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RESEARCH ARTICLE :

Isolation, characterization and identification of epiphytes from *Curcuma longa*

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SUMMARY : Several herbal plants have active compounds that are believed to be influenced by the coexistence of microbes within this plant. The habitat above ground where microbes grow is called the phyllosphere and microbes that grow on the plant's surface are called epiphytes. Exploitation of beneficial properties of plant associated microbes is of great relevance 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. In the case of Turmeric (*Curcuma longa* L.), the presence of rhizome is expected 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 epiphytes associated with this plant may be helpful for agriculture purposes as biocontrol agent and plant growth promoting activities. The present is focused on isolation, characterization and identification of epiphytes from turmeric. As such three isolates were characterized morphologically, biochemically and identified with partial DNA sequencing method. They were identified and the sequence were deposited in Genebank.

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BACKGROUND AND OBJECTIVES

Plants sustain a complex micro ecosystem that harbours a diverse array of bacteria, able to colonize different plant organs and tissues, including roots, leaves, flower clusters, seeds and fruits. In so doing, 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 and 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.

Several herbal plants have 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 (Whipps *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 and Stapleton and Simmons 2006).

Therefore the present research work is proposed in a view to study and isolate epiphytic and 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.

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).

Epiphytic bacteria have been defined as populations that can survive and multiply on the surface of plants. Thus, they develop survival strategies in protected positions such as the trichomes base, inside substomatal chambers, hydathodes and especially, in between the depressions along the junctions of adjacent epithelial cells (Monier and Lindow, 2005). Epiphytics as biological control agents are still misused, especially compared to rhizobacteria and endophytic bacteria. However, the biocontrol of diseases affecting several crops by those microorganisms have been increasingly researched (Halfeld-Vieira *et al*., 2008). Notably, the interest in the study of those microbes is related to their capacity to occupy ecological niches on the phylloplane that could be occupied by pathogens (Monier and Lindow, 2005), and to their broad antagonistic effect against pathogens. Biosurfactants, antibiotics, bacteriocins and volatile organic compounds (VOCs) synthesis, siderophores and competition for space and nutrients are related to the antagonistic effects of epiphytic bacteria on the phytopathogen growth. Recent surveys demonstrate that epiphytic bacteria also act as elicitors of the induced systemic resistance (ISR) in plants (Morris and Kinkel, 2002 and Halfeld-Vieira *et al.*, 2006).

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.

RESOURCES AND METHODS

Collection:

The *Curcuma longa* leaves were collected from

agriculture farmlands crop near Nanded district (M.S.). 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:

To isolate the leaf-associated bacteria, 100 grams of mixed leaf samples from plants collected from one field were placed in sterile Erlenmeyer flasks with 500 ml 0.1 M potassium phosphate buffer (pH 7.0) and sonicated for 10 min. The resulting mixture was briefly centrifuged at 800 g to remove leaf debris. The suspension was then serial diluted to tenfold and spread over nutrient agar plates supplemented with cycloheximide 0.1g/l to suppress fungal growth. The plates were incubated at 25 ^{0}C for 48 hrs. Pure colonies were transferred to Nutrient agar and King's B medium followed by incubation of 72 hrs. Purified isolates were stored at -80 °C in LB liquid broth with 30% glycerol until further processing (Anzhou *et al.*, 2013).

Morphological evaluation:

The potential isolates were studied for colony shape, and colony colour. Pure culture of selected isolates were streaked on nutrient agar medium separately for colony development. The individual colonies were examined for colony colour and shape following standard micro-biological (Smibert and Krieg 1995 and 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 were also evaluated. All biochemical tests were done as per Aneja (2006).

Carbohydrate utilization profile:

Carbohydrate utilization profile was obtained using Hi Media 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'-AGAGTTTGATCCT GGC TCAG-3') and reverse primer 1492R (5'- GGTTACC TTGTTACGACTT-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 programme 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).

Table A: General morphological and biochemical characteristics of isolates

Sr. No.	Test	L5	L9	L14
1.	Colony form	Circular	Circular	Irregular
2.	Margin	Entire	Entire	Undulate
3.	Elevation	Raised	Umbonate	Flat
4.	Surface	Smooth, Glistering	Smooth, Glistering	Wrinkled
5.	Opaque/transparent	Translucent	Translucent	Opaque
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 B : Carbohydrate utilization profile of the isolates

Sr.No.	Test	L5	L9	L14
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 C: Identification of isolates

Sr. No.	Isolate No	Identification	Genebank No
1.	L5	*Agrobacterium larrymoorei*	KU257663.1
2.	L9	*Bacillus licheniformis*	KU257656.1
3.	L14	*Bacillus pumilus*	KU257649.1

OBSERVATIONS AND ANALYSIS

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. There are few reports on endophytic community in turmeric however, the study on epiphytes is not observed. In the present investigation three epiphytes were isolated from the leaves of turmeric collected from 5 months old plant from agriculture farmlands near Nanded district (MS).

Isolate L5 was found to have circular colony with entire margin and raised elevation. KOH test was positive and it showed siderophoreproduction test positive. It was able to utilize dextrose, ONPG, esculin and identified as *Agrobacterium larrymoorei* by partial DNA sequencing method. The sequence was deposited in Genebank (KU257663.1). The phylogeny is given in Fig 1. Bouzar and Jones (2001) reported the *A. larrymoorei* as strict aerobe gram negative rod and was isolated from aerial parts of *Ficus benjamina*. Isolate L9 showed circular colonies with entire margin that were translucent and glistering. KOH test was negative while starch hydrolysis, catalase and siderophore production was observed. Good antifungal activity was observed with isolate L9. It was observed to utilize almost all carbohydrates except, adonitol, ONPG and malonate. It was identified as *Bacillus licheniformis* and the sequence was deposited in Genebank (KU257656.1). *Bacillus licheniformis* is a Gram-positive, spore-forming soil bacterium that is used in the biotechnology industry to manufacture enzymes, antibiotics, biochemicals and consumer products (Rey *et al.*, 2004). Isolate L14 was found to have irregular colony with undulated margin, flat elevation and wrinkled surface.It showed positive amylase, catalase, skimmed milk and antifungal activity. Among evaluated carbohydrate utilization L14 utilized many sources except Adonitol, Arabitol, Erythritol, α-Methyl-D-glucoside, Rhamnose, Melezitose, Á-Methyl-D-mannoside, Xylitol, ONPG, D-Arabinose and Sorbose. It was identified by partial DNA sequencing as *Bacillus pumilus* and the sequence was deposited in gen bank (KU257649.1). Roberto *et al*. (2010) reported the presence of *B pumilus* on tomato and observed its biopesticide potential to control *Xanthomonas vesicatoria* and *Alternaria solani* disease severity in tomato plants. They concluded that these epiphytic bacteria are able to inhibit the growth of tested phytopathogens *in vitro* and efficiently colonize the phylloplane of tomato plants.

Conclusion:

Three different bacterial cultures were isolatedfrom the agriculture farmland grown turmeric leaves. They were characterized and their carbohydrate utilization

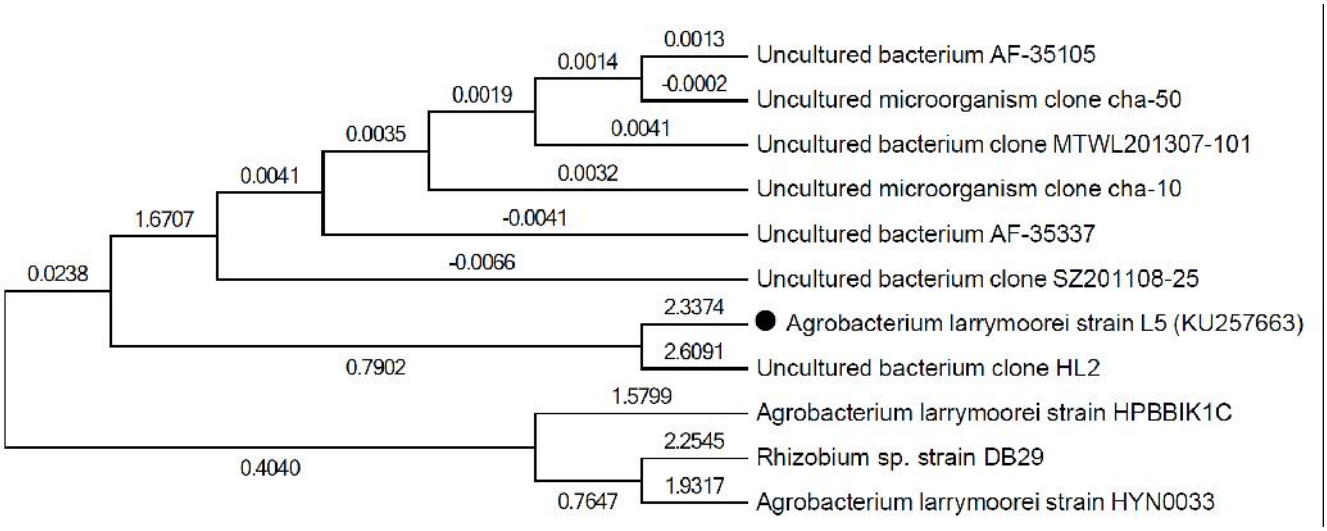

Fig. 1: Phylogenetic analysis of L5 *i.e. Agrobacterium larrymoorei*

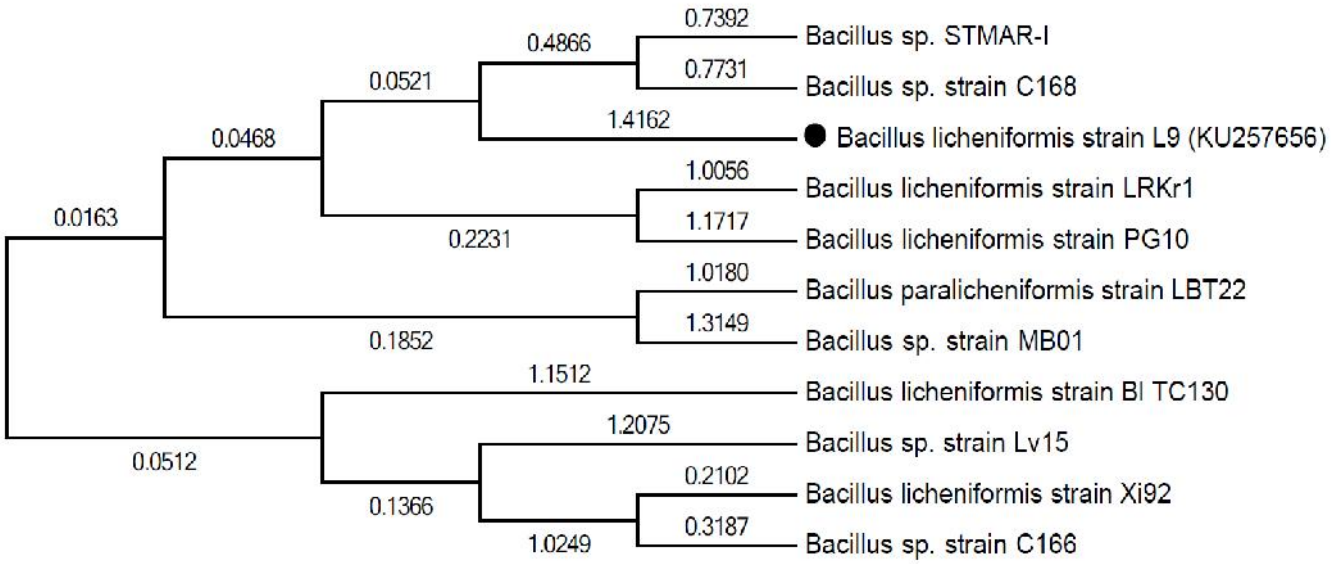

Fig. 2 : Phylogenetic analysis of L9 *i.e. Bacillus licheniformis*

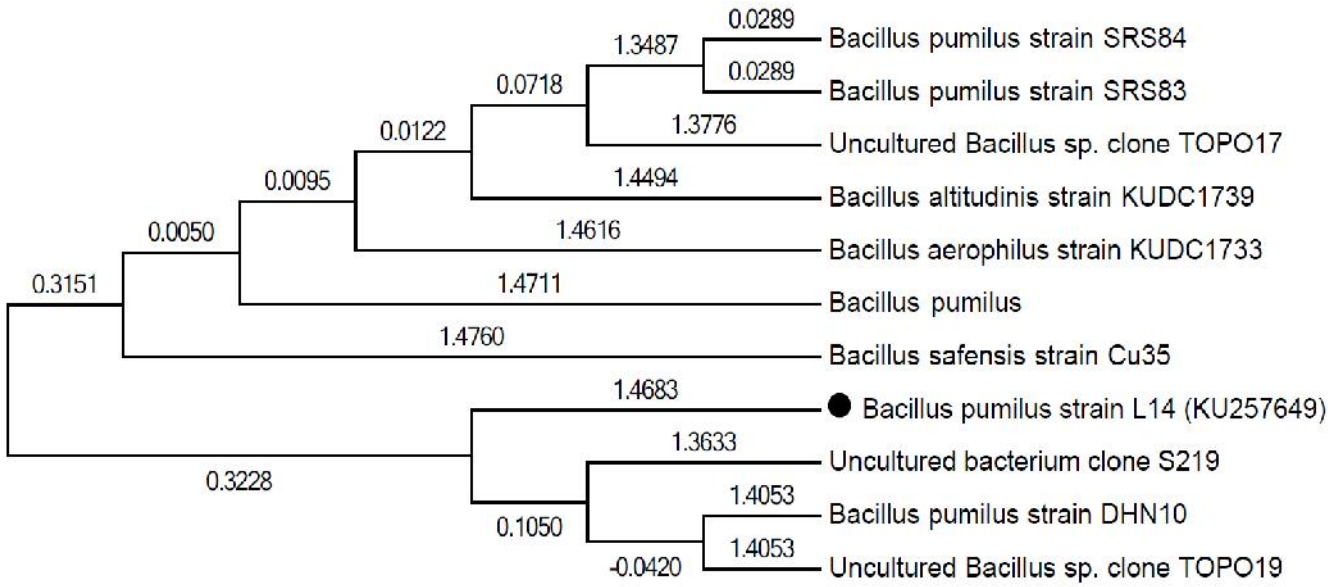

Fig. 3: Phylogenetic analysis of L14 *i.e. Bacillus pumilis*

profile was evaluated. The isolates were identified and the sequence were 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 epiphytes as industrially important organisms and in agriculture to reduce pathogenicity of many microprganisms.

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