conidiophores bearing sterigmata and chains of conidia of ***Aspergillus*** **spp.** isolated in the present study

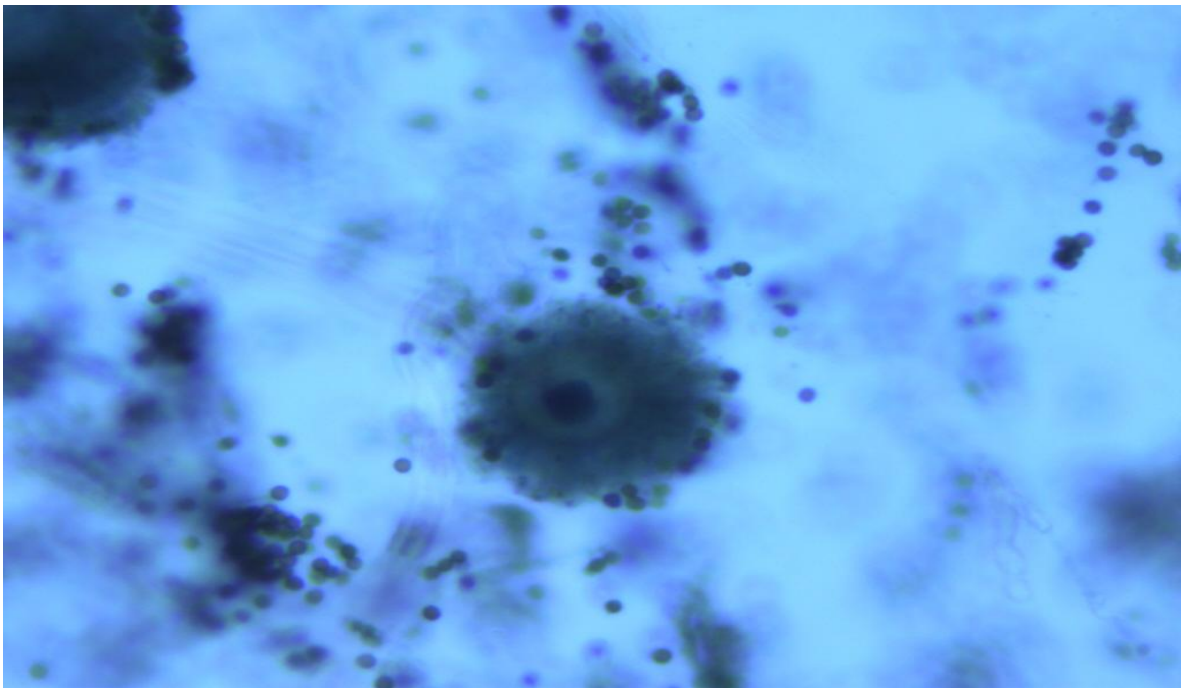
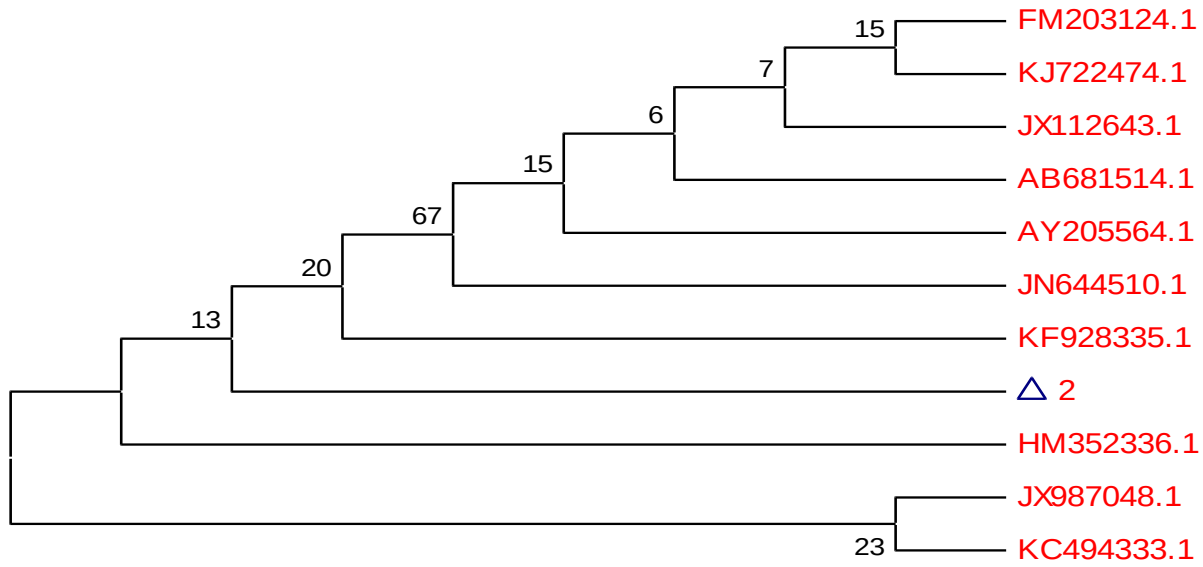


Figure.6 Depicting conidia of ***Bipolaris*** **spp.** isolated in the present study

Figure.7 Evolutionary relationships of 11 taxa



It was observed that 62.75% of the bacterial endophytes isolated from the plant Amaranthus spinosus were able to fix nitrogen. Roots of the plant possessed maximum nitrogen fixers followed by stem and leaves. It was also observed that 45.10% were able to soluibilize phosphate. Leaves of the plant possessed maximum phosphate solubilizers followed by roots and stem. The phosphate solubilization efficiency was found to be highest for the fungal genera Aspergillus.

Endophytes in tropical areas constitute a diverse but poorly investigated group. Moreover, the area of Sanganer town, which was selected for the present study was heavily contaminated with the textile effluents creating an environment difficult for the plants to survive. The presence of the selected plant species in the area and its population outnumbering the other plant species reveals a major contribution of the endophytes which are found in large number within the selected plant species. The present study also clearly revealed that it was essential to look for the plants growing under unique environmental conditions so as to isolate novel bacterial and fungal endophytes that could be utilized for the biodegradation of toxic azo dyes in the textile effluents. Further studies on endophytes harbored within plant Amaranthus spinosus promises to reveal their potential applications for the benefit of mankind and eco- friendly approach of environment conservation.

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