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Original Research Article

Isolation, Characterization and Identification of Endophytes from *Curcuma longa*

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Endophytes, *Curcuma longa*, Biochemical characterization and identification

A B S T R A C T

Virtually all plants are inhabited by diverse bacteria known as endophytes. The secondary metabolites produced by these plants are believed to be influenced by the coexistence of microbes within this plant. Utilization of beneficial properties of plant associated microbes is of great significance at an applied level, either to increase production yields of agricultural crops, control of plants diseases or pests, adapt plant to suitable growth conditions, or in reforestation activities. In India, turmeric has been used traditionally for thousands of years as a remedy for stomach and liver ailments, as well as topically to heal sores, basically for its supposed antimicrobial property. Turmeric (*Curcuma longa* L) rhizome is likely to provide a specialized habitat for the association of a diverse group of bacteria with potential impact on plant growth. This makes studies on isolation and characterization of bacteria from turmeric much more interesting and informative. Thus the exploration of endophytes associated with this plant may be helpful for agriculture purposes including plant growth promoting activities and biocontrol agent. The present work is focused on isolation, characterization and identification of endophytes from turmeric. As such four isolates were characterized morphologically, biochemically and identified with partial DNA sequencing method. They were identified and the sequence was deposited in Genebank.

Introduction

A diverse array of bacteria and fungi is able to colonize different plant organs and tissues, including roots, leaves, flower clusters, seeds and fruits, thus forming a complex micro ecosystem in the plants. While doing so, these plant-associated bacteria can affect crop health, due to their capacity to suppress or stimulate the colonization of tissues by plant pathogens (Hallman *et al.*, 1997; Gray and Smith 2005). The plant associated microbes lives in varying relation with the host, the host provide nutrients to the microbes and in turn the plant get benefited from the associates by promoting plant growth, increase yield, vigour tolerance to a list of biotic and abiotic stress such as increased resistance against plant pathogens and parasites, tolerance against pH, temperature, drought, salinity etc. Production of active metabolites by the

associates contributes much to the host plant. Exploitation of beneficial properties of plant associated microbes is of great relevance at an applied level, either to increase production yields of agricultural crops,

The herbal plants have several active compounds that are believed to be influenced by the coexistence of microbes within this plant. These microbes can produce active compounds with the potential to act as medicine. Microbes that coexist with the plant can live on the surface of the plant or in the plant's system. The habitat above ground where microbes grow is called the phyllosphere and microbes that grow on the plant's surface are called epiphytes. Microbes that grow in the plant's system are called endophytes. Endophytes are symbiotic and exist in the plant's system without causing it any harm. Nutrients needed by the phyllosphere microbe to grow, for instance carbohydrates, organic acid, and amino acid, come from the plant (Whips *et al.*, 2008).Bacteria are considered to be the dominant microbial inhabitants of the phyllosphere, although archaea, filamentous fungi, and yeasts may also be important (Yadav *et al.,* 2005; Stapleton and Simmons 2006).

Therefore the present research work is proposed in a view to study and isolate endophytic bacterial community associated with one of the most important Indian medicinal plant i.e. turmeric (*Curcuma longa* L family Zingiberaceae). In India, turmeric has been used traditionally for thousands of years as a remedy for stomach and liver ailments, as well as topically to heal sores, basically for its supposed antimicrobial property. In the Auyurvedic system (since c. 1900 BCE) turmeric was a medicine for a range of diseases and conditions, including those of the skin, pulmonary, and gastrointestinal systems, aches, pains, wounds, sprains, and liver disorders. A fresh juice is commonly used in many skin conditions, including eczema, chicken pox, shingles, allergy, and scabies. Thus the exploration of bacterial community associated with this plant may be helpful for agriculture purposes as biocontrol agent and plant growth promoting activities. Endophytes are organisms inhabiting in plant tissues whether they are neutral, beneficial or detrimental to hosts (Sturz *et al.*, 2000). In recent years, many researches have focused on the bioactivities of endophytes (Cui *et al.,* 2003).The plant kingdom is colonized by diverse range of endophytic microorganisms. Some microbes form non-pathogenic relationships with their host, where they colonize the internal tissues of the host plant and form a range of associations including symbiotic, mutalistic, commensalistic and trophobiotic relationships. Endophytic microorganisms can promote plant growth and yield and can act as strong biocontrol agents against various diseases including insect-pest. Endophytes can stimulate plant growth hormones, increase disease resistance, improve the plants ability withstand environmental stress conditions (e.g. drought, pH, temperature etc.) or enhance N2 fixation and increase in nutrient supply. They contribute to biotic and abiotic stress tolerance of the plants (Mamota *et al.*, 2012). A large variety of plant species are shown to be endophytically associated with bacteria such as *Pseudomonas*, *Bacillus*, Azospirillum, etc. (Chanway, 1996). Several genera of endophytic bacteria have been isolated from different plants, including *Aerobacter*, *Aeromonas*, *Agrobacterium*, *Chryseomonas*, *Curtobacterium*, *Enterobacter*, *Erwinia*, *Flavimonas* and *Sphingomonas* (Sturz *et al.,* 1997). Studies also showed the presence of endophytic diazotrophs, *Acetobacter diazotrophicus,*

Herbaspirillum sp., *Burkholderia sp*., *Enterobacter sp*. and *Klebsiella sp*., associated with sugarcane plants (Boddey *et al.,* 2003).

Turmeric (*Curcuma longa* L) is a rhizomatous herbaceous perennial plant of the ginger family, Zingiberaceae. It is native to tropical Indian Subcontinent and needs temperatures between 20 °C and 30 °C (68 °F and 86 °F) and a considerable amount of annual rainfall to thrive. Plants are gathered annually for their rhizomes, and propagated from some of those rhizomes in the following season. India and Pakistan are significant producers of turmeric. Turmeric has been used in India for thousands of years and is a major part of Ayurvedic medicine. It was first used as a dye and then later for its medicinal properties. The most important chemical components of turmeric are a group of compounds called curcuminoids, which include curcumin (diferuloylmethane), demethoxycurcumin, and bisdemethoxycurcumin. The best studied compound is curcumin, which constitutes 0.3-5.4% of raw turmeric. In addition there are other important volatile oils such as turmerone, atlantone, and zingiberene. Some general constituents are sugars, proteins, and resins (Miquel *et al.*, 2002).

There are several reports on presence of endophytes in turmeric, however the exploration of epiphytic community is not reported much. Therefore this is an effort to isolate, characterize and identify bacteria from the plant leaf surface.

Materials and Methods

Collection

The *Curcuma longa* leaves were collected from agriculture farmlands crop near Nanded District (MS). The plants were uprooted in the month of November and the approximate age was 5 months. The plants were uprooted, cleaned from the soil and debris and were put in brown paper bag and processed further within 24 hrs.

Isolation and purification

Fresh leaves and rhizomes were cleaned in tap water. The sample is then surface sterilized with 70%ethanol for 1 min, rinse with tap water and then with 0.1 % w/v $HgCl_2$ for 3 min. Again wash with distilled water for 5 min. Surface sterilization efficiency of the sterilizers was checked by inoculating surface sterilized unsliced rhizome on nutrient agar plate, prior to inoculation of endophytic bacteria. The leaves and rhizomes are cut to uniform size and placed aseptically on nutrient agar supplemented with benomyl. The plates were incubated for 72 hrs. The bacterial colonies surrounding the tissue section were picked and streaked on the fresh nutrient agar for the selection of clone (Sun *et al.*, 2008).

Morphological evaluation

The potential isolates were studied for colony shape, and colony color. Pure culture of selected isolates was streaked on Nutrient agar medium separately for colony development. The individual colonies were examined for colony color and shape following standard microbiological (Smibert and Krieg 1995; Sneath, 2001).

Biochemcial characterization

Biochemical tests *viz*., Starch hydrolysis, citrate utilization, catalase activity, nitrate reduction, oxidase test and IAA production were done. Antibacterial and antifungal activity was also evaluated. All biochemical tests were done as per Aneja (2006).

Carbohydrate utilization profile

Carbohydrate utilization profile was obtained using HiMedia kits as per manufacturer's protocol.

Identification

Bacterial DNA was isolated following protocol given by Cheng and Jiang 2006. The PCR amplification was performed according to Rahman *et al.,* (2013) with some modifications. The amplification was done by using forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer 1492R (5'-GGTTACCTTGTTACGACTT-3'). The amplified DNA was purified using the Qiaquick PCR Purification Kit (Qiagen) and sent for sequencing at Progene Life Sciences, Pune (MS). The amplified 16S rDNA sequences were compared with the nucleotide sequence database in the GenBank using the standard BLASTn tool at the NCBI server (https://blast.ncbi.nlm.nih.gov/Blast.cgi).
The alignment of the sequences was done using the CLUSTALW program from the EMBL - EBI website (http://www.ebi.ac.uk/Tools/msa/clustalw2). From the aligned sequences neighbour-joining dendrogram was constructed with MEGA 5 software (Rahman *et al.,* 2013).

Results and Discussion

There are good reasons for isolating plant associated microorganisms, e.g. for their characterization, for studying population dynamics and diversity, use of microbial inoculants to improve plant growth and plant health, and as sources of novel biologically active secondary metabolites. Though there is much work on epiphytes and endophytes especially on biological activity and diversity analysis; the data on microbes associated with turmeric plant is scarce. Such work is mainly concentrated in countries like China, Indonessia, Thailand and India where the use of medicinal plants is much higher. In the present investigation four endophytes were isolated from the rhizomes of turmeric collected from 5 months old plant from agriculture farmlands near Nanded district (MS).

The four isolates were identified by partial DNA sequencing method. Isolate TR4-1 was found to have circular colony with entire margin and pulvinate elevation. The colonies were transparent with smooth and glistering surface. KOH test was positive (Gram -) and it showed catalase and siderophore production test positive. There was no evidence of auxin production, casein hydrolysis, antibacterial and antifungal activity.

Amongst all the tested carbohydrates for utilization, only three carbohydrates such as cellobiose, esculin and malonate were utilized. It was identified as *Stenotrophomonas maltophilia* by partial DNA sequencing method. The sequence was deposited in Genebank (KU257661.1). Isolate TR13-3 showed circular colonies with lobate margin that were flat, wrinkled and white opaque. KOH test was negative (Gram +) while starch hydrolysis, catalase and skimmed milk agar test were positive. Siderophore production, auxin production, antibacterial and antifungal activity were not observed. It was observed to utilize few carbohydrates including L-arabinose, mannose, rhamnose, cellobiose, melezitose, ONPG, esculin and D-arabinose. It was identified as *Bacillus safensis* and the sequence was deposited in Genebank (KU257665.1).
Isolate TR19-1 was found to have irregular colony with lobate margin and flat elevation. The colonies were opaque with wrinkled

surface. It showed positive catalase, skimmed milk and auxin production. Among evaluated carbohydrate utilization Tr19-1 utilized many sources except A-Methyl-D-mannoside, D-Arabinose, Citrate and Sorbose. It was identified by partial DNA sequencing as *Brevibacterium halotolerans* and the sequence was deposited in gen bank (KU257658.1).

Isolate TR26-1 was found to have irregular colony with lobate margin, flat elevation, and smooth surface. It showed positive catalase, skimmed milk and siderophore production activity. KOH reaction was –ve (Gram +).Among evaluated carbohydrate utilization TR26-1 utilized many sources including Xylose, Maltose, Fructose, L-Arabinose, Mannose, Cellobiose, ONPG, Esculin, Citrate and Malonate. It was identified by partial DNA sequencing as *Bacillus pumilus* and the sequence was deposited in gen bank (KU257646.1). The phylogeny of all these isolates is given in Fig1-4.

It has been rationalized that plants having an ethnobotanical history and exploited for human use in traditional medicine may harbor an endophytic population which may produce a plethora of microbial metabolites related closely to the plant biochemistry (Strobel *et al.,* 2004). Recent studies have established that secondary metabolites elaborated by these microbial endophytes could serve as prospective resources of antimicrobial substances, antioxidants, cytotoxic compounds, growth hormones and hydrolytic enzymes of biotechnological applications (Yu *et al.,* 2010). There are several reports of bioactivity potential of the endophytes. The antibiotic-producing potential of endophytic populations from medical plant of *Salvia miltiorrhiza* was examined. A total of 63 isolates were screened against five fungal and three bacterial species for the production of antimicrobial compounds. It showed that more isolates were antagonistic to fungi than to bacteria (Xia *et al.,* 2011). A total of 18 endophytic bacteria and 32 phyllosphere bacteria were isolated from the herbal plants of *Citrus* sp., *Plucheaindica*, *Curcuma longa*, *Nothopanaxscuttelarium*, *Piper crocatum*, and *Andrographispaniculata*. About 72% of endophytic bacteria isolates have proteolytic activity and about 11% have lipolytic activity. On the other hand, about 59% of phyllosphere bacteria isolates have proteolytic activity and about 19% have lipolytic activity (Yogiara *et al.,* 2012).

Several studies have reported that microbes which coexist with plants can produce secondary metabolites that are beneficial to treat ailments like tumors, bacteria, fungi, and compounds to regulate plant growth. Gayathri *et al.*, (2010) stated that endophytic bacteria isolated from the swamp mangrove plant could produce the enzymes protease, inulase, and invertase.

In the present study four isolates from rhizomes of *Curcuma longa* were characterized and identified by partial DNA sequencing method. Isolate TR4-1 i.e. *Stenotrophomonas maltophilia* is an uncommon, aerobic, non-fermentative, gram negative bacterium; motile due to polar flagella, catalase-positive, oxidase-negative. It was reported that though *S. maltophilia* is an aerobe, it can still grow using nitrate as a terminal electron acceptor in the absence of oxygen (Crossman *et al.,* 2008). There is ubiquitous distribution of *S. maltophilia* strains in the environment with regard to habitat and geography: often associated with roots of many plant species (Ryan *et al.,* 2009).

Table.1 General morphological and biochemical characteristics of isolates

SN	Test	TR4-1	TR 13-3	TR 19-1	TR 26-1
1	Colony Form	Circular	--	Irregular	Irregular
2	Margin	Entire	--	Lobate	Lobate
3	Elevation	Pulvinate	--	Flat	Flat
4	Surface	Smooth, glistering	--	Wrinkled	Smooth
5	Opaque/Transparent	T	--	O	O
6	KOH Test	-	-	-	+
7	Amylase	-	+	-	-
8	Catalase	+	+	+	+
9	Citrate	-	-	-	-
10	Siderophore	+	-	-	+
11	Skimmed milk test	-	+	+	+
12	Auxin Production	-	-	+	-
13	Antibacterial activity	-	-	-	-
14	Antifungal Activity	-	-	-	-

Table.2 Carbohydrate utilization profile of the isolates

SN	Test	TR4-1	TR 13-3	TR 19-1	TR 26-1
1	Lactose	-	-	+	-
2	Xylose	-	-	+	+
3	Maltose	-	-	+	+
4	Fructose	-	-	+	+
5	Dextrose	-	-	+	-
6	Galactose	-	-	+	-
7	Raffinose	-	-	+	-
8	Trehalose	-	-	+	-
9	Melibiose	-	-	+	-
10	Sucrose	-	-	+	-
11	L-Arabinose	-	+	+	+
12	Mannose	-	+	+	+
13	Inulin	-	-	+	-
14	Sodium Gluconate	-	-	+	-
15	Glycerol	-	-	+	-
16	Salicin	-	-	+	-
17	Dulcitol	-	-	+	-
18	Inositol	-	-	+	-
19	Sorbitol	-	-	+	-
20	Mannitol	-	-	+	-
21	Adonitol	-	-	+	-
22	Arabitol	-	-	+	-
23	Erythritol	-	-	+	-
24	α-Methyl-D-glucoside	-	-	+	-
25	Rhamnose	-	+	+	-
26	Cellobiose	+	+	+	+
27	Melezitose	-	+	+	-
28	A-Methyl-D-mannoside	-	-	-	-
29	Xylitol	-	-	+	-
30	ONPG	-	+	+	+
31	Esculin hydrolysis	+	+	+	+
32	D-Arabinose	-	+	-	+
33	Citrate Utilization	-	-	-	+
34	Malonate Utilization	+	-	+	+
35	Sorbose	-	--	--	--

Table.3 Identification of isolates

SN	Isolate No	Identification	Genebank No
1.	TR4-1	*Stenotrophomonas maltophilia*	KU257661.1
2.	TR 13-3	*Bacillus safensis*	KU257665.1
3.	TR 19-1	*Brevibacterium halotolerans*	KU257658.1
4.	TR 26-1	*Bacillus pumilus*	KU257646.1

Fig.1 Phylogenetic analysis of TR4-1 i.e. *Stenotrophomonas maltophilia*

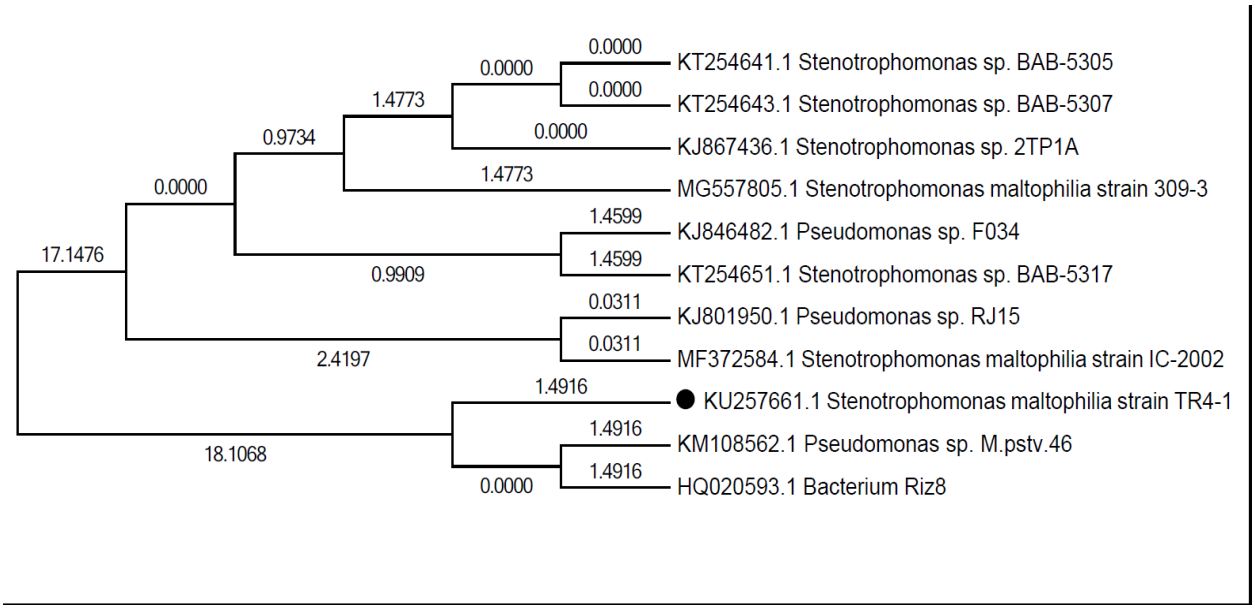


Fig.2 Phylogenetic analysis of TR 13-3 i.e. *Bacillus safensis*

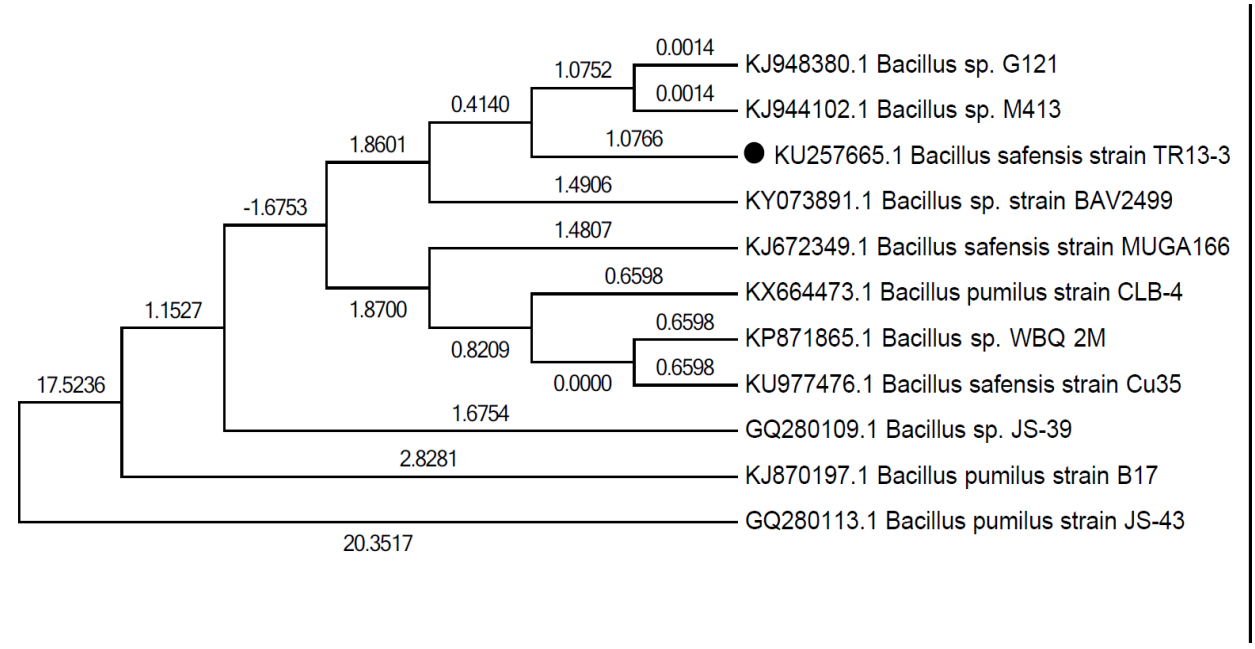


Fig.3 Phylogenetic analysis of 19-1 i.e. *Brevibacterium halotolerans*

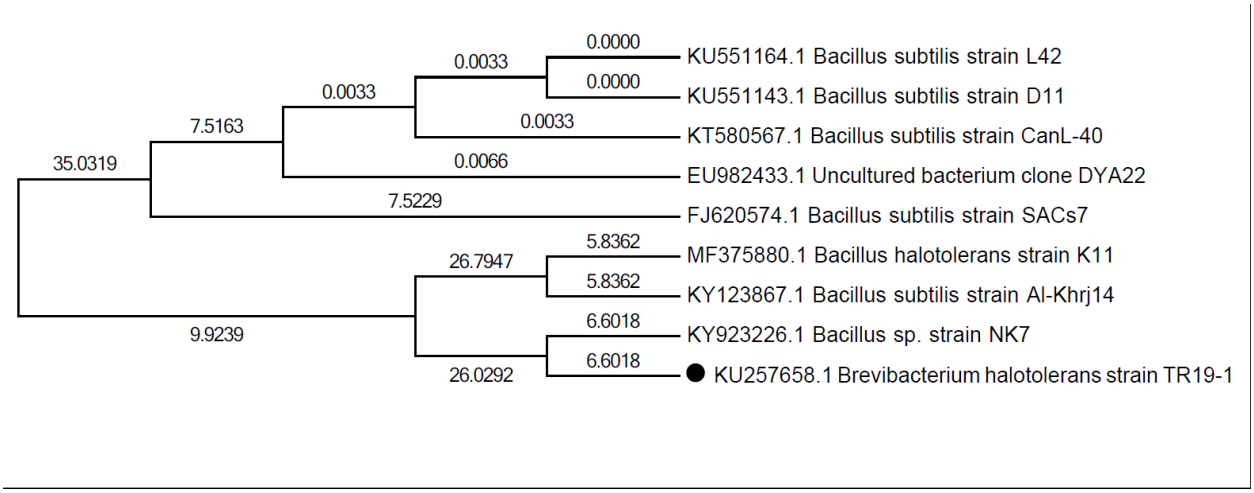
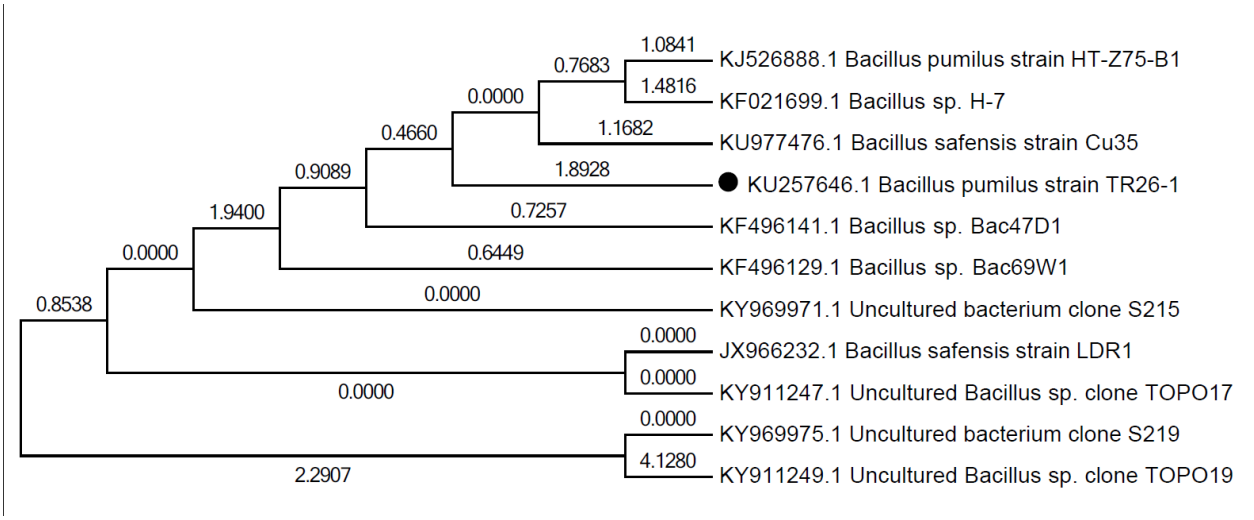


Fig.4 Phylogenetic analysis of TR 26-1 i.e. *Bacillus pumilus*



S. maltophilia have been documented as a potential source for several novel bioactive compounds useful as bio control agents due to their antifungal, antibacterial and insecticidal properties but also have widespread applications as plant growth promoting substances (PGPR). Rhizobacteria introduced in the rhizosphere of tomato, pepper, melon or bean was found to increase growth of roots/shoots (Elad *et al.,* 1987). Though reported in the literature, the strain of *S maltophilia* isolated in the present study lacks auxin production and antimicrobial activity. *B safensis* i.e. isolate TR13-1 is a Gram-positive, aerobic, mesophilic, chemo-heterotrophic and sporeforming, bacterium. It is documented as a rod-shaped and motile bacterium with high tolerance for salt, heavy metals, and ultraviolet and gamma radiations (Kothari *et al.,* 2013). It colonizes wide range of habitats, some of which are extreme environments including spacecraft and associated environments, saline desert, oil polluted sites, industrial effluents, rhizosphere, plant body, human and animal

excreta, soil and others. It is one of the most widespread species of the *B. pumilus* group (Branquinho *et al.,* 2014a). It has also been isolated as endophytic and plant growth-promoting rhizobacterium (Bibi *et al.,* 2012; Chakraborty *et al.,* 2013; Kothari *et al.,* 2013; Edelman and Lin 2014).

Isolate 19-1 i.e. *B halotolerans* is a Gram+ and an alkaliphilic bacterium (Takami and Horikoshi, 1999) that can grow well at pH 7–10.5 in saline environments. *B. halodurans*is well characterized physiologically, biochemically, and genetically (Horikoshi, 1999).

It is reported to have some antimicrobial activity and can inhibit the growth of both Gram-positive and Gram-negative pathogens such as (Wietz, M. *et al.,* 2010), however such activity was not observed in the present study. The fourth isolate TR26-1 i.e. *Bacillus pumilus* is a Gram-positive, spore-forming rod-shaped bacteria, and aerobic. It resides in soils and some colonize in the root area of some plants where *B. pumilus* has antibacterial and antifungal activity (Thomas 2004). The proteases from *B. pumilus* have been used in various industries. Food, chemical, detergent, and leather industries have benefited from the proteases from *B. pumilus* (Pan *et al.,* 2004).

Four different bacterial cultures were isolated from the agriculture farmland grown turmeric rhizomes. They were characterized and their carbohydrate utilization profile was evaluated. The isolates were identified and the sequence was submitted to Genebank. Further evaluation of these isolates is required for their potential biological activity and plant growth promotion activity. Literature supports the potential of endophytes as industrially important organisms and in agriculture to reduce pathogenicity of many pathogens.

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