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Endophytic microbial diversity and population dynamics in wild and cultivated rice genotypes

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ABSTRACT

Diversity and dynamics of endophytic heterotrophic (HB), spore forming (SFB), spore-crystal forming (SCB), nitrifying (NB), denitrifying (DNB), phosphate solubilizing (PSB) bacteria, actinomycetes (ACT) and fungi (FUN) in leaf, stem and root of leaf folder tolerant (PTB-12 and Nivara) and susceptible (Naveen and Tapaswini) rice (*Oryza* spp.) genotypes were analyzed which are unattained to date. Tapaswini roots produced all 8 types endophytes but that of Nivara, Naveen and PTB12 produced 6, 5 and 4 types of communities but stems and leaves had lower diversity. The HB, SFB and SCB were universal but NB, DNB, PSB, ACT and FUN had discrete occurrence. The SCB (*Bacillus thuringiensis*) was recorded first time from the cultivated rice genotypes. Quantum ($x10^2$ cfu/g dr. wt.) of endophytic microbes in different parts of the rices were 0.05-53.14; higher population of HB was in *O. nivara* stem (4.23) and root (53.14), Naveen leaf (10.40), and SF (44.11) or SCF (41.91) in Tapaswini root. At least one part of each plant had endophytic FUN (0.05-0.88 $x10^2$ cfu/g) but ACT (0.33-1.09 $x10^2$ cfu/g) was present in root of most rices, DNB (0.05-1.33 x 10^2 cfu/g) was less pronounced, NB (0.05-4.10 x 10^2 cfu/g) was undetectable in leaf, and PSB (1.00 x 10^2 cfu/g) was present in Tapaswini root only. Broadly, the endophytic microbes were 2-4 exponent lower than the native soil microbial pool. Wide dynamics and diversity of beneficial endo-microbial communities would variously help growth and development of the rice genotypes, and the *B. thuringiensis* would intrinsically suppress rice pests and diseases.

1. Introduction

Alike *ex-planta* plant growth promoting microbes (PGPM), endophytes have been reported from almost all plant groups including monocots and woody plants (Bent and Chanway 1998, Shishido *et al*., 1999). The beneficial endophytic microbes are universally associated with plants (Azevedo *et al*., 2009) which have superioreffect than superficial microbiome (Ladha *et al*., 1998). Rice (*Oryza sativa* L.) genotypes are also natural reservoirsof hundreds of endo-microbes (Barraquio *et al*., 1997) and each plant harbours more than two types of organisms (Strobel *et al*., 2004; Senthilkumar *et al*., 2011). The plant growth promoting (PGP) and biocidal microbes viz. *Aeromonas, Berkholderia, Bacillus, Pseudomonas, Methylobacterium, Curtobacterium, Flavobacterium, Herbaspirillum, Pantoea, Klebsiella, Azospirillum, Enterobacter, Streptomyces, Penicillium, Fusarium* spp. etc. have been recorded as natural phytonic (rhizo-/phyllo-/endo-/ectospheric) residents of various wild and cultivar rice genotypes (Mano and Morisakai, 2008, Francis *et al*., 2010, del Castillo *et al*., 2015). Besides nutrient supplement (Mano and Morisakai, 2008; Francis *et al*., 2010; Bashan *et al*., 2014), they metabolize various growth regulators which support growth and development of plants (Duran *et al*., 2014), as well as, combat biotic and abiotic stresses (Azevedo *et al*., 2000; Sturz *et al*., 2000; Compant *et al*., 2005; Francis *et al*., 2010; Mitter *et al*., 2013).

Endophytic population (10^2 to 10^9 cells/g) has been assessed from several plant species, which would vary with genotype or environmental conditions (Lamb *et al*.,1996,

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Hallmann *et al*., 1997, Overbeek *et al*., 2008, Chi *et al*., 2005), as well as, the endophytic diversity decreases from root upwards *i.e.* stem and leaves (Lamb *et al*., 1996). Numerical abundance of endospheric microbes (10^3-10^6cfu/g) recorded lower than ectosphericones (10^6-10^8cfu/g) in rice, cotton, corn etc. (Lindow and Brandl, 2003; Bashan *et al*., 2014) and the polyvalent plant growth promoting bacteria (PGPB) (8.91 x 10^1 to 7.24 x 10^6 cells/g) of cultivated (Sabita, Swarna, Swarna *Sub1*) and wild (*Oryza eichingeri*) rices also differed widely (Banik *et al*., 2016, 2017). However, except for N-fixing endo-PGPB (Mano and Morisaki, 2008; Francis *et al*., 2010; Banik *et al*., 2015, 2016), quantum of endospheric microbial dynamics and diversity of cultivated and wild rices have been unsystematically investigated (van der Lelie, 2012). Considering poor knowledge on diversity and dynamics of rice (cultivar and wild) endophytes and significance of their functionalities on overall rice improvement, the endo-microbial guilds and pool sizes in some wild and cultivar rice genotypes were investigated to understand their significance in relation to the host rice genotypes.

2. Materials and methods

2.1. Experimental site and soil physico-chemical characters at transplantation

The experimental rice (*Oryza sativa* L. and *O. nivara* Sharma & Shastry) genotypes were grown in the rice field of ICAR-National Rice Research Institute, Cuttack, Odisha located at 20°312 232 2 N and 85°472 172 2 E. The experimental field soil is deltaic sediment, sandy (52.5%)-clayey (25.9%)-loam (21.6%) with pH 6.28, electrical conductivity (EC) 0.53dS/m, total C 5.01 g/kg, 0.52g/kg total N, 18.52 mg/kg Olsen Pand 120.49 mg/kg available K. The field was prepared following standard agronomic practices supplemented with 5t/ha farm yard manure.

2.2. Collection of pre-transplantation soil samples for microbial analysis

Five subsurface (1-5 cm depth) soil samples (25 g each) from 5 locations at 5 m apart were collected from the pre-transplanted field before subplot preparation. The soilsamples were mixed and the composite soil wasused to assess the microbial guilds.

2.3. Cultivation, selection and collection of plant genotypes

Leaf folder (LF) tolerant genotype i.e. *O. sativa* cv. PTB-12 and *O. nivara* (wild species), and susceptible genotypes *O. sativa* cv. Naveen and *O. sativa* cv. Tapaswini were selected for analysis of endo-microbial dynamics and diversity.The cultivars viz. *O. sativa* var. PTB-12, Naveen and Tapaswini were grown fertilized with NPK @ 60:40:40 kg/ha. The wild sp. *O. nivara* was grown with periodic weeding only. Healthy plants were uprooted at panicle initiation (PI) stage, stored in polythene bags, brought to the laboratory and processed immediately or stored at -80°C for isolation of endophytic bacteria.

2.4. Microbial diversity and dynamics of rice endophytes and in soil of experimental field

Healthy tillers were thoroughly washed under running tap water to remove adhered soil particles, thereafter individually washed in sterile (autoclaved at 1.1 kg/sq. cm pressure, 121°C) distilled water and surface sterilized with 0.1% (w/v) $HgCl_2$or 1% chloramine T for assessment of the endophytes (Barraquio *et al.,* 1997). For endophyte isolation from leaf, stem and root, each part was cut into 1cm pieces andsix segments were used for endophyteisolation while another six parts were used for gravimetric assessment of dry wt. Under a laminar air flow, eachtype of plant pieces were sterilized washed twice in 90% ethanol, followed by sterile distilled water followed by surface sterilized with 0.1% $HgCl_2$ for 2 min and washed with sterile distilled water. The samples weremacerated in 1 ml sterile distilled water, removed the fibrous materials and the macerates were used for enumeration of eight different microbial guilds (Collee and Miles, 1989, Collins *et al*., 2004).

The composite soil of the experimental field prior to transplantation was optimally dried within sterile (mentioned elsewhere) blotting papers, 1 g blotted soil was suspended in 10 ml sterile distilled water and diluted to 10^{-3} level for microbial analysis following standard methods (Collee and Miles, 1989, Collins *et al*., 2004). Soil dry weight was estimated gravimetrically.

2.5. Viable count of different endophytic and soil microorganisms

The macerates (200µl) of the plant parts and soil suspensions (100 ìl, 10^{-3} dilution) were individually mixed with different media, poured into 5 plates and incubated at 28±0.1°C for 3-5 days or more (if required) to assess the microbiome (Collee and Miles, 1989; Collins *et al*., 2004). Total population was counted and expressed as colony forming units (cfu)/g dr. wt. To estimate heterotrophic bacterial guild, 100 mlnutrient agar (NA) medium (g/l: peptone 5.0, beef extract 3.0, NaCl 3.0, pH 7.0, agar 20) was mixed with macerate/soil suspension and colonies were counted after 72h incubation. For spore, the macerate/soil suspension were heated at 60 ± 0.1°C for 1h, mixed separately with 100 ml NA, plated and colonies were counted after 3d as spore (depicting reflecting structure under phase objective) producing bacteria. For spore-crystal producers (tentative *Bacillus thuringiensis*), heated macerate/soil

suspension was plated in NA containing 0.25M Na-acetate (Das and Dangar, 2008), after 3d the colonies were observed under 100x phase objective and those produced crystal inclusion bodies (bright structures) along with spore were counted as spore-crystal former.

The nitrifying (NH_4^+oxidizer) and denitrifying (NO_3^- reducer) bacteria were determined using the macerate/soil suspension mixed with 100 ml Winogradsky medium (g/l: K_2HPO_4 1, NaCl 2, $MgSO_4.7H_2O$ 0.5, $FeSO_4.7H_2O$ trace, $CaCl_2.2H_2O$ 0.02, pH 8.5, agar 18) containing separately filter (0.22ìm) sterilized 1g/l $(NH_4)_2SO_4$ and KNO_3 or $NaNO_3$, respectively and incubated for 25-30d and 7d, respectively. To determine the nitrifying/denitrifying bacterial population, sulfanillic acid reagent (eqi-volume mixture of 0.8% sulphanillic acid and 0.5% á-naphthyl amine, both in 5M acetic acid) was poured into plates and the pink colonies were counted.

Glucose asparagine (GA) medium (g/l: glucose 10, asparagine 0.5, K_2HP0_4 0.5, pH 7.0, agar 15) was used for actinomycetes enumeration. To 100 ml of the medium, macerate/soil suspension were mixed separately, plated and the dry chalky colonies were counted after incubation for 10d or more.

The macerate/soil suspension were mixed with calcium phosphate agar medium (g/l: $Ca_3(PO_4)_2$ 10, $MgSO_4.7H_2O$ 0.2, $FeSO_4.7H_2O$ 0.01, $Ca(NO_3)_2.4H_2O$ 0.01, glucose 10, $(NH_4)_2SO_4$ 0.1, agar 18) and the colonies encircled with halo zones were noted as P-solubilizers.

The fungal population was estimated in mycological agar (MBA) medium (g/l: peptic digest of soybean meal 10, dextrose 40, pH 7.0, agar 18) plated with the macerate/soil suspension and the wooly colonies were counted after 3-5d growth.

Population of the microbial guilds were estimated following: Population (cfu/g. dr. wt.) = (Average population count x dilution factor x 1000)/Dr. wt. (mg) of tissue or soil.

2.6. Phenotyping of spore-crystal forming bacteria

The spore-crystal forming bacteria were characterized by cultural, morphological, staining, physiological and biochemical characters following standard techniques (Collee and Miles, 1989; Collins *et al*., 2004). The vegetative cells, spores and crystals, and motility were recorded under a phase contrast microscope (100X) from 6-96h old shake cultures. Gram staining of 6-8 h old vegetative cells, malachite green staining of spores and amido black 10B staining of crystals from 72-96 h old cultures were observed under 100X light microscope.The organisms were identified using the diagnostic phenotypic characters (Logan and de Vos, 2009).

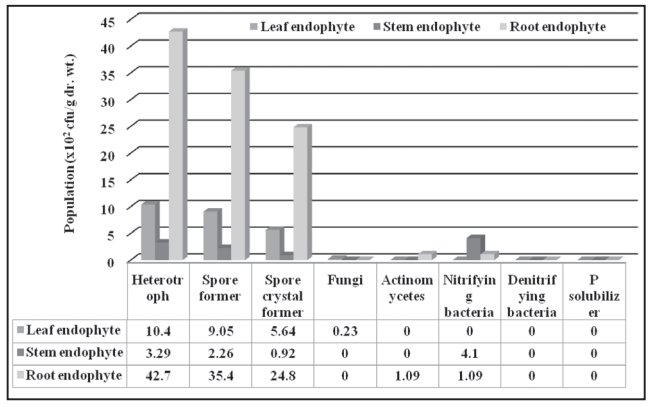
3. Results and discussion

Diversity of endophytic microbial groups (types) viz. heterotrophic (HB), spore forming (SFB), spore-crystal forming (SCB), nitrifying (NB), denitrifying (DNB), phosphate solubilizing (PSB) bacteria, actinomycetes (ACT) and fungi (FUN) in root, stem and leaf of LF tolerant (PTB-12 and Nivara) and susceptible (Naveen and Tapaswini) rice genotypes are presented in Figs. 1-4. Endo-microbe diversity revealed that the roots of Tapaswini accommodated optimum i.e. 8 diverse types of microbes (Fig. 3) followed by Nivara (6 types), Naveen (5 types) and PTB12 (4 types) (Figs. 1, 2, 4). Relative to roots, the stems and leaves of the rice genotypes possessed fewer viz.3 (Nivara) to 5 types of bacterial communities (Figs. 1-4). However, HB, SFB and SCB resided in the endospheric niches of all rices but other groups i.e. NB, DNB, PSB, ACT and FUN discretely inhabited in different parts of the rice genotypes and microbe community types had no systematic relation either with plant parts or genotype (Figs. 1-4) but the reasons thereof could not be explained form the present study. Nevertheless, presence of SCB (tentatively *Bacillus thuringiensis i.e*. Bt) in all plants suggested that Bt would be universal endophyte of rice which, however, been recorded from cultivated rices for the first time but known in wild rice *O. brachyantha* (Acharya *et al*., 2017). Quantum of endophyte diversity was greater (5-8 types) in various parts of Tapaswini compared to those in the counterparts of the remainder rices (Figs. 1-4). Presence of various microbe communities in all genotypes suggested that like environmental and ectospheric microbes, endogenous microbes also regulate diverse biogeochemical processes within the rice varieties and supports occurrence of different microbial guilds in various plants (rice, cotton, corn etc.) including cultivated and wild rices (Sabita, Swarna, Swarna *Sub1* and *O. eichingeri*) (Hallman *et al*., 1997; Bashan *et al*., 2014; Banik *et al*., 2016, 2017; Acharya *et al*., 2017). The results also proved that endogenous microbial diversity was more in roots of all varieties than either in leaves or stems (Figs.1-4).Generally the endophytic microbes colonize from soil mainly through rootsystem which would result in more microbial communities in root than upper plant parts of both wild and cultivated rices (Reinhold-Hurek and Hurek, 1998; Banik *et al*., 2015, 2016, 2017; Acharya *et al*., 2017). Nevertheless, to conclude the reason of presence of all microbial guilds in different parts of Tapaswini, indifferent relations of the microbiomes with different genotype and plant parts needs to be studied thoroughly targeting specific plant-microbe interactions.

Plant part wise, the root, stem and leaf of Tapaswini had numerically ($x10^2$cfu/g dr. wt.) more HB (6.13 - 47.42) (but Nivara stem/root), SFB (5.75 – 44.10) and SCB (5.42

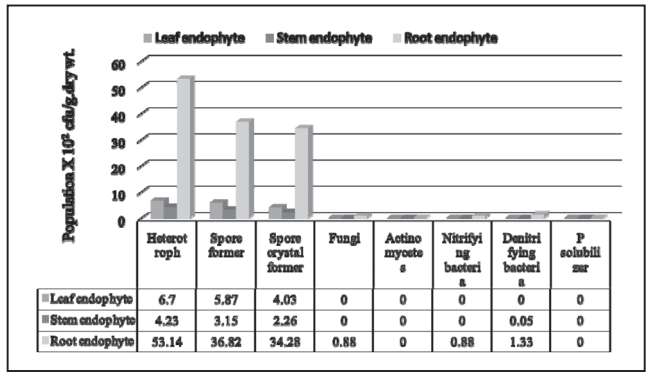
– 41.91) pool than the corresponding populations viz. 2.30-53.14, 2.05-36.82 and 0.92-34.28 of other genotypes (Figs. 1-4). All parts of different genotypes did not harbor NB, DNB, PSB, ACT and FUN, and their spatial occurrence also did not follow any general trend (Figs. 1-4). Although Tapaswini root possessed all microbe communities with pool dynamics range 0.50-1.53 x10^2cfu/g dr. wt. (Fig. 3) but leaf, leaf/stem and stem did not accommodate FUN, NF/PSB and DNB, respectively (Fig. 3). Other than roots, NB, DNB, PSB, ACT and FUN could not be obtained from different parts of other rice genotypesalso (Fig. 1, 2, 4). Microbial guild wise, endophytic HB population (x10^2 cfu/g dr. wt.) was more in stem (4.23) and root (53.14) of wild variety *O. nivara*, Naveen had maximum HB count in leaf (10.40), Tapaswini had more SF (44.11) and SCF (41.91) abundance in root (Figs. 1-4). Endophytic fungi (x10^2 cfu/g dr. wt.) occurred in the leaf of Naveen (0.23) and PTB12 (0.05); root of Nivara (0.88) and Tapaswini (0.50), and stem of Tapaswini (0.06) but actinomycetes (0.33 in PTB12 to 1.09 in Naveen) were detected in root of most rice genotypes (Figs. 1-4). Nevertheless, nitrifying bacterial population density (0.05-4.10 x 10^2 cfu/g dr. wt.) was limited to stem and root zones while undetectable in leaf, denitrifying bacteria (0.05-1.33 x 10^2 cfu/g dr. wt.) were quite low and phosphate solubilizing bacteria (1.00 x 10^2 cfu/g dr. wt.) were obtained in Tapaswini root only (Figs. 1-4). Unlikely, different authors could culture various microbial communities from all parts of different cultivated (*e.g*. Sabita, Swarna, Swarna *Sub1* etc.) and wild (e.g. *O. eichingeri*) rice genotypes (Elbeltagy *et al*., 2000; Banik *et al*., 2017). Numerical abundance (0.05-53.14x 10^2 cfu/g dr. wt.) of endogenous microbes of the rice varieties of the present study was lower than those (10^2-10^9cfu/g) of various other plants (Hallman *et al*., 1997; Bashan *et al*., 2014), as well as, rice cultivars Sabita, Swarna *Sub1*, Swarna and wild *O. eichingeri* (8.91 x 10^1 to 7.24 x 10^6cfu/g) (Banik *et al*., 2017). However, in the cultivars/wild rices of the present investigation,absence of some microbial guilds in some aerial plant parts proved that endospheric colonization of microbes would not be universal i.e. all parts of each rice variety or any one part of all genotypes might be microbe free which favoured the report of differential colonization of microbial guilds in different parts of the wild rice *O. brachyantha* (Acharya *et al*., 2017), as well as, gradual declining trend of endogenous microbes supported the proposition of Lamb *et al*. (1996).

In soil of the pre-transplanted field, numerical abundance (x10^6cfu/g dr. soil) of HB (1.42), SFB (0.99), SCB (0.45), NB (0.89), DNB (1.12), PSB (0.17), ACT (0.19) and FUN (0.84) were recorded. The results corroborated with the population dynamics (0.15-1.70 x10^6cfu/g dr. soil)



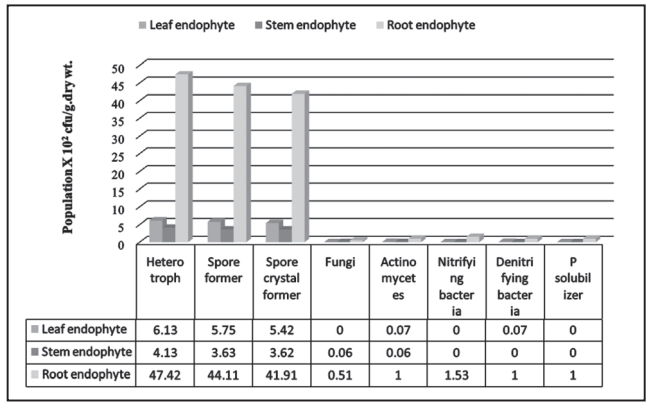
	Heterotroph	Spore former	Spore crystal former	Fungi	Actinomycetes	Nitrifying bacteria	Denitrifying bacteria	P solubilizer
Leaf endophyte	10.4	9.05	5.64	0.23	0	0	0	0
Stem endophyte	3.29	2.26	0.92	0	0	4.1	0	0
Root endophyte	42.7	35.4	24.8	0	1.09	1.09	0	0

Fig. 1: Population dynamics of *Oryza sativa* cv. Naveen



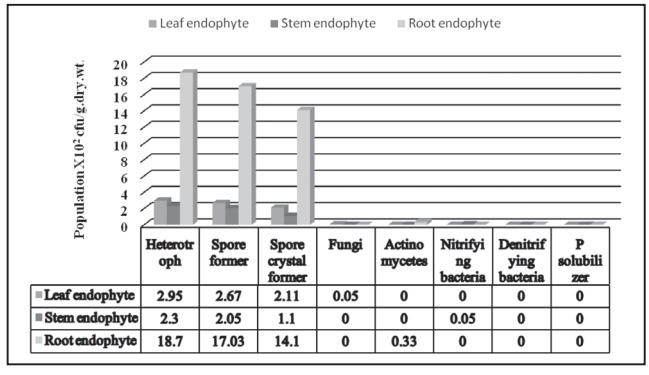
	Heterotroph	Spore former	Spore crystal former	Fungi	Actinomycetes	Nitrifying bacteria	Denitrifying bacteria	P solubilizer
Leaf endophyte	6.7	5.87	4.03	0	0	0	0	0
Stem endophyte	4.23	3.15	2.26	0	0	0	0.05	0
Root endophyte	53.14	36.82	34.28	0.88	0	0.88	1.33	0

Fig. 2: Population of endophytic microbes in *Oryza nivara*



	Heterotroph	Spore former	Spore crystal former	Fungi	Actinomycetes	Nitrifying bacteria	Denitrifying bacteria	P solubilizer
Leaf endophyte	6.13	5.75	5.42	0	0.07	0	0.07	0
Stem endophyte	4.13	3.63	3.62	0.06	0.06	0	0	0
Root endophyte	47.42	44.11	41.91	0.51	1	1.53	1	1

Fig. 3: Population of endophytic microbes in *Oryza sativa* var. Tapaswini



	Heterotroph	Spore former	Spore crystal former	Fungi	Actino mycetes	Nitrifying bacteria	Denitrifying bacteria	P solubilizer
Leaf endophyte	2.95	2.67	2.11	0.05	0	0	0	0
Stem endophyte	2.3	2.05	1.1	0	0	0.05	0	0
Root endophyte	18.7	17.03	14.1	0	0.33	0	0	0

Fig. 4: Population of endophytic microbes in *Oryza sativa* var. PTB-12

of other rice fields of experimental site (Das *et al*., 2013). Comparative results of native soil microbes and endophytic microbes of different plants revealed that the latter populations were about 2-4 exponent lower than the former ones. As endophytic microbes colonize mainly through the roots (Reinhold-Hurek and Hurek, 1998), observations of significantly lesser endophytes in the cultivar and wild rices suggest that all soil-inhabiting microbes wouldnot be rice endo-colonizers, otherwise, interaction for migration of microbes in plants is highly restricted.However, the observations supported the proposition that endophytic diversity is highly depended on host genotype, and soil and environmental conditions (Pillay and Nowak, 1997; Tan *et al*., 2003).

Phenotypic characters showed that the vegetative cells of the SCB were motile, rod shaped, gram positive; spores were elliptical formed within non-swollen sporangium, spore stained with malachite green; along with spores inclusion crystals were produced which were amido black 10B stain positive; the organisms produced protease, catalase, oxidase and nitrate reductase but none was strict anaerobe. The diagnostic phenotypic characters identified the SCB as *Bacillus thuringiensis* (Logan and de Vos, 2009). Thus the results proved that all cultivars and wild rices investigated during the study endophytically colonized Bt which conformed with the record of endo-Bt in wild rice *O. brachyantha* (Acharya *et al*., 2017).

4. Conclusion

Leaf folder tolerant (wild Nivara and cv. PTB-12) and susceptible (cv. Naveen and Tapaswini) rices have wide endomicrobial diversity, predominantly in the roots. Dynamics and diversity of various endophytic microbial communities of diverse biogeochemical groups would modulate the functionalities inside the plant like the ecto-/ environmental microbiome and support growth and development rice. Besides, the SCB (Bt) would be intrinsic biocides against pests and pathogens of rice. Exploitation of theeffective endophyticPGPB would supplement nutrition and Bt would suppress pests and diseases.

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