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Characterization of Plant Growth Promoting Endophytic Bacteria from Castor Plants [*Ricinus communis* (L.)]

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ABSTRACT

The present study was carried out in June–Janauary (2021–2022) at the Laboratory of the Department of Microbiology, Chimanbhai Patel College of Agriculture, Sardarkrushinagar Dantiwada Agricultural University (SDAU), Sardarkrushinagar, Gujarat, India. 15 promising endophytic bacterial isolates from castor plants showing higher dry matter accumulation in seedlings of wheat were subjected to detailed characterization for their molecular and plant growth promoting abilities. The bacterial isolate Erc7 performed best for plant growth promoting activities viz., ammonia and IAA production, phosphate solubilization, nitrogen fixation, HCN production and antagonistic activity against *Fusarium oxysporum* f. sp. *ricini* in-vitro which cause wilt in castor plant. Pot trials involving wheat seed inoculation (*rabi* 2021–22) with bacterial isolates showed that the treatment with endophytic bacterial isolate Erc7 showed maximum shoot and root length (47.65 cm, 10.50 cm), shoot fresh and dry weight (824.90 mg, 308.83 mg), root fresh and dry weight (122.37 mg, 36.20 mg), spike length (09.76 cm) and number of seeds in central tiller plant^{-1} (15.33). PCR amplification of 16S *rRNA* genes and ARDRA profiling using restriction enzymes *AluI, TaqI* and *HpaII* grouped the 15 isolates in 2 clusters (A and B) at a Jaccard's similarity co-efficient of 0.54. The best PGPR isolate Erc7 was gene sequenced for 16S *rRNA* gene. The sequence was submitted to the NCBI Genbank (Accession number ON514555). Upon BlastN It showed maximum similarity to *Bacillus cereus*. The isolates Erc7 showed presence of diplobacillus endospores which is a typical characteristic of *Bacillus cereus*.

KEYWORDS: *Bacillus cereus*, characterization, dendrogram, *Fusarium oxysporum* f.sp. ricini, PGPR, castor

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Data Availability Statement: Legal restrictions are imposed on the public sharing of raw data. However, authors have full right to transfer or share the data in raw form upon request subject to either meeting the conditions of the original consents and the original research study. Further, access of data needs to meet whether the user complies with the ethical and legal obligations as data controllers to allow for secondary use of the data outside of the original study.

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1. INTRODUCTION

Bacteria can positively impact plant growth and health while plants can select their microbiome through beneficial bacterial colonizers, including those living within the plant tissues (Marasco et al., 2012). An endophyte-free plant is a rare exception to what is typically found in nature (Partida-Martinez and Heil, 2011). Endophytic bacteria colonize the plants without any negative effects (Chaturvedi et al., 2016). The rhizosphere plays a 'critical gating role' for the entry of endophytes into plants (Edwards et al., 2015). Lundberg et al. (2012) concluded that only a small subset of the rhizosphere microbiome enters the plant immune system actively excludes some specific bacterial groups and shield the plant from root pathogen attack by producing biofilm around roots (Rybakova et al., 2015). Bacteria adopt various direct and indirect mechanisms and complex relationships to reproducibly improve the plant growth in a given growth condition (Marasco et al., 2012, Rashid et al., 2012). Deeb et al. (2013) and Kumar et al. (2016) isolated endophytic bacteria from plant parts like leaf, root, stem and petiole. Endophytes enter the plant through the roots that control entry of the soil bacteria from the rhizosphere and rhizoplane (Truyens et al., 2014). In direct mechanism, promotion of plant growth occurs through the production of phytohormones like indole acetic acid, cytokinin, phosphorous solubilization and biological nitrogen fixation (Nutaratat et al., 2014). Indole acetic acid production and phosphate solubilisation activity has also been observed in endophytic isolates and produce secondary metabolites i.e., siderophore, antibiotics, hydrogen cyanide (Souza et al., 2015). Endophytes enhance crop yield by producing indole acetic acid, gibberellic acid (Khan et al., 2014) ethylene (Kang et al., 2012) and auxins (Dutta et al., 2014). Diazotrophic bacteria are able to fix atmospheric nitrogen and transform it into ammonia (Rangjaroen et al., 2014). These endophytes have the capacity to produce secondary metabolites (Shweta et al., 2013) which has immense applications such as antibacterial, antifungal, antiviral, antioxidant, insecticide, anti-diabetic and immunosuppressive activities whereas some endophytes can also produce HCN (Etesami et al., 2014). Several bacterial endophytes exhibiting antibiotic production against antagonistic bacterial cells and the fungal plant pathogens (Kumar et al., 2013). PCR amplification and 16S RNA gene sequencing of the endophytic bacteria using universal primers 27F and 1541R is instrumental in ascertaining the identity of bacterial endophyrtes (Nongkhlaw and Joshi, 2015, Ullah et al., 2018). Ali et al. (2014) observed that genes common to all of the endophytic bacteria were potentially involved in endophytic behaviour, including genes encoding transporter proteins, plant polymer degradation or modification, transcriptional regulation, detoxification and functions like 2-isopropylmalatesynthase and diaminopimelate decarboxylase. Shidore et al. (2012) exposed the bacterial endophyte *Azoarcus* sp. strain BH72 to exudates of rice (*Oryza sativa* cv. Nipponbare) plants and employed whole genome microarray approach to discover the endophyte genes involved in root colonization.

Due to escalating food demand and deterioration of environmental quality and extensive use of chemical fertilizers and pesticides, there is an urgent need to seek a substitute to take over these chemicals (Sharma and Singhvi, 2017). The beneficial bacteria that inhabit inside plants can improve plant growth under natural and challenging conditions. They have the capacity to improve plant nutrient uptake, soil health, and strengthen plant defense to provide higher yield and confer sustainability in agriculture (Jain and Kumar, 2017). The PGPB colonize the endosphere after colonization in the rhizosphere. They adopt several mechanisms including motility, attachment, degradation, plant growth promotion and evasion of plant defenses (Glick, 2014; Singh, 2013).

The present study was aimed for isolation of endophytic bacteria from *Ricinus communis* and to test their potential as bioinoculant and plant growth promotion and their molecular characterization.

2. MATERIALS AND METHODS

The present study entitled "Characterization of Plant Growth Promoting Endophytic Bacteria from Castor Plants [*Ricinus communis* (L.)]" was carried out in June–Janauary (2021–2022) at the Laboratory of the Department of Microbiology, Chimanbhai Patel College of Agriculture, Sardarkrushinagar Dantiwada Agricultural University (SDAU), Sardarkrushinagar, Gujarat, India. The latitude and longitude of the sampling site was 24°19'19"N and 72°16'44"E, respectively.

2.1. Isolation of endophytic bacteria from castor plants

Freshly collected explants (roots, stems, leaves) from the castor plants were surface sterilized (Yan et al., 2018). Bacterial growth in Petri plates were carefully isolated and subcultured repeatedly to obtain pure culture.

2.2. Characterization of the bacterial isolates for plant growth promotion activities

2.2.1. Seed germination test

Total 33 endophytic bacterial isolates were screened based on the dry weight accumulated by wheat seedlings. The seedlings' fresh and dry weight, root and shoot length was recorded. On this basis, 15 best performing endophytic bacterial isolates were selected for further detailed studies.

2.2.2. Production of ammonia

The bacterial isolates were grown in peptone water (Dye,

1962) in test tubes in triplicates. Incubated the tubes at 30°C for 4 days. Added 1ml Nessler's reagent in each tube. Presence of faint yellow colour, deep yellow and brownish colour indicated low, medium and high amount of ammonia production, respectively.

2.2.3. IAA production

Inoculated loopful of bacterial culture in 25 ml Luria-Bertani broth medium amended with 50 µg ml^{-1} tryptophan in triplicates in test tubes and incubated the cultures at 28°C on rotary shaker for 24 h. Quantification of IAA produced by the isolates in test tubes was using standard IAA curve following the protocol of (Gordon and Weber, 1951).

2.2.4. Phosphate solubilization

The bacterial cultures were grown in Pikovskaya broth medium following protocol of Verma et al. (2004). After three days, the suspensions were centrifuged (10,000 g) and solubilized phosphates in the supernatant was estimated using ammonium molybdate solution and chlorostannous acid. The development of blue color indicated the quantity of phosphate solubilization. The optical density was measured at 600 nm and the quantity of phosphate solubilization was measured using KH_2PO_4 standard curve (Olsen and Sommers, 1982).

2.2.5. Nitrogen fixation abilities

The isolates were inoculated into nitrogen free semi solid medium and incubated at 30°C for 7 days. The isolates capable of forming pellicle in the subsurface were subsequently sub-cultured 5 times on the N-free medium. The isolates that retained pellicle nature in N-free semi solid medium were considered positive for nitrogen fixation (Dobereiner et al. 1995).

2.2.6. Hydrogen cyanide (HCN) production

The bacterial isolates were streaked in petriplates poured with King's B medium amended with 4.4 g l^{-1} glycine. Whatman No. 1 filter paper disc soaked in 0.5% picric acid in 2% sodium carbonate was placed in the lid of each petri plate. Sealed the petri plate with parafilm and incubated at 28±2°C for 4 days. Change of colour of the filter paper from yellow to orange to dark brown indicated HCN production (Bakker and Schipper, 1987).

2.2.7. Suppression of fungal plant pathogen

Endophytic bacterial isolates were assayed for suppression of *Fusarium oxysporum* f. sp. *ricini*, the causal agent of castor wilt. The antagonistic activity of e bacterial isolates were studied against fungal pathogen by placing actively growing fungal culture bit at the centre and streaking the bacterial isolates at a distance of 3.0 cm at 4 places on petriplates (having half concentration each of NA and PDA medium). Mycelial growth of control having only fungus and antagonist with bacteria was investigated 7 days after incubation at room temperature and the Percent inhibition of fungal growth was estimated (Vincent, 1927).

$$I=(C-T)/C\times100 \quad \ldots\ldots\ldots\ldots\ldots\ldots(1)$$

Where, I=Percent inhibition of mycelia growth.

C=Mycelial growth in control.

T=Mycelial growth in treatment.

2.2.8. Inoculation of bacterial isolates in wheat in pots under net house

The bacterial isolates were tested for their plant growth promoting ability under pot studies on Wheat (GW 496). First 15 treatments comprised of seed treatment with the 15 different isolates and the 16th treatment was maintained as control (untreated). The plant growth parameters recorded for this pot experiment were shoot length and root length (cm), fresh weight and dry weight of shoot and root (mg), spike length (cm) and number of seeds in central tiller $plant^{-1}$ after harvest. The data recorded were analysed using MSTAT-C software and DNMRT.

2.3. Molecular characterization of the endophytic bacteria

2.3.1. Genomic DNA extraction and amplification of 16S rDNA gene

DNA was extracted from the Log phase growth of bacterial pure cultures using Thermo Scientific GeneJET Genomic DNA Purification Kit (K0721, Thermo Fisher Scientific). PCR was performed using 10 pmol of forward (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse (5'-AAGGAGGTGATCCAGCC-3') primers (Weisberg et al., 1991), 2 µl of extracted DNA, 5 µl Taq polymerase, 10X buffer [10mM Tris HCl, pH 9.0, 1.5 mM $MgCl_2$, 500 mM KCl], 2.5 mM dNTPs. PCR amplification were performed at Initial DNA denaturation and enzyme activation steps at 94°C for 5 min., followed by 35 cycles of denaturation at 94°C for 50 s, annealing at 55°C for 1.45 m, extension at 72°C for 2 m and a final extension of 72°C for 10 m. The amplified PCR product was put to gel electrophoresis gel photograph was scanned using gel documentation system.

2.3.2. Amplified ribosomal DNA restriction analysis

The amplification 16S *r*RNA gene product were subjected to restriction digestion using tetra-cutter restriction enzymes (*TaqI, AluI* and *HpaII*) using manufacturer protocol (Himedia). The restriction digestion fragments of the amplified 16S *r*RNA gene were separated by electrophoresis on 2.5% (w/v) agarose gel in 1X TAE buffer and the gel pictures were scanned using fire Reader gel documentation system. The lengths of the restriction fragments were estimated by comparison against a Gene Ruler 50 bp DNA ladder.

2.3.3. 16S rRNA gene sequencing and phylogenetic analysis

The PCR amplified 16S *r*RNA gene product of the best

plant growth promoting endophytic isolate (Erc7) was sequenced using Big Dye terminator cycle sequencing kit (Applied BioSystems) and its phylogenetic relationship was established based on 16S *r*RNA gene sequence using reference strain sequences deposited in the NCBI GenBank database using BLAST search.

3. RESULTS AND DISCUSSION

3.1. Isolation of endophytic bacteria

33 bacterial endophytes were isolated in pure culture in petri plates from castor plants and out of 33, 15 bacterial isolates were screened based on dry weight of wheat seedling *in vitro*. Endophytic bacteria from plants have been isolated by several researchers for different purpose. Araújo et al. (2002) suggested that endophytes can be isolated from plants by surface-disinfection of the plant tissues and exposing the inner parts of plant tissues to the suitable nutrient medium so as to support growth of these bacteria. Lodewyckx et al. (2002) were of the opinion that endophytic bacteria could be isolated from the roots, leaves, stems, flowers, fruits and seeds. They further concluded that endophytic bacteria were abundant in the roots and the number decreased in the stem and leaves.

3.2. Characterization of the endophytic bacterial isolates for plant growth promotion activities

The test tubes used for test of ammonia production by bacterial isolates revealed that the isolates Erc5, Erc6, Erc10, Erc11 and Erc15 produced faint yellow colour and hence produced less amount of ammonia. The isolates Erc2, Erc3, Erc4, Erc9, Erc12 and Erc13 produced deep-yellow colour and hence produced medium amount of ammonia production whereas the isolates Erc1, Erc7, Erc8 and Erc14 produced brownish colour and hence produced high amount of ammonia (Table 1).

IAA production by various isolates ranged from 20.63 μg ml^{-1} (Erc5) to 68.75 μg ml^{-1} (Erc7). Erc7 was most efficient in IAA production and was followed closely by Erc8 (62.80 μg ml^{-1}) and Erc15 (55.94 μg ml^{-1}). Whereas, phosphate solubilization ranged from 08.64 μg ml^{-1} (Erc12) to 25.49 μg ml^{-1} (Erc7). Thus, Erc7 was most capable of solubilizing tricalcium phosphate. However, it was followed by the isolates Erc15 and Erc8, which respectively showed phosphate solubilization of 25.43 μg ml^{-1} and 24.60 μg ml^{-1} after an incubation of 48 h.

It was noteworthy to mention that only 2 isolates Erc7 and

Table 1: Plant growth promotion traits of endophytic bacterial isolates

Isolates	Production of Ammonia	Production of IAA (μg ml^{-1})	Solubilization of phosphate (μg ml^{-1})	Nitrogen fixation (Growth on Nfb medium and pellicle formation)	Antifungal Inhibition of *F. oxysporum* f. sp. ricini (%)
Erc1	High	36.36^{e}	20.88^{de}	No	37.78
Erc2	Medium	30.72^{g}	19.29^{efg}	No	55.55
Erc3	Medium	24.85^{h}	17.99^{g}	No	53.33
Erc4	Medium	21.36^{hi}	14.12^{h}	No	46.67
Erc5	Low	20.63^{i}	20.42^{def}	No	60.00
Erc6	Low	32.55^{fg}	23.12^{bc}	No	57.78
Erc7	High	68.75^{a}	25.49^{a}	Yes	71.11
Erc8	High	62.80^{b}	24.60^{ab}	Yes	68.89
Erc9	Medium	48.62^{d}	18.97^{efg}	No	48.89
Erc10	Low	22.25^{hi}	14.83^{h}	No	46.67
Erc11	Low	35.81^{ef}	22.22^{cd}	No	53.33
Erc12	Medium	32.20^{fg}	08.64^{i}	No	64.44
Erc13	Medium	24.16^{hi}	24.15^{abc}	No	55.55
Erc14	High	51.12^{d}	18.47^{fg}	No	60.00
Erc15	Low	55.94^{c}	25.43^{a}	No	51.11
SEm±	-	1.23	0.65	-	-
CD (p=0.05)	-	3.57	1.90	-	-

Note: (1) The values indicate average of three replications; (2) All the isolates were negative for HCN production; (3) Alphabets in superscript shows Duncan's New Multiple Range Test (DNMRT) ranking. Values represented by common alphabets are non-significant 5% level of significance

Erc8 showed positive result of nitrogen fixation because these isolates survived on Nfb medium upon repeated subculturing. However, none of the isolates showed HCN production.

The antifungal activity of the endophytic bacterial isolates was tested against *Fusarium oxysporum* f. sp. ricini *in vitro.* Co-inoculation of plant growth promoting bacterial endophytes and *Fusarium oxysporum* f. sp. *ricini* showed differential level of inhibition due to the antagonistic interaction between the 2 organisms. Erc7 proved the most efficient and showed 71.11% inhibition whereas Erc1 showed least inhibition (37.78%) of the fungal pathogen (Table 1).

Fusarium oxysporum f. sp. *ricini* causes in castor wilt, which is a destructive disease and induces serious yield losses around the world. The pathogen attacks host plant during at all stages when conditions are favourable (Punja, 1985).

3.3. Inoculation of endophytic bacterial isolates on wheat in pot study under net house

The 15 bacterial isolates selected for pot studies on wheat revealed considerable variation for their ability to promote performance of plant growth.

Among the 15 isolates, the bacterial isolate Erc7 resulted in highest shoot length (47.65 cm) and root length (10.50 cm) at harvest and were respectively, 38.90% and 67.46% higher over control. Erc7 resulted in highest fresh weight (824.90 mg) and dry weight (308.83 mg) of shoot at harvest and were respectively, 38.33% and 24.74% higher over control. The bacterial isolate Erc7 resulted in highest fresh weight (122.37 mg) and dry weight (36.20 mg) of root which was respectively 72.83% and 39.30% higher over control. The isolate Erc7 resulted in highest spike length (09.76 cm), which was 24.08% higher over control. The bacterial isolate Erc7 again resulted in highest number of seed in central tiller plant^{-1} (15.33), which was 32.61% higher over control (Table 2).

Evaluation of *Bacillus* strains for plant growth promotion and predictability of efficacy by *in vitro* physiological traits was done by Akinrinlola et al. (2018). (include in references) Bacilli are commonly used as plant growth-promoting agents but can be limited in effectiveness to certain crop and soil environments. Plant growth promoting bacteria may affect plant growth either directly through facilitation of the acquisition of nutrients like nitrogen, phosphorous and

Table 2: PGP traits of endophytic bacterial isolates on pot study under net house on wheat

Treatments (isolates)	Shoot length (cm)	Root length (cm)	Shoot fresh weight (mg)	Shoot dry weight (mg)	Root fresh weight (mg)	Root dry weight (mg)	Spike length (cm)	No. of seeds in central tiller plant^{-1}
Erc1	42.17^{cde}	6.73^{hi}	578.03^{fg}	232.40^{gh}	104.17^{bcd}	29.33^{c}	7.80^{cd}	11.67^{e}
Erc2	38.97^{ef}	6.93^{ghi}	614.43^{def}	239.97^{fgh}	101.77^{bcd}	20.07^{e}	9.28^{a}	14.67^{c}
Erc3	36.08^{fg}	7.53^{defg}	613.77^{def}	243.83^{fgh}	91.50^{ef}	28.77^{c}	8.13^{bc}	12.33^{de}
Erc4	39.60^{def}	6.87^{ghi}	638.70^{cde}	253.03^{defg}	86.90^{f}	25.47^{d}	8.55^{b}	13.67^{cd}
Erc5	42.75^{bcde}	7.83^{cdef}	598.90^{ef}	247.67^{efgh}	107.63^{bc}	30.50^{bc}	7.67^{cd}	10.33^{f}
Erc6	44.44^{abc}	7.83^{cdef}	615.07^{def}	245.73^{efgh}	100.00^{cde}	29.73^{bc}	9.41^{a}	14.67^{c}
Erc7	47.65^{a}	10.50^{a}	824.90^{a}	308.83^{a}	122.37^{a}	36.20^{a}	9.76^{a}	15.33^{a}
Erc8	46.40^{ab}	9.83^{b}	798.07^{a}	296.90^{ab}	111.13^{b}	32.67^{b}	9.55^{a}	15.00^{b}
Erc9	40.33^{de}	7.37^{fgh}	702.83^{b}	282.63^{bc}	106.23^{bcd}	30.27^{bc}	8.92^{b}	14.03^{cd}
Erc10	43.20^{bcd}	8.13^{cde}	541.83^{g}	227.23^{h}	96.83^{de}	30.27^{bc}	6.91^{e}	09.33^{f}
Erc11	39.40^{def}	7.70^{def}	687.75^{bc}	275.00^{cd}	107.20^{bc}	32.43^{b}	7.56^{cd}	10.33^{f}
Erc12	41.20^{cde}	8.47^{c}	608.67^{def}	258.30^{def}	100.73^{cde}	30.50^{bc}	8.98^{b}	14.03^{cd}
Erc13	42.67^{bcde}	7.40^{fgh}	623.20^{def}	240.77^{fgh}	86.83^{f}	28.30^{c}	9.39^{a}	14.67^{cd}
Erc14	42.80^{bcde}	8.20^{cd}	579.23^{fg}	232.67^{gh}	100.17^{cde}	31.00^{bc}	7.41^{de}	10.00^{f}
Erc15	39.73^{def}	7.47^{efg}	660.77^{bcd}	268.23^{cde}	98.97^{cde}	30.50^{bc}	7.72^{cd}	11.67^{e}
Control	34.30^{g}	6.27i	596.33^{ef}	239.97^{fgh}	70.80g	21.97^{e}	7.41^{de}	10.33^{f}
SEm±	1.192	0.214	16.434	7.143	2.949	0.920	0.186	0.432
CD (p=0.05)	3.459	0.622	47.695	20.731	8.558	2.670	0.540	1.254

NOTE: Alphabets in superscript shows Duncan's New Multiple Range Test (DNMRT) ranking. Values represented by common alphabets are non-significant 5% level of significance

iron or by modulation of plant growth through various plant hormones line auxins, ethylene or cytokinins. However, the indirect mechanisms adopted by PGPB to promote plant growth includes production of antibiotics, siderophores, ACC deaminase, cell wall-degrading enzymes, induced systemic resistance, and production of pathogen-inhibiting volatile compounds (Singh, 2013). Bacterial endophytes isolated from the Zn/Cd hyperaccumulator plant *Sedum plumbizincicola* exhibited PGPB activities. The isolates were positive for ACC deaminase activity, production of indole-3-acetic acid and siderophores, phosphate solubilization, and resistance to heavy metals like Cd, Zn and Pb.

Egamberdieva et al. (2017) stated that endophytic bacteria are an essential determinant of cross-tolerance to biotic and abiotic stresses in plants. They isolated 40 bacteria from internal root tissues of chickpea grown in salinated soil and observed that four bacterial isolates, namely *Bacillus cereus* NUU1, *Achromobacter xylosoxidans* NUU2, *Bacillus thuringiensis* NUU3, and *Bacillus subtilis* NUU4 colonizing root tissue demonstrated plant beneficial traits and/or antagonistic activity against *F. solani*. *Agrobacterium fabrum*, *Acinetobacter radioresistant*, *Brevibacillus brevis*, *Bacillus cereus*, *Bacillus subtilis*, *Paenibacillus barengoltzii*, and *Burkholderia cepacia* were identified as major bacterial endophytes from the leaves of medicinal plant *Pulicaria incisa* (Fouda et al., 2021).

Several experiments have reported increase in plant biomass in response to endophytic bacteria and thus rendering multifarious advantages to the host plants (Dutta and Thakur, 2017). *B. cereus* BI-8 and *B. subtilis* BI-10 showed high efficacy against the three phytopathogens *Fusarium oxysporum*, *Alternaria alternata,* and *Pythium ultimum*, with inhibition Percent ranging from 20%±0.2% to 52.6%±0.2% ($p \leq 0.05$) in vitro (Fouda et al., 2021). *B. cereus* BI-8 and *B. subtilis* BI-10 were good in IAA production and they produced IAA of about 117±6 and 108±4.6 µg mL^{-1}, respectively. These 2 isolates were also good in phosphate solubilization and ammonia production, and were positive for amylase, protease, xylanase, cellulase, chitinase, and catalase activity. They observed that 2 endophytic bacteria (*B. cereus* BI-8 and *B. subtilis* BI-10) when used as bio-inoculants for maize seeds, significant increase in root length as well as the fresh and dry weights of the roots compared to the control plants.

3.4. Molecular characterization of the plant growth promoting endophytic bacteria

3.4.1. Amplification of 16S rRNA gene

The DNA isolated from the bacterial isolates revealed presence of single band on 0.8% agarose gel upon electrophoresis (Figure 1). DNA concentration of the isolates ranged from 37.5–428.6 µg ml^{-1}. However, DNA concentration of all the isolates were maintained to 25.0 µg ml^{-1} with sterile deionised water.

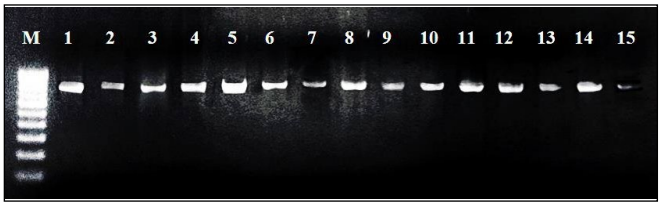

Figure 1: PCR amplification of 16S rRNA gene of the endophytic bacterial isolates (Note: Numbers 1to 15 sequentially represent the isolates Erc1 to Ecr15)

The *16S rRNA* gene amplification by polymerase chain reaction with the 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and Rd1 (5'-AAGGAGGTGATCCAGCC- 3') primers revealed a band size of about 1.5Kb on 1.5% agarose gel stained with ethidium bromide in all the bacterial isolates (Figure 1).

PCR amplification for 16S *r*RNA gene was also carried out by Bind and Nema (2019) in 20 µL reaction volume having DNA 2 µl, Taq buffer 2 µl, $MgCl_2$ 1.4 µl, dNTPs 0.4 µl, Primers 2 µl, Taq DNA Polymerase 0.4 µl and nuclease free water 11.8 µl. Reaction mixtures were put to polymerase chain reaction (PCR) at optimized temperature conditions.

3.4.2. Amplification ribosomal DNA restriction analysis (ARDRA)

PCR amplified products of 16S *r*RNA gene were then digested with restriction enzymes *Alu*I, *Taq*I and *Hpa*II to determine whether there is a restriction fragment length polymorphism that can be used to identify certain bacteria. The restriction enzyme *AluI, TaqI* and *HpaII* were able to produce diagnostic fingerprints in the isolates under investigation. *AluI* produced three fragments, *TaqI* 2–4 fragments whereas *HpaII* produced four fragments in the endophytic bacterial isolates (Table 3, Figure 2).

In Amplified DNA restriction analysis, the dendogram prepared using restriction profile obtained with all the restriction enzymes (*AluI*, *TaqI* and *HpaII*) grouped these 15 isolates of bacteria into 2 groups (A and B) at the Jaccard's similarity co-efficient of 0.54 (Figure 3). The first cluster A got divided into many sub groups which includes bacterial isolates Erc1, Erc2, Erc3, Erc4, Erc5, Erc6, Erc7, Erc9, Erc10, Erc11 and Erc12 whereas the cluster B divided into 2 sub groups; the first sub group comprised of only one isolate Erc8 and the second sub group was further divided in to 2; of which one included the isolate Erc13 and another included isolates Erc14 and Erc15.

Table 3: Fragments (bp) produced by the Amplified Ribosomal DNA Restriction Analysis of the endophytic bacterial isolates

Isolates	*AluI*	*TaqI*	*HpaII*
Erc1	650,470,330	640,415,267,175	630,400,215,160
Erc2	650,470,330	640,415,267,175	630,400,215,160
Erc3	650,470,330	640,415,267,175	630,400,215,160
Erc4	660,480,330	640,415	630,400,215,160
Erc5	660,480,330	640,415,267,175	630,400,215,160
Erc6	620,480,320	640,415,267,175	620,400,215,150
Erc7	620,480,320	640,415,267,175	620,400,215,150
Erc8	635,490,340	640,415	620,375,215,150
Erc9	670,500,330	640,415,267,175	620,400,215,150
Erc10	620,480,320	640,415	620,400,215,160
Erc11	620,480,320	640,415,267,175	630,400,215,160
Erc12	620,480,320	640,415,267,175	630,400,215,160
Erc13	670,500,340	640,415,267,175	630,400,220,170
Erc14	650,510,340	640,415,267,175	630,400,220,170
Erc15	650,510,340	640,415	630,400,220,170

Three restriction enzymes (*AluI*, *TaqI*, *HpaII*) were used for restriction digestion of the amplified 16S rRNA gene. The fragment length is expressed in terms of base pair (bp)

3.3.3. Gene sequencing and identification of the best PGPR isolate

Validation of phylogenetic position of the best performing endophytic bacterial isolate (Erc7) was done based on partial 16S *r*RNA gene sequencing. The sequence obtained was compared using the BlastN tool with the nucleotide sequences already present in the NCBI GenBank database and this sequence was submitted to the NCBI Genbank with Accession number ON514555. The results revealed maximum similarity of the isolate Erc7 to *Bacillus cereus* (Figure 4). Thus, the endophytic bacterial isolate was confirmed to belong to the bacterial species *Bacillus cereus*. This conclusion was further strengthened from the finding that the isolate Erc7 upon spore staining (Cappuccino and Sherman, 2002) showed elliptic shaped spores under microscope using oil immersion lens. The endospores were found in pairs which is the diplobacillus morphological characteristic of the *Bacillus cereus.* The isolate Erc7 also showed best result as plant growth promoting bacteria.

It is considered that endophytic colonization of plants is a complex process and requires the efficiency of bacteria to compete effectively in the rhizosphere soil so as to find an edge over others to communicate and interact with

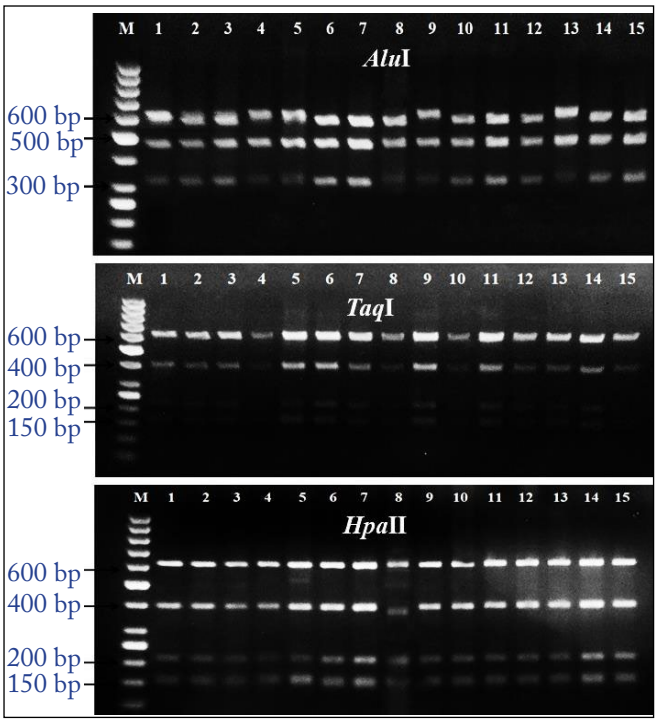

Figure 2: Restriction profile of the PCR amplified 16S rRNA gene of the endophytic bacterial isolates (Note: M=Marker (50 bp); Numbers 1to 15 sequentially represent the isolates Erc1 to Ecr15)

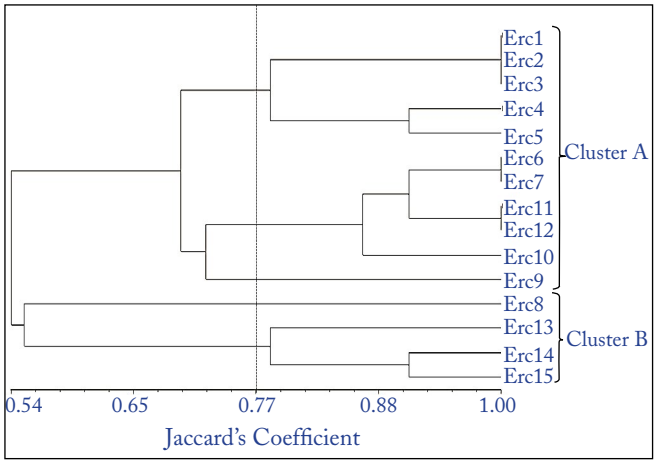

Figure 3: Dendrogram based on ARDRA pattern obtained with restriction enzyme AluI, TaqI and HpaII using Jaccard's similarity co-efficient and Unweighted Pair Group Method with Arithmetic Average (UPGMA)

the plant roots. Further, the root exudates are chemically diverse and include such molecules involved in attracting endophytes to be able to colonize the internal plant tissues. The bacterial interaction with the plants influences the growth-promotion and the efficiency of the PGP strains is possibly dependent on the genetic factors of both partners influence plant colonization and growth promotion by the endophytic bacteria (Afzal et al., 2019).

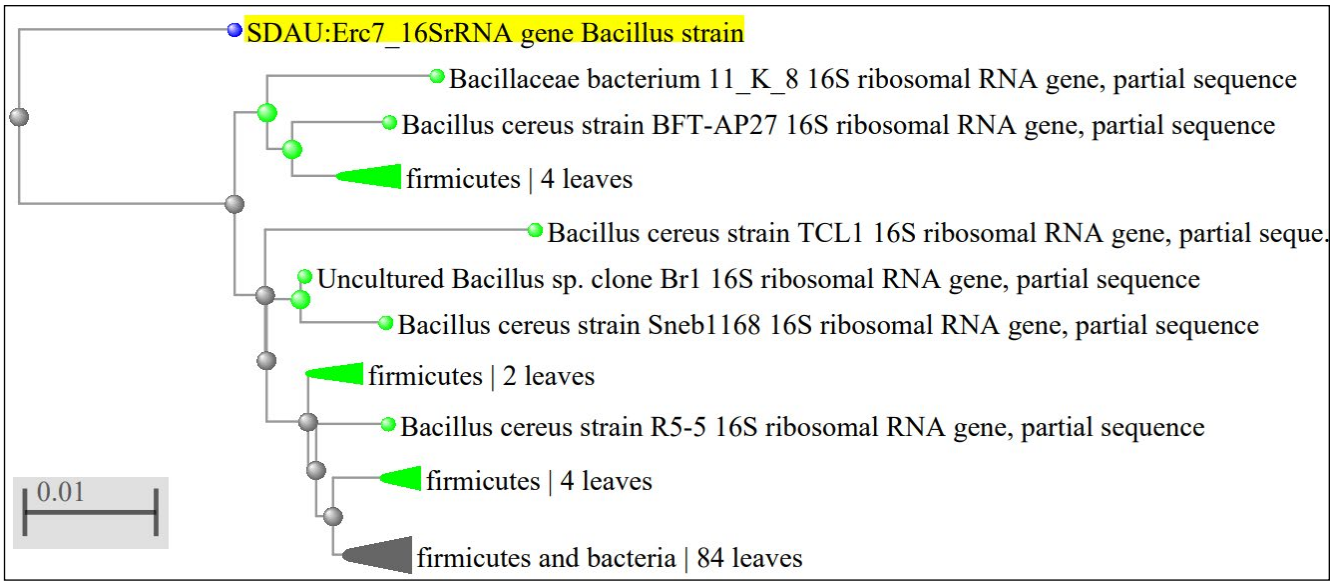

Figure 4: Phylogenetic grouping of the isolate Erc7 by Neighbour joining method

4. CONCLUSION

The isolate Erc7 was best in ammonia production, IAA production, phosphate solubilization, HCN production and antifungal activity against fungal pathogen. This isolate promoted shoot length, root length, fresh and dry weight of shoot, fresh and dry weight of root, spike length and number of wheat grains at harvest. Therefore, Erc7 is considered best for plant growth promotion and was identified as *Bacillus cereus* based upon morphological characteristics, PCR amplification and sequencing of 16S rRNA gene.

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