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ISOLATION AND MOLECULAR CHARACTERIZATION OF PLANT GROWTH PROMOTING RHIZOBACTERIUM *PAENIBACILLUS ILLINOISENSIS* STRAIN NAGOTH JAR 007 FROM SEEDS OF *CAPSICUM CHINENSE* BHUT JOLOKIA

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ABSTRACT

The present study investigated the identities of endophytic bacteria isolated from the seeds of dry fruits of *Capsicum chinense* Bhut Jolokia using PCR analysis with the view of food product quality. The bacteria were isolated from the seeds soaked in peptone broth followed by serial dilution. Standard molecular methods were used for DNA extraction (UniFlex™ DNA Isolation kit method), Polymerase Chain Reaction (PCR), electrophoresis, purification and sequencing of generated PCR products. The partial sequences obtained were deposited in the database of National Centre for Biotechnology Information (NCBI). Based on genotypic characteristics, these strains belonged to the genus *Paenibacillus*. Phylogenetic analysis based on 16S rRNA gene sequence comparison showed that they clustered with *Paenibacillus illinoisensis* (99 % similarity). It was assigned *Paenibacillus illinoisensis* strain Nagoth JAR 007 and was submitted to Genbank (KC886309). The research findings concluded that presence of the endophytic *Paenibacillus* are nonpathogenic and has a role in plant resistance to abiotic or biotic stress.

Keywords: *Paenibacillus illinoisensis, Capsicum chinense* Bhut Jolokia, endophytic, molecular characterization, phylogenetic tree

INTRODUCTION

Fruits and vegetables cultivated in natural environment are exposed to microbial contamination. The production and processing of food items with quality and hygiene are the most important criteria for the consumers. The food products eaten raw would turn into a health hazard, if contaminated with pathogenic bacteria **(Bruhn, 1995).** Most microorganisms that are originally found on whole fruit or vegetable surfaces are soil inhabitants **(Margaret Barth, 2009).** Research has proved that the use of contaminated irrigation water can increase the frequency of pathogenic microorganism from harvest **(Norman and Kabler, 1953).** The human diseases associated with fresh fruits and vegetables are mainly those transmitted by the fecal–oral route **(De Roever, 1998).** *Serratia sp.* are commonly found inhabitant of plants **(Grimont *et al.*, 1981). Jacxsens *et al.* (2003)** reported that the spoilage of mixed cut bell peppers and grated celery was related to the populations of lactic acid bacteria and yeasts. *Bacillus subtilis, Lysinibacillus sphaericus*, *Bacillus amyloliquefaciens* and *Bacillus pumilus* isolated from pepper fruits (*Capsicum annuum* L.) causes discoloration **(Liu Hai *et al.*, 2013).** The methods used to isolate spoilage microorganisms are mainly based on culture procedures by dilution method. **O'Connor-Shaw *et al.* (1994)** extracted microorganisms from the freshly cut fruits by dilution method using sterile 0.1% peptone water and 0.5% sodium chloride. **Ukuku and Fett (2002)** enumerated microbes on cut melons using plate count agar method. In this current research the isolation of microbes was carried out by serial dilution and plating method. Sequences of the 16S rRNA gene are generally used for bacterial identification **(Garcıa-Martınez *et al.*, 2001).** The Naga chilli is mainly cultivated in Kohima, Peren, Mon and Dimapur areas in Nagaland as well as in Assam and Arunachal Pradesh **(Anon, 2008).** This chilli has massive scope in domestic as well as international market due to its major high capsaicin content. In 2010–2011, the quantity of chilli exported was 2, 41,000 metric tonnes at a value of 2,144.08 crore **(Spices Board India, 2012)**. Hence the study has great impact on production of quality chilli products for the consumers. Screening of bacteria in crop plant is economically significant. In view of this, the present study was conducted to isolate and identify the bacteria present in *C. chinense* Bhut Jolokia dry fruits.

MATERIAL AND METHODS

Sample Collection

Capsicum chinense Bhut Jolokia dry fruits were obtained from Manipur, North India. The morphology of the fruit shape, color, seed color and size of the *C. chinense* were examined according to the description of **Moscone *et al.*,(2007)** and **Dias *et al.*, (2013)**.

Isolation

The dry fruits were washed with sterile distilled water and then rinsed with 1% (v/v) detergent (Tween 20, HIMEDIA, MUMBAI) for 5min. Then it was surface sterile with 0.1% (w/v) aqueous solution of Mercuric chloride (FISHER SCIENTIFIC, MUMBAI) for 2 sec followed by 4-5 rinses in sterilized double distilled water. Using sterile scalpel the fruits were cut and seeds were collected (Manzoor et al., 2013). The collected seeds were incubated by soaking in Peptone broth (HIMEDIA, MUMBAI) for 24hours. After incubation, sample was serially diluted followed by spread-plating (0.1 ml) and plates were incubated at 37°C for 24 hours. The pure colonies were isolated and sub-cultured for molecular characterization.

Biochemical and morphological characterization

Purified isolates were characterized by biochemical analysis using Nitrate reductase, Catalase test, Gelatin hydrolysis, Casein hydrolysis, Starch hydrolysis, Urease test, H_2S production, Voges–Proskauer, L-Arabinose, D-Cellobiose, D-Fructose, Citrate, D-Alanine, D-Galactose, Glucose, Lactose and Maltose (HIMEDIA, MUMBAI AND FISHER SCIENTIFIC, MUMBAI). Gram staining (KIT, HIMEDIA, and MUMBAI) and motility tests were performed as morphological tests.

Molecular Characterization of the isolate

Genomic DNA isolation

Two ml of 24 hour bacterial culture were centrifuged at 6000 rpm for 5 min. The supernatant was discarded. One ml of UniFlex™ Buffer 1 and 10 µl of RNase were added to the pellet, mixed well by pipetting and incubated for 30 min at

37ºC in a water bath. To the lysed samples 1 ml of 1:1 phenol: chloroform was added and mixed well. The samples were centrifuged at 10,000 rpm for 15 min at room temperature. The aqueous layers were separated in a fresh 1.5 ml vial. To the aqueous layer 1 ml of UniFlex™ Buffer 2 was added and mixed well by pipetting. The mixture was centrifuged at 12,000 rpm for 15 min at room temperature. The supernatant was discarded. To the pellet 500 µl of 70% ethanol was added, mixed and centrifuged at 10,000 rpm for 10 min at 4ºC. The supernatant was discarded. The pellet was air dried for about 10-15 min till the ethanol evaporated. The pellet was resuspended in 50-100 µl of UniFlex™ Elution Buffer. DNA was stored at -20ºC (UniFlex™ DNA Isolation kit method).

PCR amplification and sequencing

The 16S ribosomal RNA was amplified by using the thermocycler (EPENDORF EP GRADIENT) with *Taq* DNA polymerase and primers 8F (5' AGA GTT TGA TCC TGG CTC AG 3') and 1492R (5' ACG GCT ACC TTG TTA CGA CTT 3'). The cyclic conditions were as follows: initial denaturation at 94ºC for 3min, 35cycles at 94ºC for 1min, 54ºC for 1min, and 72ºC for 2min, and final extension at 72ºC for 10min and final hold at 4ºC. PCR amplification was detected by agarose gel electrophoresis and visualized by alpha image gel doc (Alpha Imager EC – Gel Document) with ethidium bromide staining. The PCR product obtained was sequenced by an automated sequencer (Genetic Analyzer 3130, Applied Biosystems, and USA). The same primers as above were used for sequencing. The sequence was compared for similarity with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST available at http:// www.ncbi-nlm-nih.gov/. 16S rRNA sequence was then submitted to the GenBank, NCBI, USA. The phylogenic tree was generated using neighbour – joining method. The DNA sequences were aligned and phylogenetic tree was constructed by Neighbour joining method using ClustalW. The 16s rRNA sequence was then submitted to GeneBank, NCBI, USA.

RESULTS

***Capsicum chinense* fruits morphology**

The *Capsicum chinense* dry fruits used in our study were collected from Manipur and were identified taxonomically by studying the morphology (Table 1). The Fruit was sub-conical, wrinkled with undulating surface. Fruit length, diameter and dry weight were 5.67±0.9cm, 3.8±9.1cm and 0.75±0.18 respectively. The fruits contain 51.6±16.83 seeds (depending on fruit size) and cream to yellow lenticular seeds about 3.2±3.7mm in diameter. The fruits, especially the seeds and placenta, have a biting pungent taste. The shape of the fruit is sub-conical to conical and about 2.50–2.95cm wide at the shoulders and 5.95–8.54cm in length; and fresh weight of the fruit was 12-16 g. The fruit skin is finely wrinkled with undulating surface as shown in the figure 1A. The seeds of the studied species were similar in structure and shape, but varied in size as shown in figure 1B. These seeds are campylotropous, ellipsoid, long and broad oval in longitudinal section, elliptical in cross section, and with an embryo, endosperm and a mantle consisting of a silver film. Longitudinal section of the *C. chinense* entire seed embryo axis was of circinate type showing its ellipsoid, long, broad oval shape as shown in the figure 1C.

Biochemical and morphological characterization of the isolate

The colonies were identical in the morphology on the dilution plate. The colonies were yellowish and translucent on nutrient agar plate. Four bacterial colonies were picked from each dilution and assigned CPS1, CPS2, CPS3 and CPS4. These colonies were subjected to biochemical and morphological tests. Based on the comprehensive range of phenotypic tests four isolates were identical (Table 2).

Molecular Characterization of the isolate

The isolated pure culture was subjected to isolation of genomic DNA. The isolated genomic DNA was electrophoresed on 1% agarose gel stained with ethidium bromide. The genomic DNA was observed under UV transilluminator. The isolates showed good yield of DNA. The PCR reaction mix was analyzed by agarose gel electrophoresis and the DNA of the expected size was purified and sequenced. An amplicon of 700 base pair was obtained on PCR amplification of the DNA with specific forward and reverse primers. The new isolate was identified as *Paenibacillus illinoisensis* based upon the similarity search of 16S rRNA sequence (99 % similarity) in the NCBI GenBank database. It has been assigned *Paenibacillus illinoisensis* strain Nagoth JAR 007 and was submitted in the Genbank (KC886309). A phylogenetic tree was constructed based on Neighbour-joining method showed in figure 2.

DISCUSSION

Fruits and vegetables are ideal environment for the growth of microorganisms. The isolate *Paenibacillus illinoisensis* strain Nagoth JAR 007 resists and survives in the highest pungent environment. The presence of microorganisms in plant and food are noteworthy, because not only are they involved in food spoilage but are also pathogens causing various diseases. The association of microorganisms is either beneficial or harmful to the host plant. *Serratia rubidaea* degrade the complex polysaccharides of the plant cell wall and membrane **(Samiah Al-Mijalli, 2013)**. The genus *Erwinia sp*. cause major microbial spoilage of whole vegetables that causes soft rot **(Liao, 2005; Lund, 1983).** On the other hand the presence of non pathogenic endophytic bacteria in *Capsicum annuum* plays an important role in plant vitality and resistance to abiotic or biotic stress. *Arthrobacter sp.* and *Bacillus sp.* resulted in a significantly reduced upregulation or even downregulation of the stress-inducible genes CaACCO and CaLTPI in green chilli **(Sziderics, 2007)**. The presence of microorganisms in the seeds and ovules of herbaceous plants were reported by **Orvin Mundt, (1976).** It has been isolated from potato seeds **(Hollis, 1951),** corn and pea seeds **(Samish, 1963)** and seeds of *Triticum sp.* **(Dueggeli, 1904).** The research findings predict that the bacteria might have entered the seeds through the vascular system **(Schuster, 1974),** the germ tube of the pollen grain and the hilum of ripened seeds **(Baker, 1966).** The occurrence of epiphyseal flora within tissue of fruits and vegetables are through various pathways. According to **Samish *et al*. (1962)** in green pepper fruits bacteria are unevenly distributed, and entry may be from the stem scar tissue through the core and into the endocarp. The bacteria enter fruit tissue more readily in the early stages of fruit development. The primary entry of microorganisms into the crop starts in the seed itself and also during crop growth in the field, during harvesting and postharvest handling, or during storage and distribution **(Margaret Barth, 2009)**. *Bacillus alvei* and *Enterobacter aerogenes* were indentified in the rhizosphere and rhizoplane of *Capsicum frutescens* **(Oyeyiola *et al*., 1994).** Endophytic colonization was shown to result from root infection or contamination of seeds **(Tyler and Triplett, 2008).** Hence in the current study the microorganism isolated from the seed of *C. chinense* seeds may be from seed contamination in the soil and external damage such as bruising, cracks and punctures creating sites for establishment and growth of the spoilage microbes.

P. illinoisensis belonging to this genus have been found in a diversity of environments such as soil, water, rhizosphere, vegetable matter, forage and insect larvae, as well as clinical samples **(Lal, 2009; Mc Spadden Gardener, 2004; Montes, 2004; Ouyang *et al*., 2008).** *Paenibacillus* species are found to be a plant growth promoting rhizobacteria (PGPR). It has been used as biofertilizers and also as antagonists (biopesticides) of known root pathogens **(Bloemberg, 2001).** The antagonistic activity of *P. illinoisensis* KJA-424 against *P. capsici* infection has suppressed the activities of peroxidase (POD) and superoxide dismutase (SOD) in the root of pepper plant **(Jung *et al*., 2006).** Most of the endophytes are nonpathogenic and have a role in plant resistance to abiotic or biotic stress. Hence the results conclude that the presence of *Paenibacillus illinoisensis* in the seed would have contributed for the growth and protection of the host pepper plant under unfavorable condition during germination.

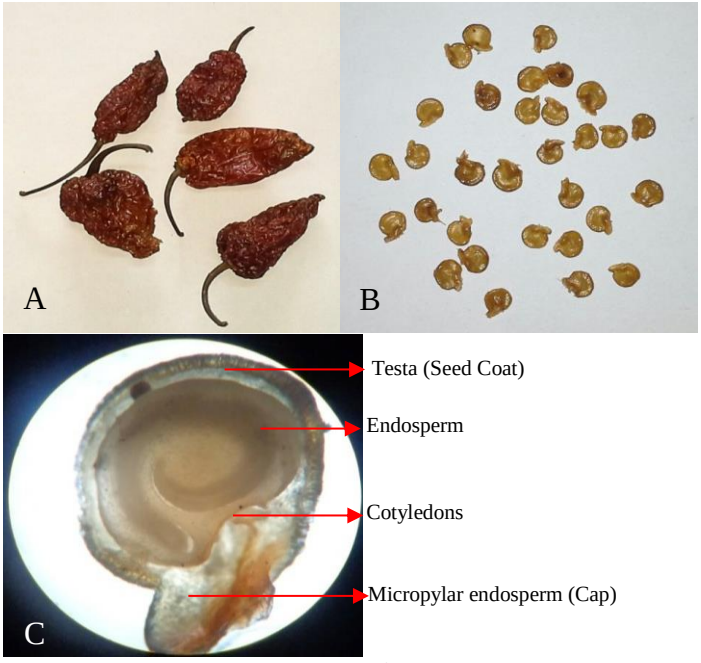

Figure 1 *Capsicum chinense*: **A-** Entire fruit**, B** –seeds and **C-** longitudinal section of *Capsicum chinense* seed

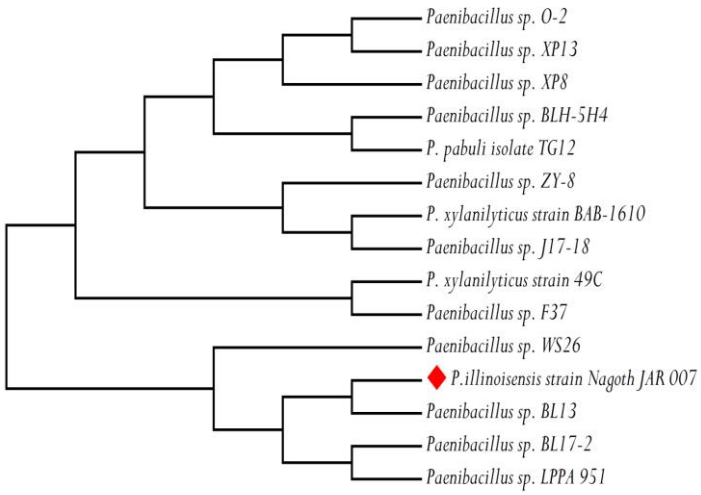

Figure 2 Phylogenetic tree constructed based on neighbor-joining method for *Paenibacillus illinoisensis* strain Nagoth JAR 007

Table 1 Morphology of *Capsicum chinense* fruit

S.NO	PARTS	**Mean with SD**
1	Fruit length (cm)	**5.67±0.9**
2	Fruit diameter (cm)	**3.8±9.1**
3	Fruit weight(gm)	**0.75±0.18**
4	Seed number	**51.6±16.83**
5	Seed size (mm)	**3.2±3.7**
6	Embryo size (mm)	**2.8±3.1**

Table 2 Biochemical characterization tests of bacterial isolates from the seeds of *C. chinense*

Morphological characteristics	CPS 01	CPS 02	CPS 03	CPS 04
Gram's staining	+	+	+	+
Motility	+	+	+	+
Biochemical characteristics				
Nitrate reductase	-	-	-	-
Catalase test	+	+	+	+
Gelatin hydrolysis	+	+	+	+
Casein hydrolysis	+	+	+	+
Starch hydrolysis	+	+	+	+
Urease test	-	-	-	-
H_2S production	-	-	-	-
Voges–Proskauer	-	-	-	-
L-Arabinose	+	+	+	+
D-Cellobiose	+	+	+	+
D-Fructose	+	+	+	+
Citrate	-	-	-	-
D-Alanine	-	-	-	-
D-Galactose	+	+	+	+
Glucose	+	+	+	+
Lactose	-	-	-	-
Maltose	+	+	+	+
Mannitol	+	+	+	+

Symbols – and + meaning negative and positive results

CONCLUSION

The identification of the endophytic bacteria present in the seeds of dry fruits of *C. chinense* was confirmed by 16S rRNA gene sequencing studies. The phylogenetic tree revealed that the organism fits into an evolutionary cluster comprising members of *Paenibacillus sp*. Finally, based on the above study it can be concluded that bacterial species can be exploited fully for the growth and protection of host pepper plants under unfavorable condition during the germination. Further research is necessary to find out the non - pathogenicity of this novel strain to human beings when the affected fruits are consumed raw or cooked.

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