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Research Article

Isolation and Identification of potential diazotrophic Endophytic Bacteria from Sweet flag *(Acorus calamus)* medicinal plant

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ABSTRACT

An endophytic bacterium was isolated from *Acorus calamus* rhizomes, which is an important medicinal plant. The rhizome is an important medicinal nutrient source of microorganisms, in the rhizosphere. The isolate was evaluated for various biochemical tests. Further the isolate PPAU01 was subjected to 16S rRNA sequencing. The isolate was identified as *Enterobacter cloacae* sub sp dissolvens which was selected for further studies. The isolate was able to exhibit the plant growth promoting traits such as IAA production, ARA activity, Phosphate solubilization and siderophore production. The ability of this isolate was tested under pot culture condition. The isolate PPAU01 treated plants recorded the maximum germination percentage (93.3%), vigour index (1156.23), plant height (61.03 cm), rhizome dry weight (37.02g) and β-asarone compound (2.26%) compared to control.

Key words: Endophytic bacteria, 16S rRNA sequencing, PGPR traits, *A. calmus*.

INTRODUCTION

Acorus calamus Linn, (Family: Araceae) commonly known as sweet flag or Bach plant which has been known for its beneficial and medicinal value in the Asia since long time. From the ancient times it is harvested on the commercial scale and mostly cultivated in the asian region. It is a most valuable plant in the medicinal sciences almost throughout the India (Rupali Singh et al, 2011). The sword like leaves of the plant is a characteristic feature to identify the plant having several therapeutic values for medicinal and pharmaceutical purpose (Satyajit Kanungo et al, 2012). Traditionally it has been used in remedies for numerous ailment such as insomnia, melancholia, neurosis, remittent fever, delirium, hysteria, head ache, migraine, muscle pain, joint pain, vascular and nerve injury associated serve inflammatory and neuropathic pain Arunachalam Muthuraman and Nirmalsingh (2012).

One of the most important medicinal plant constraints to crop productivity and prevents cultivation is low around the world (Mandhania et al, 2006). The plants accumulate proline and betaine derivatives to mitigate detrimental effects of sweet stress, by higher water potential. The osmoregulation allows additional water to be taken from the environment, and thus buffering the immediate shortage with water in *Acorus calamus* very well enhances its growth.

Plant growth promoting rhizobacteria (PGPR) are a group of bacteria that can actively colonize plant roots and enhance plant growth (Kloepper et al, 1980). These PGPRs have been reported to prevent the deleterious effects of pathogenic organisms and environmental stress such as water stagnation, nutrient content deficiency, and temperature (Bai et al, 2003). Growth promotion by rhizobacteria can be attributed to one or more of factors such as nitrogen fixation, mineral solubilization, nutrient scavenging, release of plant growth hormones, suppression of pathogenic microbes, stress alleviation and induction of systemic resistance (Kloepper et al, 1997).

Several bacterial species belonging to the genera *Pseudomonas, Bacillus, Paenibacillus, Enterobacter, Rhizobium, Azospirillum* and *Agrobacterium* have been reported to promote plant growth by one or more mechanisms.

Through the occurrence of bacterial species like *Azospirillum, Azotobacter* and *Pseudomonas* in several medicinal plants has been reported earlier (Gopal, 2004; Karthikeyan et al, 2008; Balakarthikeyan et al, 2012). To best of our knowledge this is the first report performed on diazotrophic endophytic bacterial (*Enterobacter cloacae*) in *A. calamus.* It has been suggested that these diazotrophic endophytic colonizing the plant interior might interact more closely with the host, with less competition for carbon sources and a more protected environment for nitrogen(N) fixation Reinhold and Hurek (1998). In additional to N fixation these diazotrophic entophytic bacteria may also involve in the production of phyto-hormones, Phosphate solubilization, siderophore production inhibition of ethylene biosynthesis and confer resistance to various biotic and abiotic factorsJha and Kumar (2009).

Taking this into account the present study was conducted with the following objectives, isolation and characterization of diazotrophic entophytes associated with *A. calamus.* Further the entophytic isolate of *A. calamus* tested under *in vitro* and pot culture conditions and compared with other PGPR strain for the potentiality.

MATERIALS AND METHODS

Isolation of Endophytic diazotrophs

Acorus calamus plants were up rooted from the field of Nagapattinam region of Tamil Nadu, India. The collected rhizome was washed thoroughly in running tap water. The air dried rhizome was surface sterilized using 70% ethanol and sodium hypochlorite (3% available chlorine). The rhizome was cut in to 1 cm pieces and washed with 4% sucrose solution using sterile pestle and mortar. The resulting suspension was plated in Nfb and LGI media. The plates were inoculated at 37°C for 24 hrs. The pure isolates were further analyzed for its phenotype and biochemical characterization.

Identification and characterization of endophytic bacteria

The endophytic bacterial growth was determined by planting it in two different media (N-free media such as Nfb and LGI) as described by Doebereiner (1995). Microscopic observation (wet mounts) of 72 hrs old cultures grown on LGI medium was carried out to study the bacterial shape. Gram straining was carried out as per Hucker's modification (Isenberg, 1995) .The endophytic culture was isolated from specific agar medium. The isolated strain enterobacter sp was tested for their compatibility among each other following the biochemical method (Fukui et al, 1994).

The following biochemical test was as follows [Glucose, Sucrose, Sorbitol, Raffinose, Arabinose, Inositol, Adonitol, Melobiose, Urease test, H_2S test, Indole test, Lysine decarboxylase, Arginine dihydrolase, Ornithine decarboxylase, Tryptophan deaminase, Esculinhydrolysis test, Voges-Proskauer test, Citrate test, Malonate, O-nitrophenyl-β-D-galactopyranoside, β-galactosidase activity, Oxidase activity], was performed for the endophytic isolate in basal medium according to the methods of (Pandey et al, 2005).

After phylogenetic placement of the bacterial and its possible evolutionary relationship with other *Enterobacter cloacae* isolates those exist as endophytes, was carried out considering their 16S ribosomal RNA gene sequences using MEGA 6.0 software.

Authentication of endophytic isolate 16S rRNA gene sequencing and phylogenetic analysis

Molecular identification by carried out by 16SrRNA at YaazhXenomics, Chennai. The polymerase chain reaction was run on a thermal cycler (MJ mini PTC-225 Peltier thermal cycler, BIO-RAD) in a 50µl reaction mix. The reaction mix contained 10x amplification buffer (5ml), 1.5Mm MgCl(5µl), 1µlforward primer (10 mM), 1µl reveres primer (10mM), 1µl dNTP and 0.25µl *Taq polymerase* (Invitrogen). Amplification was performed with 35 cycles at 94°C for 45 sec, 55°C for 60 sec and 72°C for 60 sec. The PCR product was purified by removing the unincorporated PCR primers and dNTPs from product of approximately 1,400bp was sequenced by using 2 Universel primer 518F forward primers, 5′-CCA GCA GCC GCG GTA ATA CG-3′, and 800R reverse primer, 5′-TAC CAG GGT ATC TAA TCC-3′. Sequencing was performed by using Big Dye terminator cycle sequencing kit (applied bio systems, USA). The sequencing products were resolved on an Applied Biosystems model 3730 XL automsted DNA sequencing system (Applied Biosystems, USA), the details with (Table-2).

Sequence analysis and phylogenetic affiliation

A BLAST search (http://blast.ncbi.nlm.nih.gov/ blat/Blast.cgi) was carried out against the complete GenBank database, (Figure-1). Sequences and their closest relatives were used to construct a phylogenetic tree. The sequence alignments and the

phylogenetic tree construction were conducted in MEGA software version-6, (Tamura et al, 2007).

The PGPR strain *Pseudomonas fluorescens*, *Azospirillum lipoferum*, and *Azotobacter vinelandii*was compared with endophytic bacteria in the present study. The *Pseudomonas fluorescens* (AU01) was used as reference strain obtained from deportment of microbiology laboratory, Faculty of Agriculture, Annamalai University, Chidambaram, Tamil Nadu and India.

Determination of plant growth promoting activities

Indole acetic acid (IAA) production

IAA production was detected by the modified method as described by **(**Brick et al,1991**)**. Quantitative analysis of IAA was performed using the method of Loper and Scroth (1986), at 100% concentration of tryptophan (100µg/ml). Bacterial cultures were grown for (*Enterobater* sp), 48 hrs on their respective media at 25±2°C. Fully grown cultures were centrifuged at 3000 rpm for 30 min. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35% of perchloric acid, 1ml 0.5 M $FeCl_3$ solution). Development of pink colour indicates IAA production. Optical density was taken at 530 nm with the help of spectrophotometer Spectronic 20 D^+. Concentration of IAA produced by cultures was measured with the help of standard graph of IAA (Hi-media) obtained in the range of 10-100 µg/ml. The quantitative estimation of IAA is performed by using Salkowski method.

Nitrogenase Activity

Nitrogenase activity of the isolate was measured by Acetylene Reduction Assay (ARA) as described previously Jhaand Kumar A (2007), and expressed in terms of nmol ethylene mg^{-1} protein h^{-1}. The assay was carried out using Chemito 7610 model gas chromatograph (GC Chemito, 7610) felted with porapak T. The flow rates of N_2, H_2 and air were adjusted to four replications was maintained for each treatment in (Table 3).

Phosphorus solubilization

Phosphate solubilizing bacteria was isolated using the Pikovskaya agar medium Pikovaskaya (1948). Many rhizome microorganisms are able to solubilize unavailable forms of bound P (Daniel et al, 1998). Visual detection and semi quantitative estimation of phosphate solubilizing ability of microorganisms is possible by plate screening methods of show clear zone around the microbial colonies in media containing insoluble mineral phosphates (Tricalcium Phosphate or hydroxyapatite) as sole P source (AMES, 1964; Rodriguez and Fraga, 1999).

Quantitative estimation of phosphate solubilization was performed using Fiske Subba Row method of colorimetric analysis by inoculating individual bacteria in Pikovasky broth and incubated for 7 days. The drop in pH was also considered for the solubilization of inorganic phosphorus.

Siderophore production

The isolate was assayed for siderophores production on the Chrome azurol S agar medium (Sigma, Ltd.) described by Schwyn and neilands (1987). Chrome azurol S agar plates were prepared and divided into equal sectors and spot inoculated with test organism (l0µl of 10^6CFU/ml) and incubated at 25±2ºCfor 48-72 h. Development of golden yellow-orange halo around the growth was considered as positive for siderophore production. Siderophores synthesized by microbial communities of soil supply iron to plants that possess the mechanisms for its uptake under iron-deficient conditions (Indiragandhi et al, 2008).

Endophytic bacteria on inoculation with *Acorus calamus* under pot studies conditions

Pot experiments were conducted to evaluate the effect of endobacterial strains on the growth of *Acorus calamus* under PGPR growth conditions. The experiment was conducted during the period of May 2013 to October 2013. The rhizome subsurface was sterilized with 70% ethanol and was sown in cement pots filled with sterilized potting soil at four weeding per pot. The cement pots (Size-30.60cm and diameter, 0.45m height, volume of soil: 0.04 m) having a pH 6.8 and 7.4. Each pot was given a basal dose of 25 mg of triple super phosphate, murrate of potash and ammonium molybdate. Where *Acorus calamus* were treated with endophytic suspension containing *Enterobacter cloacae* sub sp dissolvens isolated was used at the rate of 10 ml per pot (minimum inoculation load of $1x10^8$ CFU $g^{-1}ml^{-1}$) and five ml of enhance the adhesives. The *Pseudomonas fluorescens* (AU01), *Azospirillum lipoferum* (AU02) and *Azotobacter vinelandii* (PPAU02) strain (accession no: KF934492) treated with rhizome and planted separately in the pots. The control pot was also maintained. All the pots were watered based on field capacity. The inoculated *Acorus calamus* was maintained at 27°C and treatment was replicated five times. All pots used during the experiment were sterilized with 5 % solution of sodium hypochlorite for 20 min to maintain sterility during the experiment. The experimental design adopted was completely randomized block design. Growth parameters in terms of shoot length, and root length were recorded after 180 DAP growth period.

PGPR and alkaline content of *Acorus calamus* with *Enterobacter cloacae* sub sp dissolvens plant growth assay

The influence on *Enterobacter cloacae* sub sp dissolvens inoculation on the germination percentage, vigour index of *A. calamus* was studied under gnotobiotic condition. The collected rhizome was taken in sterilized petriplate and treated with 10 ml (NB) nutrient broth (an initial population 10^9 CFU ml^{-1}). The germination percentage was calculated from 7 to 12 DAP. The morphological parameters like shoot length and rhizome dry weight was measured on 180 DAP. The vigour index (*VI)* of the weeding was estimated as suggested by(Abdul-Baki and Anderson1973).

Growth parameter

Plant height, rhizome dry weight and β-asarone alkaloid content were determined at 180 DAP by following the method of (Singh et al, 2010)

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Statistical analysis

All data obtained were subjected to one-way analysis of variance (ANOVA) and the mean differences were compared by higher and lowest standard deviations (LSD). Each data point was a mean of three replicates (n = 5) and comparisons with P values ≤ 0.05 were considered as significantly different statistical analysis.

RESULTS

Isolation and biochemical characterization

The plant growth promoting endophytic rhizobacteria population of *Acorus calamus* (CFU 10^{-6} dilution of fresh rhizome sample) was recorded. The isolate PPAU01 showed the better growth in Nfb and LGI medium (Table-1).The isolated diazotrophic entophyte was found to be gram positive rod, mucoid, smooth margin. The endobacterial isolate was able to utilize glucose, sucrose, sorbitol, raffinose, and arabinose. The isolate was positive for Indole, Voges-Preskauer test, Citrate, lysine decarboxylase, arginine dihydrolase, Ornithine decarboxylase, Esculine hydrolysis, Malonate, O-Nitrophenyl-β-D-Galactopyranoside and β-galactosidase activity test. It showed negative for inositol, adonitol, urease, hydrogen sulphide, tryptophan deaminase and oxidase test.

16S rRNA sequence analysis and phylogenetic affiliation

The result of morphological and biochemical characteristics endophytic bacteria strain was identified by 16S rRNA sequencing analysis. Based on the high level of efficiency PGPR along with promising, the isolate PPAU01 was subjected to 16S rRNA sequencing. The isolate was identified as *Enterobacter cloacae*. The strain PPAU01, suggested that the strain belongs to *Enterobacter Cloacae* sub sp dissolvens and shares 100% of homology with *Enterobacter cloacae* dissolvent strain (Fig.1).The PPAU01 nucleotide sequence of PPAU01was deposited in GenBank (NCBI) under the accession number KF777104 (Table-2).

Determination of PGPR traits of the endophytic isolate

There was significant differences in ARA activity, IAA production, Phosphate solubilization and siderophore production efficiency of diazotrophic isolate (Table 3). Plant growth promoting activities such as IAA production (1543.26±15.22µg ml^{-1}), ARA-Acetylene Reduction Assay (480.12±12.00n mole ethylene mg protein h^{-1}), Phosphate solubilization (13.62±0.45 mg P100 mg^{-1} TCP) and Siderophore production (5.34±0.27 µg ml^{-1}) are the characteristics of plant growth promoting rhizobacteria (PGPR).

Plant growth promoting by *Enterobactercloacae* sub sp dissolvens

The effect of *Enterobacter cloacae* sub sp dissolvens on the growth parameters like plant germination percentage (%), vigour index, plant height, dry weight and alkaloid content of *Acorus calamus* was compared with other PGPR strains at 180 DAP presented in (Table 4). The highest germination percentage (93.3%) and vigour index (1156.23), plant height (61.03cm), rhizome dry weight (37.020 g) and β-asarone alkaloid content (2.26%) recorded in *Enterobacter cloacae* sub sp dissolvens followed by *Pseudomonas fluorescens*, *Azospirillum lipoferum* and *Azotobacter vinelandii*.

DISCUSSION

A wide range of plants can serve as hosts for endophytic bacteria, ranging from herbaceaes plants to woody plants. The diazotrophic endophytic bacteria are quite interesting due to its ability fix more nitrogen compared to the rhizosphere bacteria. In the present study, *Enterobacter cloacae* sub sp dissolvens entophytic bacteria able to grow on Nfb media and LGI media. Endophytic bacteria belonging to different genera such as *Azospirillum, Burkholderia, Azoarcus, Herbaspinllum, Serratia* and *Achromobacter* from rice, maize, wheat and *catharanthus roseus*and *Acorus calamus* (Singh et al., 2010; PratibhaRawal et al.2015;Baldani et al, 1986; Gyaneswar et al, 2001; Frang et al, 2006; Reiter et al, 2002)

Endophytes represent an enormous relatively in tapped source of microbial diversity which has

attracted increases attention in plant microbiology as they could play an important role in agriculture by conferring advantages to the plant. The endophytic bacteria can contribute to plant development by producing phytohormones and siderophores and antibiotic production (Burd et al, 1998; Ladha et al, 1983).

Table 1
Biochemical characterization of diazotrophic bacterial endophyte isolated from *A. calamus.*

Test	Value
Nfb	9.12±0.12*
LGI	9.54±0.23*
Morphology	Result
Gram reaction	G +ve
Cell shape	Rod
Colony morphology	Mucoid, Smooth Margin
Biochemical test	
Glucose	+
Sucrose	+
Sorbitol	+
Raffinose	+
Arabinose	+
Inositol	-
Adonitol	-
Melobiose	+
Urea use test	-
H2S test	-
Indole test	+
Lysine decarboxylase test	+
Arginine dihydrolase test	+
Ornithine decarboxylase test	+
Tryptophan deaminase test	-
Esculin hydrolysis test	+
Voges-Proskauer test	+
Citrate test	+
Malonate test	+
O-nitrophenyl-β-D-galactopyranoside	+
β-galactosidase activity	+
Oxidase activity	-

*(+) indicates positive and (-) indicates negative reactions

* Obtainable bacterial population in log CFU ml^{-1} after 24 hrs in respective growth media

Table 2
Partial sequence details of *Enterobacter cloacae* sub sp dissolvens submitted to NCBI (GenBank).

S.No	Gen Bank details	
1.	Strain Name	PPAU01 *EC*
2.	Phylogenic Affiliation	*Enterobacter cloacae* sub spdissolvens
3.	Bacterial Group and Sequence	16r rRNA
4.	Accession No	KF777104
5.	% Identity	99.00
6.	Version	KF777104.1 GI 557640835
7.	Author	P. Prakash and Karthikeyan. B

***EC- Enterobacter cloacae*

Table 3
Plant growth promoting activity exhibited by *Enterobacter cloacae* dissolvens isolate from endophtic associated with *Acorus calamus*

S.No	Strain name	Plant growth promoting activity	Results
1.	*Enterobacter cloacae* dissolvens	IAA production (µg ml^{-1})	1543.26±15.22
2.		ARA (nmole ethylene h^{-1} mg protein^{-1})	480.12±12.00
3.		Phosphate solublization (mg p100 mg^{-1} TCP)	13.62±0.45
4.		Siderophore production (µg ml^{-1})	5.34±0.27

EC- Enterobacter cloacae
Value is a mean of four replication ± SD, *IAA*-Indole Acitic Acid, *NA*-Nitrogenase activity, *PS*-Phosphate solublization, *SP*-Siderophore production.

Table 4
Effect of *Enterobacter cloacae* were plant growth parameters and alkaloid content of *Acorus calamus*.

Treatment	Germination percentage %	Vigour index	Plant height(cm)	Rhizome dry weight(g)	Alkaloid content (%)
Enterobacter cloacae	93.3[a]	1156.23[a]	61.03[a]	37.020[a]	2.26[a]
Pseudomonas fluorescens(RS AU01)	85.2[b]	1071.46[b]	58.07[b]	22.006[b]	1.46[b]
Azospirillumlipoferum	80.0[c]	1003.66[c]	55.09[c]	19.8[c]	1.30[c]
Azotobactervinelandii	72.0[d]	1054.41[d]	52.00[d]	16.741[d]	1.05[d]
Control	60.0[e]	746.54[e]	40.02[e]	7.20[e]	0.90[e]

(RS AU01) Reference strain Annamalai University 01,

Each value represented a mean of three replication ±SD. There value is a significant different at a *P* value of 0.05, as determined by DMRT.

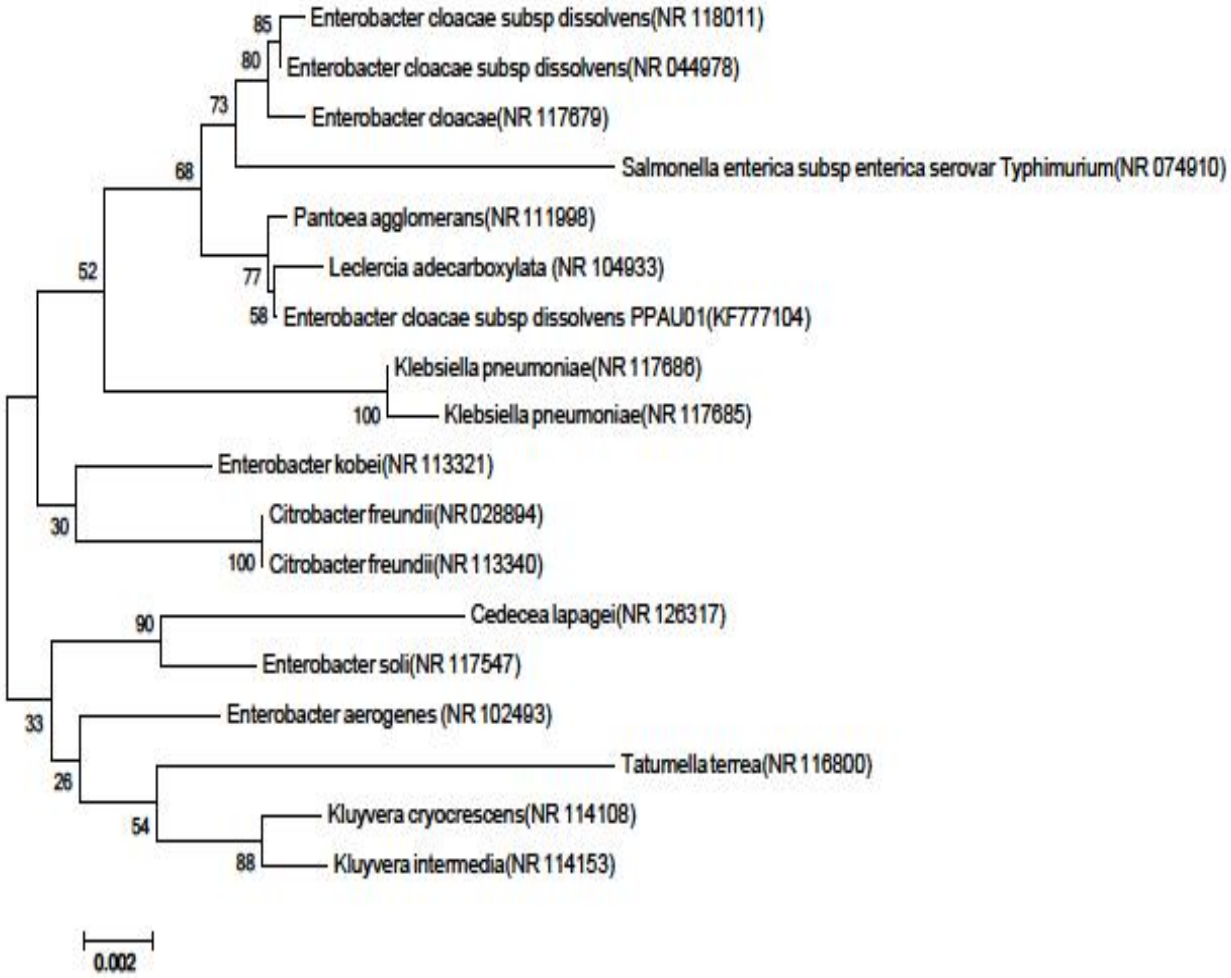

Fig.1

Phylogenic tree predicated by the neighbour joining method using 16S rRNA gene sequences. Taxa are represented by type strains with GenBank accession number. Number in bracket indicates accession number.

Several species from the genus *Enterobacter cloacae* have been reported to function as agents of plant growth promotion and growth in the several plant species. Indole acedic acid production, Nitrogen fixing ability, phosphate solubilization and siderophore production observed in our present study complies with the findings of (Archana et al, 2012;Richardson et al, 2009), who also reported that *Enterobacter* sp able to fix atmosphere nitrogen, phosphorus is known be converted from insoluble, unavailable forms to soluble readily available phosphate by organic acids by rhizospheric bacteria and siderophore production also. The presence of such diverse PGP traits in plant associated *Enterobacter* sp. Substantiates the hypothesis that reside in the plant rhizosphere may have adapted for a symbiotic plant associated life style even at the genomic level by (Forchetti et al, 2007) which supports our finding for identifying the *Entrobacter cloacae* sub sp dissolvens which posses the plant growth promoting traits.

The ability diazotrophic PGPR in exhibiting various PGP traits such as production of phytohormones, P-solubilization and siderophore production has been well documented by numerous workers (Shanker et al, 2012;Shweta Nailwal et al, 2014).

Following the endophytic bacterial inoculation and other PGPR inoculation was evaluated. The highest germination percentage, vigour index, plant height, rhizome weight and β-asarone alkaloid content recorded in *Enterobacter cloacae* sub sp dissolvens. Our finding was supported by (Shanker et al, 2012). The *Enterobacter cloacae* GSI genome was found to contain the genetic makeup of an effective PGPR as it contained genes required for motility, chemotaxis, adhesion, polysaccharide production, plant growth hormones production, phosphate solubilization and resistance to biotic and abiotic stress in the rhizosphere. The above results corroborate well with the present findings that *Enterbacter cloacae* sub sp dissolvens increased the plant growth promotion and β-asarone alkaloid content of *Acorus calamus*and there by *Enterobacter cloacae* sub sp dissolvens bacteria is a potential endophytic microorganism.

CONCLUSION

In conclusion the presented study has showed that *Enterobacter cloacae* sub sp dissolvens could be used as a suitable bio-inoculant for *Acorus calamus* and other commercially grown medicinal plants. This work opens up the possibility for better exploring plant endophytic bacterial interaction.

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