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Prospects of endophyllic *Pseudomonas aeruginosa* as a biocide against sheath blight and growth promotion of rice

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ABSTRACT

Ecofriendly ecto-/endospheric plant growth promoting microbes (PGPM) and biocides would sustain rice growth and development, save from diseases and pests to keep up rice production for food security and protect the ecosphere. As the pseudomonads are most versatile microbes, the PGP and biocidal potential of a multifaceted endophyllic pseudomonad (JE1) of rice were evaluated. Phenotyping, FAME and 16S rDNA homology confirmed identity of the organism as Pseudomonas aeruginosa. The organism possessed the plant growth promotion (PGP) functions like P solubilization (536.96 µg/ml), ACC, siderophore (7.77 mg/g dr. wt.), antimicrobial metabolite viz. HCN (0.035 ΔA625/ml) etc. and antimicrobial enzyme viz. pectinase, tributyrin hydrolase, gelatinase and cellulase. Besides, the organism inhibited growth of rice sheath blight (ShB) pathogen Rhizotocnia solani by 35.89%. The PGP and antimicrobial functionalities proved versatility of the P. aeruginosa (JE1) and potential for overall improvement/sustenance of rice health and production.

Key words: *Rice, leaf endophyte, P. aeruginosa, R. solani, biocontrol, PGP*

INTRODUCTION

The abiotic stresses like soil nutrition and water deficiency, salinity, temperature etc. and biotic stresses *i.e.,* infestation by about 120 insect pests (30 major) and 74 diseases (16 major) which causes about 30 and 10% yield losses, respectively are major constraints of rice (*Oryza sativa* L.) production (Kumar et al., 2009; Roy et al., 2013). Besides, higher N requirement for improved varieties, multiple rice cropping and lack of resistant genotypes have intensified various pest and disease infestations, especially alarmingly increased the rate of spread of sheath blight (ShB) disease of rice in global rice growing areas (Nagarajkumar et al., 2004). The ShB pathogen *Rhizoctonia solani* Kuhn. reduces up to 50% of pathogen inflicted rice production loss in different countries, as well as, infects 50 other crops viz. barley, lettuce, tomato, sorghum, maize etc. (Singh et al., 2004; Zong et al., 2007; Zhang et al., 2009; Akter et al., 2014; Yellareddygari, 2014). To uphold rice yield for food security, stress alleviation and increased use of agrochemicals which are intrinsically menaces for biological/environmental health are advocated but demands reduction or substitution of the agrochemicals by ecofriendly plant growth promoting microbes (PGPM) and biocides.

The rhizospheric, ectophytic and endophytic PGPM like *Bacillus, Enterobacter, Pseudomonas, Azospirillum, Azotobacter, Trichoderma, Beauveria* spp. etc. posses determinants for plant growth promotion (PGP) traits like nutrient (N, P, K, S, Mg etc.), plant growth regulator (PGR) (indole acetic acid (IAA), gibberellic acid (GA), cytokinin (CK), abscisic acid (ABA) and phenolic compounds), iron/other metal chelator siderophore, biocidal antimicrobial enzyme (chitinase, protease, cellulase, glycosidase etc.), antimetabolite (HCN), antibiotic (2,4-diacetylphloroglucinoal (DAPG) and fluorescent pigment (toxic pyocyanin) production; and induced systemic resistance (ISR) against various pathogens like blast (*Magnaporthe grisea*), white mold (*Sclerotina sclerotiorum*), *Fusarium solani*, *Pythium*

ultimum, ShB (*R. solani*) etc. (Deshwal, 2012; Naureen et al., 2015; Yellareddygari, 2014; Haas and Defago, 2005; Dey et al., 2014).

The rice plants also harbour various rhizo-/phyllo-/endo-/ectospheric and seed endophytic microbes like (*Azospirillum, Klebsiella, Aeromonas, Enterobacter, Bacillus, Pseudomonas, Pantoea, Herbaspirillum, Berkholderia, Streptomyces, Penicillium, Fusarium* spp.) possessing PGP and biocidal functions against some bacterial (*Xanthomonas, Erwinia* spp. etc.) and fungal (*Aspergillus, Fusarium, Rhizoctonia, Pithym, Sclerotinia* spp. etc.) pathogens (Akter et al., 2014; del Castillo et al., 2015; Deshwal and Defago, 2005; Dey et al., 2014; Reddy et al., 2008; Velusamy et al., 2006; Ferrando et al., 2012). Different PGPR and phytonic microbes like *Bacillus, Pseudomonas* and *Trichoderma* spp. have been studied thoroughly and observed to be efficient root colonizers, antimicrobial metabolite producers and potent biocides against several bacterial and fungal pathogens of different crops (O'Sullivan and O'Gara, 1992; Singh et al., 2004; Reddy et al., 2008). However, other than the endobacterial diazotrophs (*Azospirillum, Azotobacter, Klebsiella* etc.) and endofungal biocides (*Acremonium, Verticillium, Phomopis* spp.) against pathogenic *Aspergillus, Fusarium, Pyricularia, Cladosporium* spp. etc. (Gao et al., 2010), other rice endophytes (especially endophyllic bacteria) have not been investigated significantly. To date, the multiple functionalities *i.e.,* PGP and biocidal properties of endophytic microbes (especially endophyllic pseudomonads) have not been investigated at all. Therefore, the present study was undertaken to characterize, assess and exploit ecofriendly rice endophyllic, growth promoting *P. aeruginosa* for PGP and biocontrol of sheath blight (*R. solani*) of rice.

MATERIALS AND METHODS

Isolation of endophytic fluorescent bacterium (JE1) from rice leaf

The leaf endophytes were isolated (Barraquio et al., 1997) from healthy flag leaves at panicle initiation (PI) stage from the rice genotype *Oryza sativa* L. var. Naveen grown in the research field of National Rice Research Institute, Cuttack, Odisha. The leaves were washed under running tap water, cleaned thoroughly with 0.1% (w/v) tween80 to remove dust or other loosely attached particles followed by sterile (autoclaved at 121°C for 15 min) distilled water and surface sterilized with 70% ethanol for 30 sec., 0.1% $HgCl_2$ and 2% sodium hypochlorite for 5 min each, washed five times in sterile water, blotted to dryness within sterile blotting papers and cut into 1 cm long pieces under laminar air flow hood. Dried surface sterilized leaves were macerated aseptically within a mortar and pastle, suspended the paste in 2 ml sterile distilled water, the aqueous extract was mixed with 100 ml King B agar medium (g/l: proteose peptone 20, K_2HPO_4 1.5, $MgSO_4.7H_2O$ 1.5, glycerol 15 ml, agar 20, pH 7.2), poured in 5 plates and the plates were incubated at 28±2ºC for 72h. The cultured plates were observed under a UV (312/254 nm) transilluminator and the fluorescent colonies of different morphology and texture were isolated, purified and maintained on King B medium.

Phenotypic characterization of bacterial strain (JE1)

The cultural (colony colour, elevation, margin, size, consistency etc.), morphological (shape, size, motility, spore formation, staining etc.), physiological and biochemical characters (methyl red, Voges-Proskaur, indole production, citrate utilization, nitrate reduction, production of urease, oxidase, catalase etc.) were checked through standard methods and carbohydrate utilization was tested through KB009 HiCarbohydrate TM kit, Hi-MEDIA (Halt et al., 1994; Smibert and Krieg, 1995; Collins et al., 1995).

Detection of fluorescent pigment (phenazine) production by JE1

Fluorescent pigment (phenazine) production by the bacterium was checked on King B medium containing 2% glucose and grown in a BOD incubator for 3d at 30±0.1°C (Mavrodi et al., 2001). The plates were observed under a UV transilluminator 312/254 nm and those produced fluorescent diffusible pigment in 2-3d and greenish crystal deposit (Fig. 4) on 5-7d old well developed colonies were considered as phenazine (pyocyanin) producers. Solubility of the pigment crystals in water, chloroform, ethanol and methanol was checked and crystal shape was observed under a light microscope (100X).

The 16S rRNA gene sequence of JE1 analysis

Genomic DNA (gDNA) was extracted from JE1 using gDNA isolation kit (HiMedia) according to manufacturer's instructions. The 16S rRNA gene was amplified and sequenced using universal primers *viz.,* 27f (5'AGAGTTTGATCCTGGCTCAG3') and 1492r (5'GGYTAC CTTGTTACGACTT3') (Weisburg et al., 1991) and submitted to NCBI GenBank (acc. no. KJ634843).

Fatty acid methyl ester (FAME) analysis of the bacterium JE1

The organism was grown on trypticase soy agar (TSA) medium (g/l: pancreatic digest of casein 15, NaCl 5, papaic digest of soybean meal 5, agar 20) for 2d at 37±0.1°C. Whole-cell fatty acid (FA) profile was obtained through external services by resolving the fatty acid methyl esters (FAME) through an Agilent gas chromatograph and the FAs were identified by Sherlock Microbial® Identification System (MIS) (MIDI®, Microbial ID) (Sasser, 2001).

Effects of salt (NaCl), temperature and pH on growth of the bacterium

To check effect of salt stress, 50 ml NB containing 2, 4 and 6% NaCl was inoculated with 50 µl overnight grown JE1 broth, shake-cultured at 100 rpm, 30±0.1°C and green filter (A540 nm) of a colorimeter 2h intervals for 3d. Temperature (4, 42 and 45°C) effect was checked in NB recording growth at A540 nm from 3d shake-cultured broth. For pH effect, the bacterium (10^3 cfu/ml) was streaked on NA with pH 4, 9 and 11, grown at 30±0.1°C for 3d and growth as colony formation was recorded visually.

Identification of the bacterium JE1

The organism was identified by partial polyphasic taxonomy based on the phenotypic, 16S rDNA phylogeny and FAME analysis following standard methods (Halt et al., 1994; Smibert and Krieg, 1995; Oka et al., 2000; Boone et al., 2005).

Collection of sheath blight pathogen

The fungal pathogen of rice sheath blight (ShB), *Rhizoctonia solani* Kuhn. (teleomorph *Thanatephorus cucumeria* (Frank) Donk) was obtained from Pathology laboratory of the institute and maintained on potato dextrose agar (PDA) medium (g/l: potato infusion 200, dextrose 20, agar 20, pH 5.6).

Assay of antagonism of JE1 against the sheath blight pathogen *R. solani*

Antagonism of the bacterium (JE1) against *R. solani* was tested by dual culture technique (Gupta et al., 2001). A 5 mm dia. mycelial mat of *R. solani* from a 7d old PDA plate was placed at the centre of the PDA plate and the JE1was spotted at about 1 cm inside the edge of the petridish. The control plate with fungal disc was incubated without the bacterium. The plates were incubated at 28 ± 0.02ºC for 5d, growth dia. (mm) on control and treated plates were recorded by an antibiotic assay scale and inhibition was measured from the distance between the edges of the fungal mycelium juxtaposed to the antagonistic bacterium. Inhibition (%) of growth of the pathogen by JE1 was calculated from the following formula (Vincent, 1927): I(%) = (R1 - R2)/R1 x 100, where I = inhibition of mycelia growth, R1 = mycelial growth in control, R2 = mycelial growth in treatment.

Production of antimicrobial secondary metabolites

The organism (JE1) was evaluated for metabolism of antimicrobial secondary metabolites like siderophore and HCN in the laboratory.

HCN production

The organism was grown on a rotary shaker in King B broth amended with 4.4 g/l glycine. Air dried, 10 x 0.5 cm Whatman no. 1 filter paper strips saturated with 0.5% picric acid in 2% sodium carbonate were hanged inside the growth flasks avoiding contact with the medium and the flask wall. Flasks were incubated at 30±0.1ºC for 96h and change of paper colour from yellow to red due to reduction of sodium picrate by evolved hydrocyanic acid (HCN) was noted. HCN production was quantified by extracting the red colour of filter papers submerging in 10 ml distilled water followed by recording A625nm (Reddy et al., 2008).

Siderophore production

The bacterium was spotted on chromeazurol S (CAS)

agar medium composed of solution A (chromazurol 60.6 mg dissolved in 50 ml water), 10 ml solution B (1 mM $FeCl_3.H_2O$ in 10 mM HCl), solution C (CTAB 72.9 mg dissolved in 40 ml water) and 300 ml nutrient agar), incubated at 30±0.1°C for 4-5d and change in colour around the bacterial growth indicated siderophore production (Schwyn and Neilands, 1987). To quantify siderophore production, the bacterium was grown in NB for 3d, centrifuged at 10000 rpm for 10 min at 4±0.1°C, supernatant was adjusted to pH 2.0 with 1N HCl, medium colour was extracted with equal quantity ethyl acetate through a separating funnel. The solvent was air dried overnight, residue was resolved in 2 ml water, mixed with 2 ml Hathway's reagent (1.0 ml 0.1M $FeCl_3$ in 0.1N HCl added to 100 ml distilled water and 1.0 ml 0.1M potassium ferricyanide), A650 was read for dihydroxy phenols production and estimated as dihydroxy benzoic acid equivalents (Reddy et al., 2008).

Determination of antifungal enzymes

Fungal cell wall and membrane degrading pathogenicity related enzymes like chitinase, pectinase, lipase (tween 80, tributyrin and vegetable oil esterase), protease (gelatinase and caseinase), lecithinase, cellulase and amylase production by the bacterium were determined. The organism was spotted on NA plates containing 1% substrate according to the enzyme, incubated at 30±0.1°C for 3-7d in a BOD incubator and enzyme activities were recorded with or without addition of suitable reagents depending on the enzyme (Collins et al., 1995; Pointing, 1999). Activities of gelatinase and caseinase were detected by opaque circles formation around growth on flooding the plates with 0.1% $HgCl_2$ solution. Hydrolysis of carboxymethyl cellulose by cellulase was visualized from a clear area formation around the colonies after flooding the plates sequentially with 1% Congo red, 1N HCl and 1N NaOH for 5 min each. Starch metabolism by amylase was observed from clear zone formation around the growth on flooding with iodine solution (1g iodine dissolved in 300 ml water containing 2g KI). Lecithinase activity was detected by an opaque zone around the colony and associated lipolytic activity of lecithinase observed from halo formation around the opaque ring on NA plate containing 10% (final) egg yolk. Metabolism of chitin, pectin, tributyrin and vegetable oil by the respective enzymes was revealed from halo zone formation but tween esterase from sopaque circle around the colonies (Renwick et al., 1991).

Detection of antibiotic gene 2, 4 diacetyl-phloroglucinol (DAPG) of the bacterium JE1

The 2, 4 diacetyl-phloroglucinol (DAPG) gene specific primers (Phl2a 5'GAGGACGTCGAAG ACCACCA3' and Phl2b 5'ACCGCAGCATCGTGTATGAG3') were used to amplify DAPG antibiotic production gene (Mavrodi et al., 2001). PCR amplification was performed in 25 µl reaction mixture containing 25 ng template DNA, 1x DNA amplification buffer, 0.2 mM dNTP, 20 pmol each primer, 1U Taq DNA polymerase through a PCR with the cycling conditions of initial denaturation 94°C for 90s once, followed by 35 polymerization cycles each programmed for denaturation at 94°C for 35s, annealing at 53°C for 30s and extension at 72°C for 45s, and a final extension at 72°C for 10 min. The PCR product was electrophoresed on 1.2% agarose gel in 1xTris-acetate-EDTA (TAE) buffer (40 mM Tris-acetate, 1 mM EDTA pH 8.0) at 100v for 60 min, stained with ethidium bromide and visualized under a UV transilluminator.

Assessment of plant growth promotion (PGP) traits of JE1

The PGP determinants such as phosphate solubilization, indole compounds, ammonia and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase production were assessed *in vitro*.

Qualitative and quantitative assessment of inorganic phosphate solubilization

The bacterium was spotted on National Botanical Research Institute phosphate medium (NBRIP) agar medium (g/l: glucose 10, $Ca_3(PO_4)_2$ 5, $MgCl_2.6H_2O$ 5, $MgSO_4.7H_2O$ 0.25, KCl 0.2, $(NH_4)_2SO_4$ 0.1, agar 18, pH 7) and incubated for 3d at 30±0.1ºC. A clear zone around the bacterial growth indicated P solubilization, and P solubilization index (PSI) and P-solubilization efficiency (PSE) were estimated by the following formulae (Ponmurugan and Gopi, 2006).

PSI = Z/C, PSE = (Z - C)/C X 100, where, Z is clearing zone diameter and C is colony diameter.

To quantify inorganic phosphate (tricalcium phosphate, $Ca_3(PO_4)_2$) solubilization, 20 µl overnight

grown NB culture (10^3 cells/ml, A660 1.0) was inoculated in 100 ml NBRIP broth contained in 250 ml flasks and shake-cultured at 85 rpm for 3-5d at 30 ± 0.1°C along with uninoculated control. The culture was centrifuged at 10000 rpm for 10 min at 4±0.1°C, to 1 ml supernatant, 2 ml each of 2.5% sulfomolybdate solution, 0.25% p-nitrophenol indicator, 1N H_2SO_4 and 1 ml stannous chloride solution (40% w/v in HCl) were added. A660 nm was recorded and P content was estimated as µg P/ml medium and µg P/g bact. dr. wt. (Olsen, 1954). Change of pH of the culture was checked at 24h intervals for 3d through a pH meter.

ACC deaminase production

To assay ACC utilization as N source, diluted (10^3 cfu/ml) bacterial sample was streaked on Dworkin and Foster (DF) salt minimal medium (g/l: KH_2PO_4 4, Na_2HPO_4 6, $MgSO_4.7H_2O$ 0.2, glucose 2, gluconic acid 2 and citric acid 2 and trace elements: $FeSO_4.7H_2O$ 1 mg, H_3BO_3 10 mg, $MnSO_4.H_2O$ 11.19 mg, $ZnSO_4.7H_2O$ 124.6 mg, $CuSO_4.5H_2O$ 78.22 mg, MoO_3 10 mg, pH 7.2) supplemented with 3.0 mM ACC as N source and incubated for 24 h at 30±0.1°C (Penrose and Glick, 2003). Growth of the organism was considered for ACC utilization as N source.

Ammonia production

For ammonia production assay, the bacterium was inoculated in 5 ml buffered peptone water (g/l: peptone 10, NaCl 5, Na_2HPO_4 3.5, KH_2PO_4 1.5, pH 7.2) and incubated for 96h at 30±0.1°C. Development of brown colour of culture on addition of 1 ml Nessler reagent (70.83 g/l K_2HgI_4 in 2.5M KOH) indicated ammonia production (Cappuccino and Sherman, 1992).

Indole production

To test indole production, the bacterium was grown in broth medium (g/l): peptone 20, NaCl 5 and tryptophan (final) 0.1%, pH 7) and incubated at 30±0.1 °C. To the broth, 1 ml Salkowski reagent (50 ml 35% $HClO_4$ containing 0.5M $FeCl_3$) was added, mixed and change of medium to pink colour indicated indole production (Bric et al., 1991).

RESULTS AND DISCUSSION

The phenotypic characters *viz.,* cultural, morphological, physiological and biochemical properties of the organism are given in Table 1. The organism produced irregular, greenish, raised, undulate, non-gummy, 3.8 mm dia. colonies. The vegetative cells were short, Gram (-)ve, motile rods and measured 1.033 x 0.866 µm. The organism utilized citrate, dextrose, galactose, xylitol, malonate; hydrolyzed pectin, gelatin, tributyrin, cellulose, and produced nitrate reductase and catalase. However, the bacterium did not utilize lysine, ornithine and phenylalanine; did not produce lecithinase, urease, oxidase, indole and H_2S; did not hydrolyze starch, casein, chitin, tween 80, DNA, and negative for methyl red (MR) and Voges-Proskauer (VP) tests. Growth of the organism was not affected at temp. 4-42°C and pH 7-9 but growth was reduced at 45°C and pH11 and stopped below pH4. The organism produced water (sparingly) and chloroform soluble fluorescent bluish green pyocyanin pigment which diffused through NA medium, formed greenish-blue crystals over the colony (Table 1) and the pigment crystals appeared needle like under a light microscope.

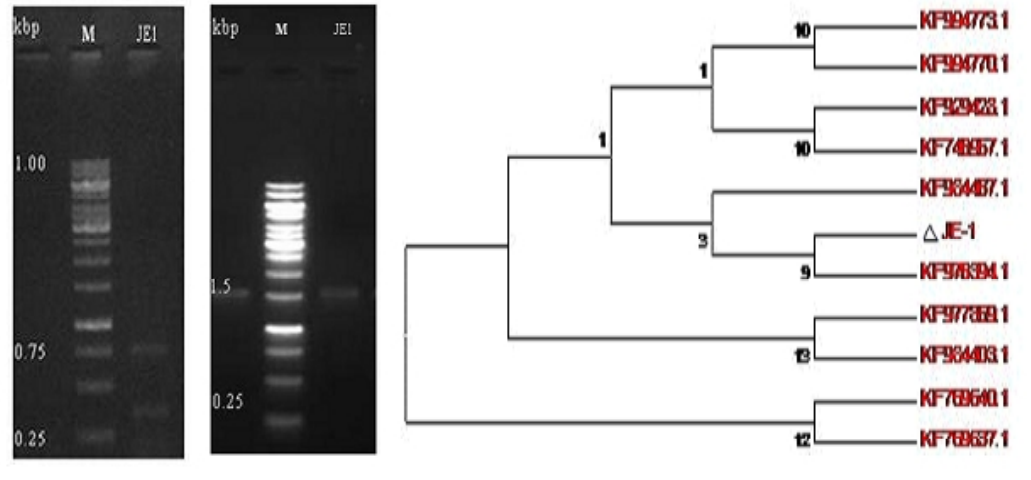
The 16S rDNA sequence of the bacterium JE1 (NCBI acc. no. KJ634843) had closest similarity (100%) with *Pseudomonas aeruginosa* strain C1501 (Acc. no. KF976394.1) (Fig. 1). Similarly, FAME profile of the organism also revealed 0.906 % similarity index with *P. aeruginosa* (Fig. 2). The phenotypic, 16S rDNA phylogeny and FAME results conformed to the characters of *P. aeruginosa* which ascertained identity of the JE1 as a member of the species *P. aeruginosa* belonging to the family *Pseudomonadaceae*, class γ-Proteobacteria, order Pseudomonadales (Halt et al., 1994; Boone et al., 2005; Boone and Garrity, 2001). The results proved that *P. aeruginosa* is a leaf endospheric microbiota of the rice genotype Naveen which favoured the records of occurrence of the pseudomonads and other microbes *viz., Bacillus Azospirillum, Klebsiella, Ochrobacterium, Herbaspirillum, Azoarcus, Methylobacterium* spp. etc. in different vegetative parts of the rice plants and *Pantoea, Bacillus, Klebsiella, Herbaspirillum* spp. etc. in seeds (Mano and Morisaki, 2008).

Growth kinetics of the organism with 2-6% NaCl (Fig. 3) depicted that multiplication of JE1 was broadly comparable (optimum OD (green filter) ~1.00) in NB and 2% NaCl but exponent growth time was almost doubled (24h) in the latter medium than that (12h)

Table 1. Phenotypc characters of the endophytic bacterium JE1.

Parameter	Character/ growth condition	Result
Culture	Colony	Irregular, greenish, raised, undulate, non-gummy, 3.8 ± 0.123 mm dia.
Morphology	Vegetative cell	Motile, short Gram (-)ve rod, size (1.033± 0.176) x (0.866 ± 0.145) µm
Pigment production	Positive	Diffusible fluorescent water soluble and green crystalline, needle shaped crystal
Physiological and biochemical tests	Positive	Citrate utilization, nitrate reduction, catalase production
	Negative	Methyl red (MR) and Voges-Proskauer (VP) test, indole, urease, H2S, oxidase production
Amino acid utilization	Negative	Lysine, ornithine, phenylalanine utilization
Extracellular enzyme production	Positive	Hydrolysis of gelatin, pectin, tributyrin, cellulose
	Negative	Hydrolysis of starch, casein, chitin, tween 80, DNA, lecithin
Carbon compound utilization	Positive	Utilization of dextrose, galactose, xylitol, citrate, malonate
	Negative	Lactose, xylose, maltose, fructose, raffinose, trehalose, melibiose, sucrose, L-arabinose, mannose, inulin, sodium gluconate, glycerol, salicin, dulcitol, inositol, sorbitol, mannitol, adonitol, arbitol, erythritol, α-Methyl-D-glucoside, rhamnose, cellobiose, melezitose, ?-Methyl-D-Mannoside, ONPG, esculin hydrolysis, D-arabinose and sorbose utilization
Growth at temp. (°C)	4	Positive (A540 0.23-0.30)
	42	Positive (A540 0.33-0.48)
	45	Negative (A540 0.00-0.01)
Growth at pH	4	–
	9	+
	11	–
Phenotypic identity of the organism		*Pseudomonas aeruginosa*

in the former medium. Although growth of the organism (optimum OD (green filter) ~0.8 in 24h) with 4% NaCl nominally differed from that with 2% salt, but 6% salt deterred growth by 2 fold (optimum OD (green filter) ~0.5 at 44h) of the organism (Fig. 3). As the bacterium was isolated from the rice leaf cultivated in normal mesophilic non-stress soil, therefore, its intolerance to salt stress would be plausible. Nevertheless, exceptions do exist, for example 9-12% salt tolerant *B. thuringiensis* could be isolated from the mesophilic soil of Srinagar, India (Das and Dangar, 2008).



a. Amplified DAPG gene b. Amplified 16S rDNA c. Phylogram of the bacterium JE1 (*Pseudomonas aeruginosa*)

Fig. 1. Amplified DAPG (0.75 kbp) and 16S rRNA gene(1.5 kbp detection), and construction of 16S rDNA based phylogram of JE1.

Table 2. Plant growth promotion (PGP) functions of the bacterium JE1.

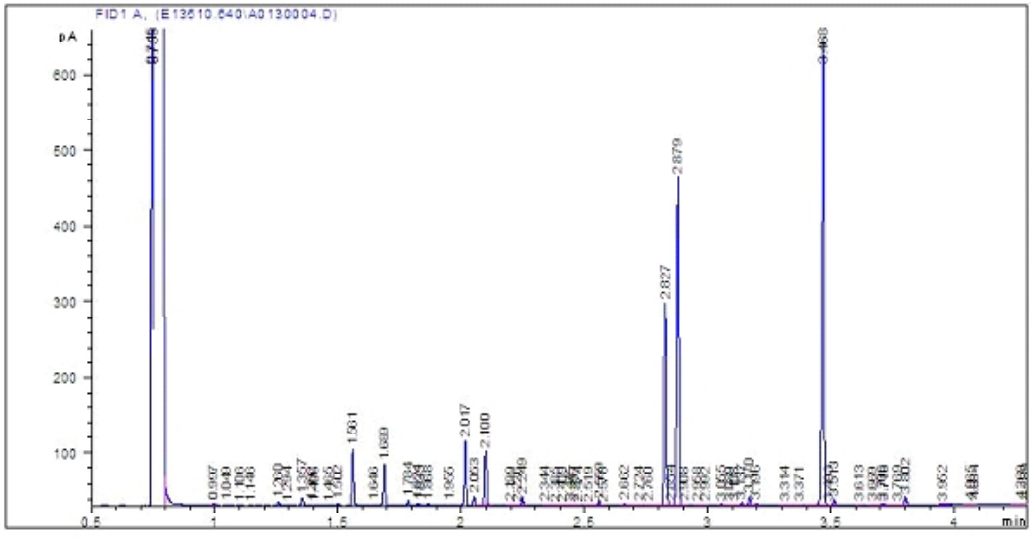
PGP function	Activity
Qualitative phosphate solubilization efficiency (PSE) on plate assay*	37.5%
Quantitative phosphate solubilization in broth culture	536.96 ± 3.61 µg/ml
Siderophore production	7.77 ± 0.52 mg/g dr. wt.
HCN production (ΔA625 nm/ml)	0.035 ± 0.001
ACC deaminase production	+
Ammonia production	+
Indole production	-

*Derived from colony dia. 16 mm and colony + halozone dia. 22 mm.

Response of JE1 to salt, pH and temperature suggests that the organism may survive in saline, alkaline and wide temp. regimes.

Antagonism of JE1 against sheath blight pathogen *R. solani*

Rice endophyllic (leaf endophyte) fluorescent *P. aeruginosa* (JE1) inhibited ShB pathogen *R. solani* and reduced growth by 35.89% (Fig. 4) which proved that the organism would be able to control rice ShB infection which was not disclosed to date. The information implicated that phytonic fluorescent *P. aeruginosa* is generally a versatile biocide which is favoured by inhibition of rice pathogen ShB by phylloplanic/phyllospheric/endophytic (Akter et al., 2014; Dey et al., 2014), as well as, *Aspergillus* sp., *Fusarium oxysporum* sp. *ciceri* and *R. solani* by rhizo-/ecto-/endospheric spp., especially pseudomonads and bacilli of different plants. Nevertheless, 35.89% inhibition of ShB by JE1 proved it to be superior than 7 *P. fluorescence* strains (3-33%) but inferior than 3 other *P. fluorescence* strains (42-85%) recorded by Reddy et al. (2008). The JE1 metabolized secondary antimicrobial compounds like siderophore (7.77 mg/g dr. wt) and HCN (0.035 ΔA625) (Table 2) which would enable it to inhibit ShB of rice and supported control of fungal pathogen by *P. aeruginosa* through inhibition of Fe availability by siderophore and cellular respiration by interrupting cytochrome C mediated electron transfer (Katiyar and Goel, 2004; Sturz and Christie, 2003; Bais et al., 2004). Nevertheless, HCN synthesis by JE1 (0.035 ΔA625/ml) was more than 6 *P. fluorescence* strains (0.01-0.03 ΔA625/ml) but lesser than that (0.09±0.01) by *P. aerugonisa* FP6 (Reddy et al., 2008; Bhakthavatchalu et al., 2013). The JE1 produced catalase (Table 1) which suggested that it would endure different stresses like other catalase producing stress tolerant microbes (Kumar et al., 2012). The fungal cell wall constituents are chitin, chitosan and structural



Library	Sim Index	Entry name
RTSBA6 6.20	0.906	*Pseudomonas aeruginosa* GC subgroup A

Fig. 2. GC spectrum of fatty acid methyl ester (FAME) of cellular lipids of the bacterium JE1.

proteins (mannoproteins and glycoproteins) etc. cross linked by glycosidic bonds *i.e.,* α-1,3 and/or α-1,4 linkages through α-glucan and β-1,3 or β-1,6 linkages through β-glucan along with the associated metabolic enzyme proteins (Roy et al., 2013; Albert and Anderson, 1987). Different rhizospheric and phytonic bacterial biocides like *Pseudomonas, Berkholderia, Bacillus, Serratia, Aeromonas* spp. etc. suppress the pathogenic fungi of rice and other plants through antifungal pathogenicity related enzymes that hydrolyze the fungal cell wall components like chitinase, protease, α-1,3 and 1,4/β-1,3 and1,6 glycosidic bonds by amylase, cellulase, glucanase etc., and cell membrane by lipase, protease, lecithinase etc. (Jaharamma et al., 2009; Inber and Chet, 1991; Albert and Anderson, 1987; Roy et al., 2013; Shyamala and Sivakumaar, 2012). For example, *Stenotrophomonas maltophilia* could control *Pythium ultimum* by protease and *P. fluorescens* could control *F. oxysporum, A. alternate, Macrophomina phaseolina, Rhizoctonia solani, Sclerotinia sclerotiorum* by α-glucanase and β-1, 3 glucanase (Yu et al., 2017). The endophyllic *P. aeruginosa* (JE1) also produced pectinase, lipase (tween 80, tributyrin and vegetable oil esterase), protease (gelatinase), cellulase (Table 1) which would antagonize the ShB pathogen *R. solani* of rice (Fig. 4), as well as, other fungal pathogens like antifungal protease and amylase producing fluorescent *Pseudomonas* sp. (Jaharamma et al., 2009; Roy et al., 2013; Inber and Chet, 1991; Albert and Anderson, 1987; Shyamala and Sivakumaar, 2012). Besides, IAA production by JE1 (Table 2) would antagonize ShB alike mycelial growth suppression of rice blast pathogen *Pyricularia oryzae* by IAA, siderophore and hydrolytic enzyme producing *P. fluorescens* (Albert and Anderson, 1987; Shyamala and Sivakumaar, 2012). The 2, 4 diacetyl-phloroglucinol (DAPG) antibiotic gene, yet un-reported from rice endospheric microbiome, could be amplified from the endophyllic JE1 by Phl2b primers (Fig.1) which indicated abundance of DAPG gene in pseudomonads of rhizospheric and phytonic microbes of plants (Ahmadzadeh et al., 2006). The JE1 produced the fluorescent pigments pyocyanin etc. (Fig. 4) which possesses the antimicrobial credential indicating that it would inhibit ShB and support the biocidal activity of fluorescent phenazine, pyrrolnitrin and pyoluteorin producing *Pseudomonas* spp. (Haas and Defago, 2005). The results of endophyllic JE1 proved that similar to the rhizospheric pseudomonads, it can control the fungal pathogens rice.

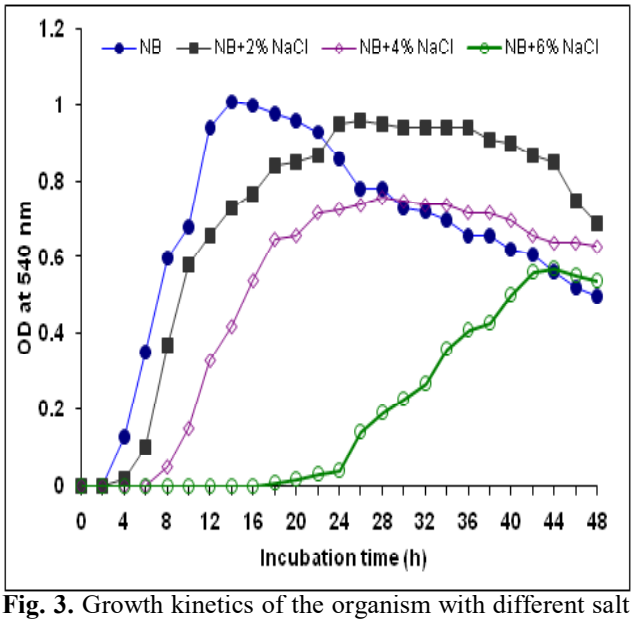
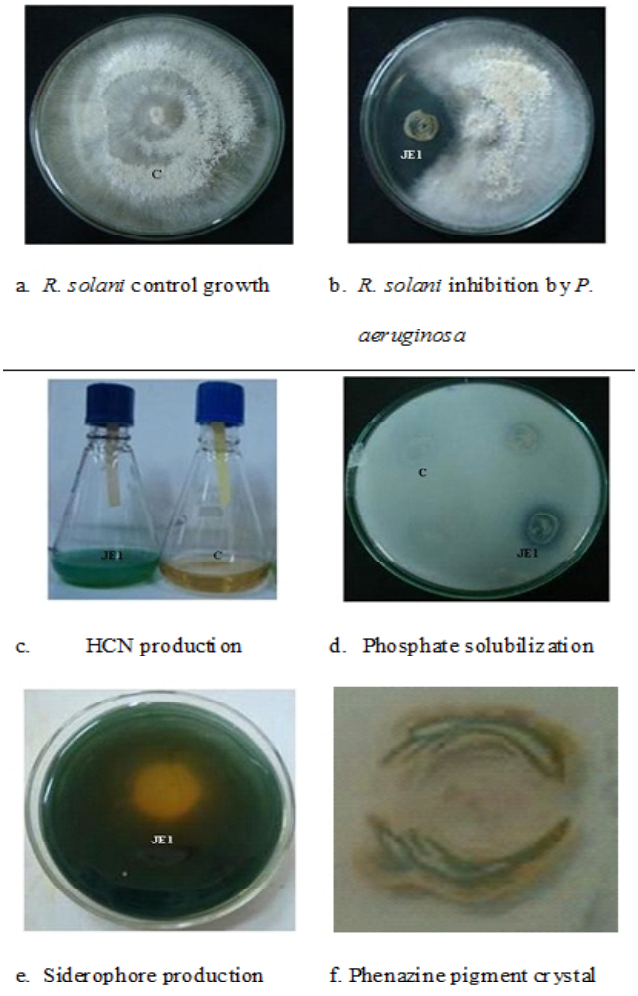


Fig. 3. Growth kinetics of the organism with different salt concentration.

Assessment of plant growth promotion (PGP) traits of JE1

The PGP determinants *i.e.,* phosphate solubilization, as well as, indole acetic acid, ammonia and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase were produced by the rice endophyllic *P. aeruginosa* (Table 2, Fig. 3) which was not (probably) recorded to date. Qualitatively, the *P. aeruginosa* (JE1) solubilized $Ca_3(PO_4)_2$ (efficiency 37.5%), and produced ACC deaminase, IAA and NH_3 (Table 2, Fig. 4) and quantitatively mineralized 536.96 µg/ml phosphate (Table 2, Fig. 4) which would incite growth promotion of rice. The results corroborated phosphate metabolism, NH_3 and ACC deaminase production by the PGPR and endophytic microbes, especially *Pseudomonas* and *Bacillus* spp. of different plants (Naureen et al., 2015, Yellareddygari, 2014; Mano and Morisaki, 2008). Nevertheless, efficiency of P mobilization (536.96 µg/ml), siderophore and HCN (7.77 mg/g dr. wt. and 0.0353 ΔA625/ml) production by JE1 were superior over *P. fluorescence* strains 1-5, 7, 9 and 10, inferior than *P. aerugonisa* FP6 but comparable to other *P. fluorescence* strains (Reddy et al., 2008). Diverse PGP and biocidal functions of the endophyllic *Pseudomonas*



a. *R. solani* control growth b. *R. solani* inhibition by *P. aeruginosa*

c. HCN production d. Phosphate solubilization

e. Siderophore production f. Phenazine pigment crystal

Fig. 4. Biocidal and plant growth promotion traits of the bacterium JE1.

sp. (JE1) proved it to be a versatile endosymbiont and can be exploited in alkaline and wide temp. regimes for rice improvement/sustenance of rice production.

The investigation concludes that the endophyllic *P. aeruginosa* of rice var. Naveen which possesses polyvalent PGP function and biocide against *R. solani* can be exploited for both protection from sheath blight and sustain productivity.

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