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RESEARCH ARTICLE

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CHARACTERIZATION OF ENDOPHYTIC DIAZOTROPHIC BACTERIA FROM WILD RICE OF SUNDARBANS FOR THEIR INCLUSION IN INM PACKAGE FOR RICE

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ABSTRACT

Method for selection of endophytic diazotropic isolates for use as nitrogen bio-fertilizer in rice cultivation is developed and standerdized. Ten endophytic diazotropic bacterial isolates were collected from roots of a wild rice species, *Oryza rufipogon*, from Sunderban area of West Bengal. These isolates were characterised on the basis of nitrogenage activity, siderophore & IAA production capacity. These isolates were also tested for contribution in yield attributing parameters in rice in pot culture. Based on these studies, 4 isolates were selected for field trials in coastal saline and red & laterite zones of West Bengal. In the trial 25% (20 – 22.5 kg) of recommended chemical nitrogen dose was deducted and supplemented by endophytic diazotropic bacteria application. Two isolates for each zones were selected on the basis of their contribution in 1000 grain weight and total yield in rice. These isolates are recommended for inclusion in INM package in rice cultivation.

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INTRODUCTION

Exploration of microbe-based symbiosis in plants is one of the effective ways in the development of sustainable agriculture (Marella, 2014). Bacterial endophytes can colonize ubiquitously in the inner plant tissues without causing any morphological change or disease symptom in the host plant. Barraquia *et al.* (1997) from IARI, Philippines, isolated diazotrophs from wetland rice and also successfully developed rice – endophytic diazotroph combinations. The endophytes promote plant growth through nitrogen fixation, production of growth promoting substances and protecting the host plants against phytopathogenesis (Ryan, 2008). Yu *et al.*(2016) isolated a number of IAA producing endophytic bacteria from corn and soybean root and demonstrated their enhancing effect on crop growth. Phetcharat and Duanga paeng (2012) got 26 isolates of endophytic bacteria from organic rice and among them, 4 were found to produce IAA over 10 µg per ml. Jasim *et al.* (2014) reported that growth promoter Indole 3-Acetic Acid production by endophytic bacteria is induced by the photochemical of host plant.

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Siderophores are are small, iron-chelating compounds of high affinity which are secreted by many bacteria and fungi. It acts as plant growth promoter as well as biocontrol agents in soil. Many workers reported the production of siderophore by endophytic bacteria and their potential role in plant growth promotion (Vendan *et al.*, 2010; Pahari *et al.*, 2017; Loaces *et al.*, 2011). It is known that rice plants can form natural association in roots with various types of nitrogen fixing (Diazotrophic) microorganisms. These microorganisms are responsible for supply of fixed nitrogen to the rice plant in addition to growth promotion through other activities. For successful colonization of endophytic diazotrophs in rice roots, their survival in soil in absence of host plant is an important factor. But the survival of effective microorganism in soil depends upon several biotic and abiotic factors in soil (Olivera *et al.*, 2004; Bashan *et al.*, 1995; Bashan and Vazquez, 2000). The biotic factors includes, presence of root exudates in soil, presence of weeds to function as alternative host, presence of organic matter in soil, *etc.* The abiotic factors includes, soil texture, soil moisture, pH , geographical location of the field. It has been speculated that wild rice is likely to harbour unique population of nitrogen fixing bacteria that differ from those in modern varieties of cultivated rice. Reports are available to demonstrate that wild rice harbour qualitatively and quantitatively better endophytic diazotrophs than cultivated rice.

Koomnok *et al*. (2007) isolated higher population of endophytic diazotrophic bacteria with higher nitrogen fixing activity from wild rice varieties *Oryza rufipogon* and *O. nivara*. Junior *et al*.,(2013) reported that an wild rice *Oryza glumaepatula* harbour diverse diazotrophic community in Brazilian Amazon. Genetically diverse endophytic diazotrophic strains were isolated from roots of wild rice, *Oryza alta.* (Chaudhary *et al.,* 2011, Liu *et al*., 2020)). Thus in the present endeavour it was hypothesized and proven that wild rice harbour endophytic diazotrophs having better nitrogenase activity, and these bacteria can be isolated from the roots and stems of wild rice. Isolated bacteria, after purification, may be tried for successful colonization in roots of cultivated rice and consequent nitrogen fixation leading to reduction in the use of external nitrogen fertilizer, and some of the bacteria are expected to be selected for further exploitation for the use as nitrogen biofertilizer in sustainable agriculture.

MATERIALS AND METHOD

Sample collection and isolation: Ten endophytic bacteria were isolate from roots of wild rice, *Oryza rufipogon* Giff, a prominant wild rice variety in Sunderban area. Roots of wild rice variety were collected from many locations of Sunderban (Table 01). For surface sterilization, fresh roots were immersed in 70% ethanol for 5 min and then in 1% sodium hypochloride for 2 min followed by five times washing in sterilized distilled water as described by Kuan *et al*., 2016. The sterilization was confirmed by streaking the roots on solid nutrient agar plates in incubation for 72 hr in 28 $\pm$ 2^0 C. About 1- 2 mm piece of sterilised roots were immersed in nitrogen free semi solid LBI medium and incubated for 72 h at 28 $\pm$ 2^0 C. Yellowish pellicles were picked out and purified by streaking on LG1.

Table 1. Isolates of endophytic diazotrophs from roots of wild rice *Oryza rufipogon*, from different locations of South 24 Parganas District (Sundarbans), West Bengal, India

Isolate No	village	Block	Latitude	Longitude
VIB ENB001	Nimpith	Joynagar-II	22.156705	88.437040
VIB ENB002	Kaikhali	Kultali	22.022719	88.615339
VIBENB0 03	Nimpith	Joynagar-II	22.156580	88.436702
VIBENB0 04	Rajatjubilee	Gosaba	22.092535	88.873338
VIBENB005	Rajatjubilee	Gosaba	22.097108	88.873338
VIBENB006	Rajatjubilee	Gosaba	22.094113	88.867113
VIBENB007	Rajatjubilee	Gosaba	22.095931	88.884677
VIBENB0 08	Tulshighata	Joynahar-II	22.150614	88.455002
VIBENB0 09	Dosea Bhagabanpur	Joynagar-II	22.155793	88.435923
VIBENB010	Nimpith	Joynagar-II	22.156212	88.438018

Nitrogenase activity: The Acetylene Reduction Assay (ARA), as described by Hardy *et al.* (1968) was followed for evaluating nitrogenase activity. Bacterial isolates were grown in 10 ml nitrogen free Jensen medium (Jensen, 1951) for 72 h in 20 ml airtight rubber cap tube. The tubes were injected by 10% (v/v) acetylene and incubated for 24 h Ethelene evolution was measured in Gas Chromatograph (Young Lin, YL6100GC, South Korea). The production of ethylene was expressed as nmols /ml of culture with 10^x number of cells/ 24 h.

Siderophore production: Microbial isolates were grown in LGI medium for 72 h. The cuture was centrifused at 15000 rpm for 5 min. 0.5 ml of supernanent was mixed with 0.5 ml CAS reagent (Pyne *et al.,* 1994). Absorbance was measured at 630 nm against a reference consisting of 0.5 ml of uninoculated medium broth. Siderophore production was calculated by the formula:
% siderophore unit = Ar –As/Ar x 100

IAA production: LB medium containing 0.5 mg L-Tryptophan /L was use for production and estimation of IAA as described by Rahman *et al.,* (2010). The inoculated culture was grown in continuous shaking at 125 rpm for 48 h at 28^0C and then centrifused at 15000 g for 1 min. Pink color was developed by adding 2 ml of Salkowski's reagent to 1 ml aliquot of supernatant and incubating in dark in room temperature for 20 min. IAA production was measured by reading the absorbance of the mixture at 530 nm against blank (uninoculated medium and reagent) and detection the concentration from the standard curve prepared from pure IAA.

Pot culture: The colonization efficiency and contribution on growth of collected isolates were studied in pot experiment as described by Youseif (2018) . Rice seeds (var *Gotra bidhan)* was surface sterilized and then washed by sterilized distilled water. Germinated seeds were treated with suspension of different isolates (10^8 cfu/ml) for 1 h and allowed to grow in pots containing 1 Kg sterilized soil. The pots were kept in polyhouse for growth. After 35 days the plants were taken carefully, the roots were washed and measured for plant height, fresh weight of whole plant, fresh weight of roots, root length, dry weight of whole plant and dry weight of roots.
The endophytic diazotrophs were recovered from the roots by inserting surface sterilized root pieces in 10 test tubes semi soil LG1 medium and observing the positive tubes after 72 h. Four isolates were selected for field experiment on the basis of these studies.

Field experiment: The selected cultures were subjected to field trials in Coastal saline zone (South 24 Parganas district) and Red & Laterite zone (Jhargram district) of West Bengal, India. The experiment was designed in RBD with treatments: Full NPK, 75%N FullPK and 75% N Full PK + Bacterial isolates. The pre soaked and germinated seeds were treated with respective bacterial culture for at least 1 h. The treated seeds were broadcasted in seed beds where no chemical nitrogen was applied. The performance of isolates were evaluated on the basis of 1000 seed weight and total yield.

RESULTS AND DISCUSSION

10 endophytic diazotrophic bacteria are isolated and purified from roots of wild rice *Oryza rufipogon* of different location of Sundarbans (Table 01). These ten isolates were analysed for the nitrogenase activity (acetylene reduction assay- ARA), siderophore production and IAA production. It was observed that 3 of the isolates VIBENB001, VIBENB003 and VIBENB010 showed good performance in ARA, VIBENB001, VIBENB002, VIBENB003, VIBENB006 and VIBENB010 are efficient in siderophore production, while VIBENB001, VIBENB003, VIBENB004, VIBENB006, VIBENB008 and VIBENB010 have good IAA production capacity in artificial medium supplimented by L-tryptophan (Table 02).

Table 02. Plant growth promoting characters of endophytic diazotrophs

Isolate No.	ARA (nmol of acetylene / ml culture of 10^8 cells/ml)	% Siderophore	IAA (µ g /mL)
VIBENB001	12.46	30.74074	8.5
VIBENB002	0.43	26.24339	2
VIBENB003	5.6	27.51323	7.0
VIBENB004	0.44	18.09524	8.5
VIBENB005	0.36	16.77249	4.8
VIBENB006	0.07	23.06878	5.4
VIBENB007	1.18	16.50794	4.6
VIBENB008	0.17	16.34921	5.2
VIBENB009	0.11	16.24339	4.8
VIBENB010	5.56	26.77249	7.5

In pot experiment, isolates VIBENB001, VIBENB002, VIBENB003 and VIBENB010 contribute significantly on plant height, fresh and dry weight of whole plant and root (Table 03). The roots from all the pots were subjected for recovery of endophytic . From all the treated pots endophytic diozotrophic bacteria were recovered but VIBENB001, VIBENB002, VIBENB003 and VIBENB010 showed 90-100% recovery. On the basis of the above studies, the four isolates were selected for field demonstration. Two isolates VIBENB003 and VIBENB010 showed good performance in field in Coastal saline region, while 2 isolates VIBENB001 and VIBENB003 perform better in Red and Laterite region having significant positive effect on 1000 seed weight as well as total yield.

Table 03. Effects of seed inoculation with different isolates of endophytic diazotropic bacteria on yield attributing parameters in rice

Treatment	Plant Height (cm)	Fresh weight whole plant (gm)	Fresh Weight Root (gm)	Root length (cm)	Dry wiight Whole plant (gm)	Dry weight root (gm)
CONTROL	54.67	1.72	0.18	10.67	0.6030	0.0687
VIBEN001	48.67	2.72*	1.00*	22.33*	0.9890*	0.2873*
VIBEN002	59.33	2.88*	0.68*	22.00*	1.1666*	0.317*
VIBEN003	54.33	2.86*	0.66*	16.00*	0.9990*	0.3733*
VIBEN004	56.67	2.53	0.45	17.33*	0.5783	0.1240
VIBEN005	51.33	1.76	0.35	16.00	0.7067	0.1533
VIBEN006	57.67	2.06	0.24	17.67	0.6467	0.1550
VIBEN007	52.33	1.71	0.19	16.00	0.5863	0.1257
VIBEN008	55.33	1.79	0.25	16.67	0.6680	0.1363
VIBEN009	53.33	1.60	0.21	12.50	0.5560	0.1190
VIBEN010	62.33*	2.47	0.66*	23.33*	0.9666*	0.2837*
SEM	41.16	0.59	0.15	18.62	0.07	0.2
$CD_{0.05}$	8.25	0.98	0.49	5.55	0.33	0.18

Significant at 5% level

Table 04. Recovery of bacteria from rice root after 35 days of growth

	No. of tubes from 10 tubes showed growth			
Treatment	R1	R2	R3	Average
CONTROL	0	0	0	0
VIBEN001	9	10	10	9.666667
VIBEN002	10	10	10	10
VIBEN003	10	9	9	9.333333
VIBEN004	7	7	10	8
VIBEN005	7	9	8	8
VIBEN006	3	7	8	6
VIBEN007	5	6	5	5.333333
VIBEN008	7	7	8	7.333333
VIBEN009	2	2	2	2
VIBEN010	10	10	8	9.333333

Table 05. Field performance of selected endophytic diazotropic isolates in coastal saline and red & laterite Zones of West Bengal.

Treatment	Coastal Saline region		Red & laterite region	
	1000 seed weight (gm)	Total yield (Qntl/ha)	1000 seed weight (gm)	Total yield (Qntl/ha)
Full NPK	26.33	33.83	33.475	32.73
75% N Full PK	22.33	16.70	24.5166667	26.58
75% N Full PK +VIBEN001	24.67	18.33	36.4583333*	33.92**
75% N Full PK +VIBENB002	26.67**	21.27	32.15	30.33
75% N Full PK + VIBEN003	27.33**	32.50**	39.5083333*	37.88**
75% N Full PK + VIBEN010	26.67**	31.67**	32.675	30.73
CD0.05	2.45	6.53	11.84	4.66
Cd0.01	3.85	10.23	18.57	7.30

Significant at 5% level
** Significant at 1% level

Dobereiner (1992) first coined the term “Endophytic Diazotrophs” to designate those nitrogen fixing bacteria which grows interiorly in the plant tissues and provide nitrogen to the host plant. Since then large number of studies have been done by several workers which suggest that these bacteria can act as nitrogen biofertilizer for highly N_2 demanding crops like sugarcane, maize and rice (Puri *et al.,* 2018). Banik *et al.* (2019) successfully applied root endophytic diazotrophic strain of *Azotobacter* sp in rice and found root biofilm containing the bacteria with associated enhanced chlorophyll content in flag leaves and yield. Similarly, enhancement of growth and yield of rice plant was observed after applying probiotic endophyte by Shabanamol *et al.*(2020). Our observation keeps pace with that by Gyaneshwar *et al.* (2001) who successfully isolated endophytic diazotrophic bacteria *Serratia marcescens* from four different rice varieties and Reports are available where it was shown that these bacteria can promote plant growth through activities other than nitrogen fixation. It is evident that production of siderophore by endophytic bacteria inhibits the growth of microorganisms including the pathogenic ones. This not only confirms the competitive advantage to the endophytic bacteria for its colonization but also prevent the plants the infection of soil borne pathogens (Loaces *et al* 2011). Evidences are also available for antagonistic activities of these bacteria for rhizosphere or rhizoplane phytopathogenic bacteria or fungi by depriving them from ironthrough production of siderophore (Pahari *et al.,* 2017). Several workers demonstrated that IAA producing bacteria, when applied in soil, promote the growth of the plants.

In an experiment, Rangjaroen *et al.,* (2017) reported enhanced growth of rice plant by applying diazotropic bacteria *Novosphingobium* which was seen to produce high amount of IAA in cultural condition. Saheb et al (2009) observed some phosphate solubilising bacteria enhances plant growth which cannot be explained merely by phosphate solubilisation, later they found these bacteria produces significant amount of IAA in addition to phosphate solubilisation. A two fold increase in growth parameter like fresh and dry weight of shoot and roots were observed by applying IAA producing rhizobacteria in tomato plant (Myo *et al.,* 2019). Our results clearly keep parity with the above findings where nitrogen fixing, siderophore producing and IAA producing bacteria enhances plant growth. Among the four isolates VIB ENB001, VIB ENB002, VIB ENB003 and VIB ENB010, all except VIBENB002 are efficient in nitrogenase activity, siderophore production and IAA production. VIBENB002 is only efficient siderophore producer. But the result clearly shows that mere siderophore production cannot contribute significantly to the yield of rice . On the basis of the result it is concluded that isolate VIBENB003 and VIBENB010 may be used as nitrogen biofertilizer during formulation of Integrated Nutrient Management package for paddy cultivation in Sundarban region while isolates VIBENB001 and VIBENB003 may be use in same purpose in red and laterite region of West Bengal, India. The responses of the selected diazotrophs along with other bacteria like Phosphate, potash and zinc solubilizing bacteria and development of complete INM package for paddy cultivation, will require further study.

CONCLUSION

Form this experiment it is concluded that these 3 isolates of endophytic diazotropic bacteria from *Oryza rufipogon* may be used as nitrogen biofertilizers in rice cultivation. Further experiment is required for the development of functional consortia of effective microorganism like phosphate potash and zinc solubilising bacteria for the development of complete Integrated Nutrient Management package for rice cultivation.

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