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Structure and function of phytonic *Bacillus thuringiensis* of wild rice genotype *Oryza brachyantha* A. Chev. and Roehr.

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ABSTRACT

Endospheric microbiome of unexplored wild rice, Oryza brachyantha (OB), was studied. Endogenous heterotrophic, spore forming, spore-crystal forming, nitrifying, denitrifying, P solubilizing bacteria, actinomycetes, and fungi in leaves, stems and roots were (0.07-4.48, 0.06-4.08, 5.00-4.00, 0.56-1.40, 0.06-0.40, 0.07, 0.03 and 0.14-8.00) x10^2 cfu/g, respectively and endo-colonizers were 5-6 times more in roots than other plant parts. Phenotypic characters, cry$^+$/cyt$^+$ in 6 SCF and 16S rDNA phylogeny of 2 potent SCF ascertained their identity as Bacillus thuringiensis (Bt). For the first time, 6 polyvalent Bt with biocidal and plant growth promotion (PGP) functions were identified from the wild rice OB. All endo-Bt possessed diverse anti-lepidopteran cry genes which predicted their virulence against rice leaf folder (LF), Cnaphalocrocis medinalis Guenee. Only cry2 or cyt primers amplified expected sized amplicons from Bt 18, 20 and 21. Bt18 and Bt21 killed 60% LF larvae in vitro by 2.65x10^4 and 7.56x10^4 cfu/ml and the former one was more virulent. They produced siderophore, amylase, protease etc. which would induce natural tolerance of OB against bacterial and fungal pathogens. Besides, the Bt had other PGP traits too viz., nitrate reduction, ammonia production, P solubilization etc. Therefore, the endo-microbiome of OB would modulate endospheric functionalities, promote growth and development, and impart natural endurance to OB against pests and diseases.

Key words: *Bacillus thuringiensis, Cnaphalocrosis medinalis, endophyte, leaf folder, Oryza brachyantha, phytonic microbe*

INTRODUCTION

Interaction of phytonic microbes and plants would be neutral, beneficial or detrimental. Beneficial ecto- and endospheric plant growth promoting microbes (PGPM) like *Pseudomonas, Azotobacter, Klebsiella, Azospirillium, Herbaspirillum, Pantoea* spp. etc. can augment in situ N_2, PO_4, NH_3, growth regulator etc. metabolism or drought and salinity tolerance (Francis et al., 2010; Bashan et al., 2014; Duran et al., 2014). The endo-PGPM or biocides (*Pseudomonas, Bacillus, Beauveria, Metarihizium, Trichoderma* spp. etc.) also counter the pests and diseases through toxin, inhibitor, antibiotic etc. metabolism in various plants (Mano and Morisakai, 2008; Vega, 2008; Naik et al., 2009; Francis et al., 2010). However, comprehension on innate resilience mechanisms of tolerant plants to low-nutrient and abiotic/biotic stress imparted by in *planta* microbiome and their co-evolution is lacking. Nevertheless, barring dicots, plant growth promotion (PGP) functions of phytonic microbiome (other than N-fixers) of monocots like rice (*Oryza* spp.) (particularly wild counterparts) is feebly resolved and biocidal function analysis is totally neglected.

The endo-/ectospheric PGPM *viz., Bacillus, Pseudomonas, Herbaspirillum, Pantoea, Klebsiella, Methylobacterium, Azospirillum, Curtobacterium, Flavobacterium, Enterobacter, Streptomyces*, *Penicillium, Fusarium* spp. etc. have been identified

from wild and cultivated rice genotypes (Elbeltagy et al., 2000; Mano and Morisakai, 2008; Naik et al., 2009; Francis et al., 2010; del Castillo et al., 2015). Except for N-fixing endo-PGPM (Mano and Morisaki, 2008; Naik et al., 2009; Francis et al., 2010), functional interactions of microbiome and plant (particularly rice), and latter's adaptive mechanism to adverse ecosphere are vague (Ryan et al., 2008). In rice, cotton, corn etc. numerical abundance of ecto-microbes were recorded to be 10^6-10^8 cfu/g (Lindow and Brandl, 2003), endo-microbes were 10^3-10^6 cfu/g (Hallman et al., 1997, Bashan et al., 2014) and polyvalent PGP endophytes of rice cultivars Sabita, Swarna Sub1, Swarna and wild genotype *O. eichingeri* were 8.91 x 10^1 to 7.24 x 10^6 cfu/g (Banik et al., 2016, 2017).

Antagonism of various pests and pathogens (*Rhizoctonia, Fusarium, Xylella* spp. etc.) of sunflower, bean, potato, cotton, sorghum, coffee, cocoa etc. (Azevedo et al., 2000; Francis et al., 2010; Gao et al., 2010; Lacava and Azevedo, 2014; Kambrekar, 2016) by endogenous biocides *Beauveria, Metarhizium, Trichoderma, Streptomyces, Pseudomonas, Flavobacterium, Bacillus, Nocardia, Clavibacter* spp. etc. and mold fungi of *Chelidonium* sp. by 11 *Bacillus thuringiensis* (Bt) were reported by different investigators (Goryluk et al., 2009). Bt is ubiquitous across ecospheres/habitats like soil, water, plant, lower animals, insects etc. and >130 insect species, several fungi and bacteria are controlled by it (Raddadi et al., 2008; Zhou et al., 2008; Dangar et al., 2010; Ghosh et al., 2016). Although no endo-Bt of rice has been studied thoroughly to date, 71 serotypes and 84 serovars of non-endospheric Bt have been recognized (Zhou et al., 2008). Bt produces entomocides like exotoxins (4 types) *i.e.,* α (lecithinase C), β (thuringiensin), γ (phospholipase) and Vip (4 types) like vegetative insecticidal protein, and endotoxins (2 types) *viz.,* δ-endotoxin 74 types (n=307) and cytolysin 3 types (n=14); antimicrobial factors like AHL lactonase, β-1,3-glucanase, zwittermicin A, autolysin, bacteriocin, chitinase etc. and several PGP (nutrient and plant growth regulator metabolism) properties (deBarjac and Fracon, 1990; Boucias and Pendland, 1998; Raddadi et al., 2008; Zhou et al., 2008; Crickmore et al., 2016). Multipotent biocidal and PGP properties implied that endogenous microbes (amid Bt) would modulate intrinsic resistance/tolerance of plants to pest and diseases, and nutrient dependency of associated plant.

Rice is staple food of about 50% world population and enhancement/sustenance of production is needed to feed about 8.1 billion people by 2025 (Zeigler and Barclay, 2008). About 120 insects and 74 diseases effect 30 and 10% yield loss, respectively (Roy et al., 2013). The major pest, rice leaf folder (LF) (*Cnaphalocrocis medinalis* Guenee) may share up to 80% loss of insects and to date lacks resistant variety to assure crop security (Satish et al., 2007). The wild rice genotype *Oryza brachyantha* A. Chev. and Roehr. (OB) has in-built LF tolerance (Ramachnadran and Khan, 1991) but the mechanism is unknown. Revelation and exploitation of the LF tolerance mechanism of OB would guide to improve tolerance of sensitive genotypes against LF and sustain production. Globally, endogenous Bt of rice has not yet been studied and no virulent endophytic Bt against LF is known to date. Therefore, diversity and dynamics of edophytic bacteria and characters of in planta *B. thuringiensis* of *O. brachyantha* have been undertaken for the first time to conjecture plant-microbe relationship for LF resistance intended to exploit the endospheric microbes, mainly Bt, to sustain/improve rice productivity.

MATERIALS AND METHODS

Collection of plant samples

Healthy tillers of the wild rice, *Oryza brachyantha* A. Chev. and Roehr. (OB) was uprooted at panicle initiation (PI) stage from unmanaged (but weed free) Oryza garden of NRRI, Cuttack, Odisha. In wild, OB grows in swampy low land, photosensitive, 90-105 cm tall, life span <100d and panicles initiate (PI) by about 65 days after transplantation (DAT). The uprooted tillers were washed to remove soil, sealed in polybags, taken to laboratory and processed immediately or preserved at -80 ± 0.1°C up to 2 month.

Structural dynamics and isolation of ecto-/ endospheric spore-crystal forming microbes

The tillers were rigorously washed under running tap water, blotted to dryness followed by washed in sterile (autoclaved at 121 ± 0.1°C, 15 min) distilled water. Further processing was done under the laminar airflow cabinet. The plant samples were rewashed in sterile water, blotted within sterile blotting paper and the leaves, stems and roots were cut into 1.5 cm pieces, washed

twice with sterile distilled water. For phyllospheric and rhizospheric microbe enumeration, 6x1.5 cm plant explants were put in 10 ml sterile wash solution (aqueous 0.001% Tween 80 with 0.01M $MgSO_4$ and 0.15% glycerin), agitated at 120 rpm at 30 ±1°C for 4h on a rotary shaker and the washings were used to isolate the epiphytic microbes (del Castillo et al., 2015; Kinkel et al., 1995). For endophytic microbe isolation, washed plant pieces were separately surface sterilized 2 min. each with 0.1% (w/v) $HgCl_2$ or 1% chloramine T followed by 90% ethanol, washed thrice with sterile water, dried within sterile blotting papers, macerated in sterile mortar and pestle, macerate suspended in 2 ml sterile distilled water and aqueous suspension was used (Barraquio et al., 1997). Six leaf/stem/root pieces were blotted to dryness, recorded fr. wt., put in an oven at 40 ± 0.1°C for 3d, followed by 80 ± 0.1°C up to a stable dr. wt. and recorded it. Untreated plant washings or extracts were used, to enumerate various microbes but for spore formers heated (60 ± 0.1°C for 30 min) samples were used. To enumerate different microbial guilds, 50 µl plant extracts were mixed separately with 100 ml different media (as per organism category), plated in 5 plates, incubated in BOD incubator at 30 ± 0.1°C for 2-5d (21d for denitrifies) for aerobic microbes, populations were counted and expressed as colony forming units (cfu)/g dr. wt. of tissue (Collee and Miles. 1989; Cappuccino and Sherman, 1999; Collins et al., 2004). To assess heterotroph (HB) and spore former (SF) bacteria, nutrient agar (NA) (g/l: peptone 5.0, beef extract 3.0, NaCl 3.0, pH 7.0, agar 20) were used. The nitrifying (NF) and denitrifying (DNF, grown in anaero-jars) bacteria were plated on 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) with 1 g/l $(NH_4)2SO_4$ or KNO_3, respectively, sulphanillic acid reagent (equivolume mixture of 0.8% sulphanillic acid and 0.5% α-naphthyl amine each in 5M acetic acid) was added and pink colonies were recorded after for 5 min. The samples were plated 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 halo forming colonies were noted as P-solubilizers (PS). Potato dextrose agar (PDA) (g/l: potato 200, dextrose 20, pH 6.5, agar 18.0) and glucose asparagine agar (GA) (g/l: glucose 10, asparagine 0.5, K_2HP0_4 0.5, pH 7.0, agar 15) media were inoculated for fungi (FU) and actinomycetes (AC) count, respectively.

To count/isolate spore-crystal formers (SCF), the heat-treated samples were incubated on NA plates with 0.25M sodium acetate (Lacey, 1997; Boucias and Pendland, 1998), each colony was checked under a phase-contrast microscope (100x), the bacteria possessing both spore and crystal were recorded and bacteria were isolated from dissimilar colonies, purified and maintained. To isolate ectophyllic SCF, the leaves were removed from wash solution, blotted, washed 5 times in sterile water, blotted to dryness and placed on separate places of NA plates for 2h each for dorsal and ventral sides (Kinkel et al., 1995; del Castillo et al., 2015), incubated in a BOD and the overlapped colonies were dilution (10^{-3}) plated on NA containing 0.25M Na-acetate, isolated colonies were checked under microscope and dissimilar colonies were isolated, purified and maintained.

Phenotyping of the spore-crystal forming bacteria

Forty three biochemical tests were conducted for phenotyping the bacteria. Cultural (colony colour, elevation, margin, size, consistency etc.), morphological (shape, size, motility, spore formation, staining etc.), physiological and biochemical characters (carbon source utilization (n =35), NaCl/Na acetate tolerance, methyl red, Voges-Proskaur, indole production, citrate utilization, nitrate reduction, urease production, oxidase, catalase, amylase, protease, antibiotic (n = 20) sensitivity, growth on PDA etc.) of the bacteria were studied following standard methods and KB009 HiCarbohydrate TM kit, Hi-MEDIA was used for carbohydrate utilization test (Holt et al., 1994; Smibert and Krieg, 1995; Lacey, 1997; Boucias and Pendland, 1998; Cappuccino and Sherman, 1999; Collins et al., 2004).

Amplification of 16S rDNA of total genome of spore-crystal forming bacteria

Total DNA of the spore-crystal forming bacteria was isolated following colony PCR technique (Janssen, 1994; Bravo et al., 1998; Sambrook and Russell, 2001). A loop full of overnight grown (32 ± 0.1°C) bacteria on NA plates was transferred into sterile micro-centrifuge tubes containing100 µl sterile distilled water and vortexed. The tubes were frozen at -70°C for 20 min and heated in water bath for 10 min to lyse the bacteria. The lysates were pulse (1-2s) centrifuged at 10000 rpm

and the supernatant was used as DNA template for PCR. The universal primers *viz.,* 27F (5' AGAGTTTGATCCCTGGCTCAG3') and1492r (5'AAGGAGGTGATCCAGCCGCA3') were used for 16S rDNA amplification (Janssen, 1994, Sambrook and Russell, 2001). The amplicons of the two selected organisms Bt 18 and Bt 21 (virulent against rice LF) were sequenced and generated the phylogram using NCBI database to reveal the evolutionary linkage of the organisms and the sequences were deposited in NCBI database.

Identification of *cry* genes of spore-crystal forming bacteria

The Lepidoptera specific cry genes of SCF bacteria were determined using the primers (Bravo et al., 1998; Beron et al., 2005; Crickmore et al., 2016), annealing temperatures and cycles as cited in Table 1. PCR reaction was carried out in 50 µl reaction mixture (0.2-0.4 µM primer, 2U of Taq DNA polymerase, 200 µM dNTP, 10 mM Tris buffer and 15 µl DNA solution according to the following sequence *viz.,* single denaturation step at 95°C for 2 min, primer specific (Table 1) amplification cycles comprising denaturation at 95°C for 1 min, annealing temperature as per primer (Table 1) 1 min, elongation at 72°C for 1 min and a final extension at 72°C for 5 min. After PCR amplification, about 10 µl of each PCR product was developed in 1% agarose gel, DNA bands were visualized in a gel documentation system. The 16S rDNA of 2 selected virulent organisms (Bt 18 and Bt 21) was sequenced through outsourcing and phylogram was derived using NCBI database.

Identification of the spore-crystal forming bacteria

Partial polyphasic taxonomy based on phenotypic characters and occurrence of *cry* gene in the SCF bacteria, as well as, 16S rDNA phylogeny of selected virulent isolates (Bt 18 and Bt 21) were accounted for identification of the spore-crystal forming bacteria (Holt

Table 1. The cry primers used for the study.

Gene	Primer used	Expected amplicon size (bp)	Annealing temperature(°C)	No. of cycles
cry1	CJI-1:TGTAGAAGAGGAAGTCTATCCA CJI-2:TATCGGTTTCTGGGAAGTA	272-290	55	38
cry2	Un2(d):GTTATTCTTAATGCAGATGAATGGG Un2(r):CGGATAAAATAACTGGGAAATAGT	689-701	52	32
cry3	III(-):AAGTGAGAGGTAGAAGCATA III(+):AAACAGAATTAACAAGAGAC	858	53	35
cry4	Dip1A:CAAGCCGCAAATCTTGTGGA Dip1B:ATGGCTTGTTTCGCTACATC	797	59	35
cry5	gral-nem(d):TTACGTAAATTGGTCAATCAAGCAAA gral-nem(r): AAGACCAAATTCAATACCAGGGTT	474-489	52	35
cry9	I(-): AGATTCTAGGTCTTGACTA I(+): TGACGATGGGGTATTAGAT	1300	52	32
cry11	gral-cry11(d):TTAGAAGATACGCCAGATCAAGC gral-cry11(r):CATTTGTACTTGAAGTTGTAATCCC	305	59	35
cry12	gral-nem(d):TTACGTAAATTGGTCAATCAAGCAAA gral-nem(r):AAGACCAAATTCAATACCAGGGTT	474-489	52	35
cry14	gral-nem(d):TTACGTAAATTGGTCAATCAAGCAAA gral-nem(r):AAGACCAAATTCAATACCAGGGTT	474-489	52	35
cry21	gral-nem(d):TTACGTAAATTGGTCAATCAAGCAAA gral-nem(r):AAGACCAAATTCAATACCAGGGTT	474-489	52	35
cyt	gral-cyt(d):AACCCCTCAATCAACATGCAAGG gral-cyt(r):GGTACACAATACATAACGCCACC	522-525	56	35
Vip1A	Vip1A-F:GGATCCGGAGATGAAAAATATGAAGAAAAAGTTA Vip2A-R:CGTCGACTGCTACTACCTTTCTTACCAACTACGT	2.4kb	56	35
Vip2A	Vip2A-F:GGATCCGATGAAAAGAATGGAGGG Vip2A-R:GTCGACTTAATTTGTTAATAATGTTG	1.3kb	58	32
Vip3A	Vip3A-F:GGATCCATGAACAAGAATAATACTA Vip3A-R:GTCGACTTACTTAATAGAGACATCGT	2.37kb	57	35

et al., 1994; Smibert and Krieg, 1995; Logan and de Vos, 2009).

Plant growth promotion traits of the spore-crystal forming bacteria

The PGP functions like siderophore, HCN, indole compounds and ammonia production of 6 potent SCF bacteria were assessed *in vitro*. HCN production was tested in King B broth (g/l: protease peptone 20, $MgSO_4$ 1.5, K_2HPO_4 1.5 with 4.4 g/l glycine) contained in flasks capped with 10 x 0.5 cm Whatman no. 1 filter paper strips (dry, saturated in 0.5% picric acid in 2% sodium carbonate) avoiding contact with medium and flask, incubated at 30±0.1ºC for 96h. Change of paper colour from yellow to orange for HCN evolution was noted (Reddy et al., 2008). For siderophore production, the organisms were spotted on chromazurol S (CAS) agar medium (chromazurol 60.6 mg in 50 ml water, 1 mM $FeCl_3.H_2O$ in 10 ml 10 mM HCl, CTAB 72.9 mg in 40 ml water and 300 ml nutrient agar), incubated at 30 ±0.1 °C for 4-5d and colour change around colony indicated siderophore production (Schwyn and Neilands, 1987). Bacteria were incubated 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) for 96h at 30 ±0.1°C and browning of broth on addition of 1 ml Nessler's reagent (70.83 g/l K_2HgI_4 in 2.5M KOH) indicated ammonia production (Cappuccino and Sherman, 1999). For indole production, bacterial broth (g/l: peptone 20, NaCl $_5$ and tryptophan (final) 0.1%, pH 7) cultured at 30 ±0.1°C for 3-5d was mixed with 1 ml Salkowski reagent (50 ml 35% $HClO_4$ containing 0.5M $FeCl_3$) and pink colouration of medium indicated indole production (Bric et al., 1991).

Assay of virulence of the spore-crystal forming bacteria

Cut leaf bioassay was carried out on 2nd, 3rd and 4th instar larvae of *C. medinalis* against the bacterial isolates (Dangar, 2008). Rice var. Naveen leaf pieces were soaked in 0.001% tween 80 to soften the leaf tissues and the leaves were immersed separately in each of 10^4, 10^5, 10^6, 10^7, 10^8 cfu/ml bacterial suspensions for 15-20 min. Different larval instars of rice leaf folders (LF) (10 larvae/test) were placed in test tubes and covered with an insect proof cloth and mortality of the larvae was recorded up to 72 h.

RESULTS AND DISCUSSION

Populations of various endospheric microbes of leaf, stem and root are given in Table 2. Populations (x 10^2 cfu/g) of endophytic heterotroph (HB), spore former (SF), spore-crystal former (SCF), actinomycetes (AC), nitrifying (NF), denitrifying (DNF), P solubilizing (PS) bacteria and fungi (FU) of leaves, stems and roots of OB varied between 0.07-4.48, 0.06-4.08, 5.00-4.00, 0.56-1.40, 0.06-0.40, 0.07, 0.03 and 0.14-8.00, respectively. Each type of endophytic guild was 5-6 times more in roots ($0.33x10^1$-$8.00x10^3$ cfu/g) than leaves ($0.70x10^1$ - $6.94x10^2$ cfu/g) or stems ($0.56x10^1$ - $4.48x10^3$ cfu/g). Numerical abundance of fungi, actinomycetes, NF, DNF and PS were at least one exponent lower than other guilds and endo-microbes were grossly lesser in stem than leaf (nominally) and root. The results proved that structure and function of the endophytes (primarily bacteria) were more pronounced in the roots than other plant parts. As the soil microbes colonize primarily through the roots, they would be more in the roots and supported by the results of more microbiome dynamics/diversity/metabolism in wild genotype (*O. eichingeri*) in general and roots of both wild or cultivated rices although colonization site or patterns differ with host (Reinhold-Hurek and Hurek, 1998; Banik et al., 2015, 2016, 2017). Nevertheless, the endogenous microbiome of the wild genotype *O. brachyantha* corroborated the 10^3-10^6 cfu/g population density of rice, cotton, corn etc. (Hallman et al., 1997; Bashan et al., 2014), as well as, 8.91 x 10^1- 7.24 x 10^6 cfu/g multifaceted PGPM in rice cultivars Sabita,

Table 2. Population of different endophytic microbial guilds of *O. brachyantha*

Organism type	Population count (x10^2 cfu/g dr. wt.)							
	Heterotroph	Spore former	Spore-crystal former	Fungi	Actinomycetes	Nitrifying bacteria	Denitrifying bacteria	P solubilizer
Leaf endophyte	6.94	6.49	5.26	0.14	0.14	0.07	ND	ND
Stem endophyte	6.84	5.66	5.07	ND	0.06	0.06	ND	ND
Root endophyte	44.81	40.82	40.00	0.80	ND	0.40	0.075	0.03

ND - Not detected

Table 3. Cultural characters of spore-crystal forming bacteria of *O. brachyantha*

Bact. no.	Habitat	Form	Colour	Elevation	Margin	Consistency	Size (mm)
Bt 8	Rhizoplane	Circular	White	Umbonate	Entire	Gummy	12.41
Bt 9	Phyllosphere	Irregular	Off white	Flat wrinkled	Lobed	Gummy	8.50
Bt 18	Root endophyte	Irregular	White	Raised	Lobate	Gummy	11.03
Bt 19	Leaf endophyte	Circular	White	Raised	Entire	Gummy	9.61
Bt 20	Stem endophyte	Irregular	Off white	Raised	Curled	Gummy	12.61
Bt 21	Phylloplane	Irregular	white	Raised	Lobate	Gummy	10.72

Table 4. Characters of vegetative cells of spore-crystal forming bacteria.

Isolate no.	Shape	Motility	Gram stain	Length (µm)			Breadth (µm)		
				Range	Mean	SE (±)	Range	Mean	SE (±)
Bt 8	Rod	Motile	+ve	3.60 -3.80	3.67	0.09	1.85 -1.97	1.91	0.05
Bt 9	Rod	Motile	+ve	3.95 - 4.20	4.08	0.10	2.20 - 2.50	2.37	0.12
Bt 18	Rod	Motile	+ve	3.65 -4.12	3.89	0.19	1.95 -2.00	1.97	0.02
Bt 19	Rod	Motile	+ve	3.98 -4.01	3.99	0.01	2.21 -2.22	2.21	0.01
Bt 20	Rod	Motile	+ve	4.20 -4.21	4.20	0.01	2.35 -2.37	2.36	0.01
Bt 21	Rod	Motile	+ve	4.10 - 4.30	4.22	0.09	1.95 - 2.11	2.05	0.07

Swarna Sub1, Swarna and *O. eichingeri* (Banik et al., 2016, 2017).

From the heat-treated suspensions inoculated in Na-acetate supplemented NA plates, 23 spore-forming dissimilar colonies were selected, the bacteria were checked under the phase-contrast microscope for insecticidal crystal protein (ICP) production, 6 spore-crystal forming bacteria (SCF) were obtained and studied in details. The organisms produced whitish to off-white and irregular colonies of 8.50-12.61 mm dia. (Table 3). The 6 SCF endophytes were tested for 43 physiological and biochemical tests and the results are given in Tables 4-8. The vegetative cells were motile, gram positive rods, l x w measured 3.67-4.22 x 1.91-2.37 µm (Table 4). The crystals produced by the organisms were spherical 0.69-1.18 µm dia. and stained with crystal stain (Table 5). Along with crystals, the isolates produced spores which were elongated or oval, and 1.83-2.07 x 0.60-1.10 µm in l x w (Table 6). The metabolic tests of the organisms indicated that Bt 8, Bt 9, Bt 18, Bt 19, Bt 20 and Bt 21 were positive for 14, 14, 18, 16, 19 and 18 tests, respectively (Table 7). The Bt 18, 20 and 21 endured 10% NaCl and Bt 8, 9 and 19 sustained in 5% NaCl; Bt 8 and 9 grew with 0.3M and others with 0.5M Na-acetate (Table 7). The organisms produced amylase, protease, catalase, nitrate reductase and oxidase but negative for VP test and could grow on PDA (barring Bt 19) (Table 7). Besides, all of them utilized trehalose, glycerol and glucose utilization; did not metabolize lactose, fructose, raffinose, melibiose L-arabinose, mannose, inulin, dulcitol, sorbital, inositol, mannitol, adonitol, arabitol, erythritol, α-methyl D-glucoside, rhamnose, cellobiose, melezitose, α-methyl D-mannoside, xylitol, ONPG, D-arabinose, sorbose as energy source, although other results differed among the organisms (Table 7). Out of 20 antibiotics, the

Table 5. Characters of crystals of spore-crystal forming bacteria

Isolate no.	Shape	Position	Crystal stain	Diameter (µm)		
				Range	Mean	SE(±)
Bt 8	Spherical	Sub-terminal	+	0.74 -0.78	0.76	0.02
Bt 9	Spherical	Terminal	+	0.80 -0.96	0.87	0.07
Bt 18	Spherical	Terminal	+	0.79 -0.86	0.82	0.03
Bt 19	Spherical	Sub-terminal	+	0.69 -0.70	0.69	0.01
Bt 20	Spherical	Terminal	+	0.82 -0.86	0.84	0.02
Bt 21	Spherical	Terminal	+	1.00 -1.30	1.18	0.13

Table 6. Characters of spores of spore-crystal forming bacteria.

Isolate no.	Shape	Spore stain	Length (µm)			Breadth (µm)		
			Range	Mean	SE (±)	Range	Mean	SE (±)
Bt 8	Rod with round ends	+ve	1.80 -2.10	2.07	0.162	1.10 -1.35	1.10	0.155
Bt 9	Rod with round ends	+ve	1.95 -2.00	1.98	0.024	0.95 -1.05	1.00	0.045
Bt 18	Rod with round ends	+ve	1.75 -1.90	1.83	0.061	0.59 -0.62	0.60	0.012
Bt 19	Rod with round ends	+ve	1.87 -2.10	1.97	0.095	0.85 -0.96	0.91	0.045
Bt 20	Rod with round ends	+ve	1.81 -1.89	1.85	0.03	0.82 -0.85	0.83	0.01
Bt 21	Rod with round ends	+ve	1.88 -1.92	1.89	0.01	0.89 -0.99	0.93	0.04

Table 7. Biochemical characters of spore-crystal forming bacteria.

Carbon source utilization	Spore-crystal forming isolate					
	Bt 8	Bt 9	Bt 18	Bt 19	Bt 20	Bt 21
Xylose	-	-	+	-	-	-
Maltose	+	±	-	+	+	+
Dextrose	+	-	+	+	+	+
Galactose	-	-	+	+	+	+
Trehalose	+	+	±	±	+	+
Sucrose	±	-	-	-	-	-
Sodium gluconate	-	-	+	+	+	-
Glycerol	+	+	+	+	+	+
Salicin	-	+	-	-	-	-
Esculin	-	+	+	+	+	+
Citrate	+W	+	+	+	+	+
Malonate	+	-	+	+	+	+
Glucose	+	+	+	+	+	+
NaCl (%):						
5	+	+	+	+	+	+
10	-	-	+	-	+	+
15	-	-	-	-	-	-
20	-	-	-	-	-	-
Na-acetate (M)						
0.1	+	+	+	+	+	+
0.3	+	+	+	+	+	+
0.5	-	-	+	+	+	+
Voges Proskauer	-	-	-	-	-	-
Oxidase	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Arginine dihydrolase	-	+	-	-	+	+
Amylase (activity zone)	+ (12)	+ (29)	+ (IC)	+ (14)	+ (16)	+ (17)
Nitrate reductase	++	+	+	+	+	+
Protease	+	+	+	+	+	+
Lecithinase	+	-	+	+	+	+
Growth on PDA	+	+	+++	-	++	++

+ = positive, - = negative. ± = variable. IC = intracellular. Amylase activity zones are given in parentheses. All organisms utilized trehalose, glycerol and glucose utilization. Negative for lactose, fructose, raffinose, melibiose L-arabinose, mannose, inulin, dulcitol, sorbital, inositol, mannitol, adonitol, arabitol, erythritol, ?-methyl D-glucoside, rhamnose, cellobiose, melezitose, α-methyl D-mannoside, xylitol , ONPG, D-arabinose and sorbose.

isolates were resistant to nystatin, amphotericin B, methicillin and ketoconazole; sensitive to nalidixic acid, erythromycin, kanamycin, tetracycline, chloramphenicol, vancomycin, streptomycin, norfloxacin, gentamicin, rifampicin and polymyxin B while other antibiotics had variable effects (Table 8). The 16S rDNA sequence based phylograms of the 2 virulent SCF bacteria (Bt 18 and 21) against rice LF (>60% mortality) cladded Bt 18 (NCBI acc. no. KY921957) with *Bacillus cereus* and Bt 21 (NCBI acc. no. KY784649) with *Bacillus thuringiensis* (more akin to Bacillus mycoides) (Fig. 1, 2). The Bt along with *B. cereus*, *B. anthracis* and *B. mycoides* (*B. cereus* group) belong to the Group I *Bacillus* spp., share several common phenotypic, H-antigenic and 16S rDNA characters but only crystal production distinguishes the ICP positive Bt from other members, thereby, hetero-species phylogenetic clustering among the *B. cereus* group of *Bacillus* spp. (amid Bt) is not unusual (Smibert and Krieg, 1995; Turnbull et al., 2004; Zhou et al., 2008; Logan and de Vos, 2009). Besides, all SCF bacteria possessed different Lepidoptera specific Cry toxins *i.e., cry* genes (Table 9). Cultural, morphological, physiological and biochemical characters and antibiogram (Tables 2-8) based phenotyping (Tables 10, 11), as well as, molecular characters like *cry*+ and 16S rDNA phylogeny of potent Bt (Table 9 and Fig. 1, 2) proved that the endo-SCFs belong to Group I of genus *Bacillus* and species *thuringiensis i.e., Bacillus thuringiensis* (Table 10)

Table 8. Antibiotic sensitivity of spore-crystal forming bacteria.

Isolate	Antibiotic response (Inhibition zone, mm)																			
no.	NS	AZM	NA	AMP	E	AP	K	TE	TR	C	P	VA	MET	S	NX	GEN	KT	R	PB	AMC
Bt 8	R	20	18	12	22	R	19	27	20	24	10	17	R	16	19	19	R	17	17	17
Bt 9	R	21	25	11	24	R	18	29	24	28	10	23	R	21	24	24	R	31	17	22
Bt 18	R	R	17	R	20	R	18.5	24	6	22	R	14	R	16	22	21	R	12	17	R
Bt 19	R	R	23	R	25.1	R	20.5	25	R	28	R	18	R	18	24	21	R	16	9	R
Bt 20	R	25	23	R	36	R	18.5	26	R	34	R	18	R	19.5	23.5	20.5	R	15	9	R
Bt 21	R	19	21	R	29	R	21	26	R	27	R	20	R	16.5	28	2	R	16	17	R

R= resistant. Sensitivity results are given as inhibition zone. Nystatin 100 µg/disc (NS), azithromycin 15 µg/disc (AZM), nalidixic acid 30 µg/disc (NA), ampicillin 10 µg/disc (AMP), erythromycin 15 µg/disc (E), amphotericin B 50 µg/disc (AP), kanamycin 30 µg/disc (K), tetracycline 30 µg/disc (TE), trimethoprim 5 µg/disc (TR), chloramphenicol 30 µg/disc (C), penicillin G 10 IU (P), vancomycin 30 µg/disc (VA), methicillin 10 µg/disc (MET), streptomycin 10 µg/disc (S), norfloxacin µg/disc 10 (NX), gentamicin 10 (GEN), ketoconazole 5 (KT), rifamycin 5 (R), polymyxin B 300 (PB300) and amoxicillin 30 ?g/disc (AMC30).

and corroborated their homology with other members of *B. cereus* group (Holt et al., 1994; Smibert and Krieg, 1995; Zhou et al., 2008; Logan and de Vos, 2009; Logan et al., 2009). However, differences of some phenotypic characters, *cry* composition and differential affinity of Bt 18 and 21 with other members of *B. cereus* group proved diversity of the endo-Bt of *O. brachyantha*. The Bt spp. are classified into tentative serological groups (serovar.) using phenotypic characters (pending serology) accepting the limitations of theoretical serotyping (de Barjac, 1981; de Barjac and Frachon, 1990; Lacey, 1997). Phenotype based serotyping of the endo-Bt failed to ascertain serovar. of Bt 8 but identities of other organisms were *B. thuringiensis* serovar. *gallriae* (Bt 9), *toumanoffi* (Bt 18), *israelensis* (Bt 19 and 20) and *sotto* (Bt 21) (Table 11) (de Barjac, 1981; de Barjac and Frachon, 1990) which proved subsp. level structural diversity of the Bt pool in the rice endosphere.

The endo-Bt were resistant to nystatin, amphotericin B, methicillin and ketoconazole (Table 8) which corroborated that alike rhizospheric/ environmental counterparts, Bt are generally resistant to antifungal (nystatin) and ampicillin group of antibiotics (Das and Dangar, 2008; Das et al., 2013). Lepidoptera specific 8 Cry toxin (*cry* gene) primers (Crickmore et al., 2016) (Table 1) could amplify at least one cry gene in all organisms *e.g.,* 6 genes (*cry1, cry3, cry5, cry12*, *cry21*and *cry14*) of Bt 8, 2 genes (*cry3* and *cry4*) of Bt 9 and 1 gene (*cry 11*) of Bt 19 but smaller than predicted gene sizes *viz.,* 350 bp and 280 bp amplicons of *cry2* and cyt genes, respectively, of Bt 18, 20 and 21 (Table 9). The size discrepancy of some amplicons would result from mismatch of primer and template or improper PCR protocol (Janssen, 1994; Sambrook and Russell, 2001; Konstantinidis and Tiedje, 2005; Reyes-Ramirez and Ibarra, 2005). The *cry1-5, 7-9, 11, 12, 14*, *15, 21, 22, 51* etc. and cyt1genes are established anti-lepidopteran genes (Frankenhuyzen, 2009; Crickmore et al., 2016) and therefore, presence of *cry1, 3, 5, 11, 12, 14, 21* and *cyt* genes (Table 9) proved wide/universal distribution of anti-lepidopteran crystal toxin genes in natural endo-Bt populations of OB like environmental samples (Dangar et al., 2010), as well as, all habitats amid endosphere.

The endo-Bt of OB produced spherical crystals (Table 5) but possessed different anti-lepidopteran *cry* genes (Table 9) which supported that *cry* gene (*i.e.,* Cry toxin) does not determine crystal morphotype and multiple *cry* containing Bt would produce diverse (broad

Table 9. Identification cry, cyt and vip genes in the isolates.

Strain no.	*cry1*	*cry2*	*cry3*	*cry4*	*cry5*	*cry9*	*cry11*	*cry12*	*cry14*	*cry21*	*cyt*	*vip1A*	*vip2A*	*vip3A*
Bt 8	+	-	+	-	+	-	-	+	+	+	-	-	-	-
Bt 9	-	-	+	+	-	-	-	-	-	-	-	-	-	-
Bt 18	-	+ (350 bp)	-	-	-		-	-	-	-	+ (280 bp)	-	-	-
Bt 19	-	-	-	-	-		+	-	-	-	-	-	-	-
Bt 20	-	+ (350 bp)	-	-	-		-	-	-	-	+ (280 bp)	-	-	-
Bt 21	-	+ (350 bp)	-	-	-		-	-	-	-	-	-	-	-

Table 10. Phenotyping of spore-crystal forming bacteria up to genus and species identity.

Isolate no.	Rod shape of bacteria	Length of bacteria	Width of bacteria (mm)	Sporangium not swollen (<2.50 mm)	Spore production	Crystal production	Gram stain	Catalase activity	Oxidase activity	Strict anaerobe	Nitrate reductase	Genus	Species
Bt 8	+	3.67	1.91	+	+	+	+	+	+	“	+	*Bacillus*	*thuringiensis*
Bt 9	+	4.08	2.37	+	+	+	+	+	+	“	+	*Bacillus*	*thuringiensis*
Bt 18	+	3.89	1.97	+	+	+	+	+	+	“	+	*Bacillus*	*thuringiensis*
Bt 19	+	3.99	2.21	+	+	+	+	+	+	“	+	*Bacillus*	*thuringiensis*
Bt 20	+	4.20	2.36	+	+	+	+	+	+	“	+	*Bacillus*	*thuringiensis*
Bt 21	+	4.22	2.05	+	+	+	+	+	+	“	+	*Bacillus*	*thuringiensis*

+ = positive result, “ = negative result

Table 11. Phenotyping of spore-crystal forming bacteria for serovar. identity.

Isolate no.	AMC production	Lecithinase production	ADH production	Mannose utilization	Pellicle formation	Sucrose utilization	Salicin utilization	Tween esterase	Cellobiose utilization	Citrate utilization	Starch utilization	Protease production	Esculin utilization	Chitin hydrolysis	*B. thuringiensis* serovar
Bt 8	+	+	+	“	“	“	“	“	“	+W	+	+	“	“	Unknown
Bt 9	+	“	+	“	“	“	+	“	“	+	+	+	+	“	*gallriae*
Bt 18	+	+	+	“	+	“	“	“	“	+	+	+	+	“	*toumanoffi*
Bt 19	+	+	+	+	+	“	“	+	“	+	+	+	+	“	*israelensis*
Bt 20	+	+	+	+	+	“	“	+	“	+	+	+	+	“	*israelensis*
Bt 21	+	+	“	“	+	+	“	“	“	+	±	+	+	“	*sotto*

Table 13. PGP functions of potent endo-Bt of *O. brachyantha*

Strain no.	Ammonia production	IAA production	HCN production	Siderophore production
Bt 8	+	-	-	-
Bt 9	-	-	-	-
Bt 18	+	-	-	-
Bt 19	+	-	-	-
Bt 20	+	-	-	+
Bt 21	+	-	-	+

Table 12. Assay of virulence of endo-Bt against leaf folder

Isolate no.	Inoculum ($\times 10^4$ cfu/ml)	larvae Larval mortality (%)
Bt 8	3.45	00
Bt 9	7.40	00
Bt 18	2.65	60.14
Bt 19	7.39	00
Bt 20	5.79	00
Bt 21	7.56	60.21

spectrum) δ-endotoxins which would be effective against various pests of the ecospheres including rice (Gao et al., 2008; Dangar et al., 2010). Production of the pathogenicity related enzymes like protease, lecithinase and amylase by the Bt (Table 7) indicated that they would degrade structural proteins (mannoproteins and glycoproteins), lipids (lipoproteins, phospholipids etc.) and α-1,3/1,4 or β-1,3/β-1,6 glycosidic linkages, respectively, of cell wall of bacteria and/or fungi and protect plants from the pathogens (Raddadi et al., 2008; Zhou et al., 2008; Roy et al., 2013).

Endophytic Bt of any rice genotype and associated virulence against rice LF is being recorded for the first time. In laboratory, Bt 18 and 21 killed about 60% LF larvae by 2.65×10^4 cfu/ml and 7.56×10^4 cfu/ml, respectively (Table 12) which suggested that Bt 18 was more virulent than Bt 21. Nevertheless, lethal doses of the 2 endo-Bt of OB were 4-5 fold lower than the LC_{50} of other indigenous soil Bt *e.g.,* (2.36-5.25) $\times 10^7$cfu/ml and (0.60-22.5) $\times 10^8$ cfu/ml against 2nd-5th instars *C. medinalis* larvae (Ramamourti et al., 2012; Ghosh et al., 2016) and proved superiority of endo-Bt over environmental counterparts. Thus, higher population (10^2-10^3 cfu/g) (Table 2) of highly virulent (about 10^4 cfu/ml) endogenous Bt in OB (Table 12) would impart natural immunity to the host (OB) against LF/lepidopteran pests.

The results of PGP functions showed that among 6 isolates, all reduced nitrate and produced ammonia (but Bt 9), Bt 20 and 21 produced siderophore,

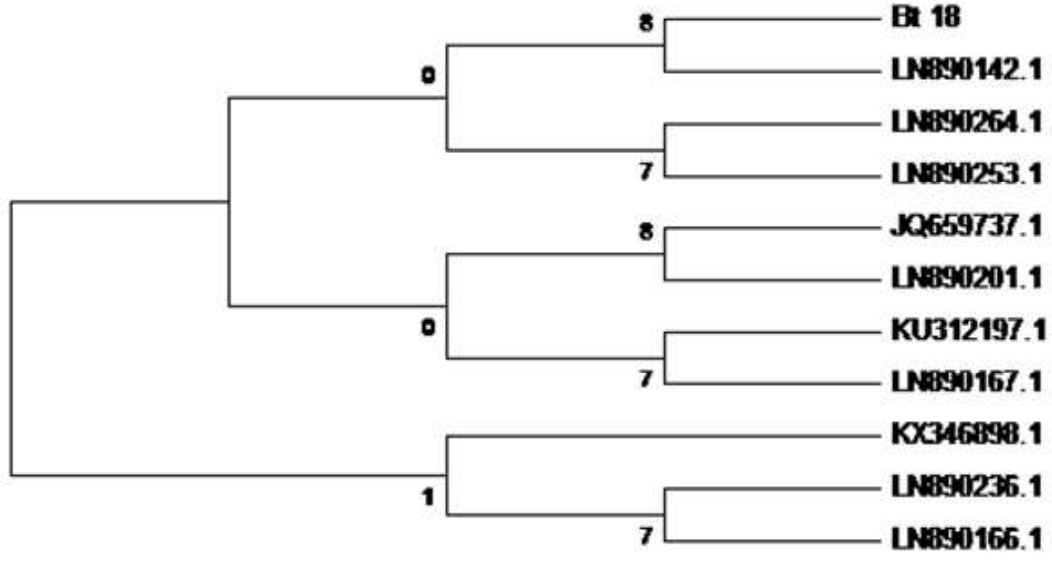

Fig. 1. Phylogram of Bt 18 based on 16S rDNA homology. LN890142.1 - *Bacillus cereus* strain M56, LN890264.1 - *Bacillus cereus* strain B88, LN890253.1- *Bacillus cereus* strain B77, JQ659737.1- *Bacillus cereus* strain R5-339, LN890201.1 - *Bacillus cereus* strain B25, KU312197.1 - *Bacillus cereus* strain CP133, LN890167.1 - *Bacillus cereus* strain M81, KX346898.1 - *Bacillus cereus* strain sys1, LN890236.1- *Bacillus cereus* strain B60, LN890166.1- *Bacillus cereus* strain M80.

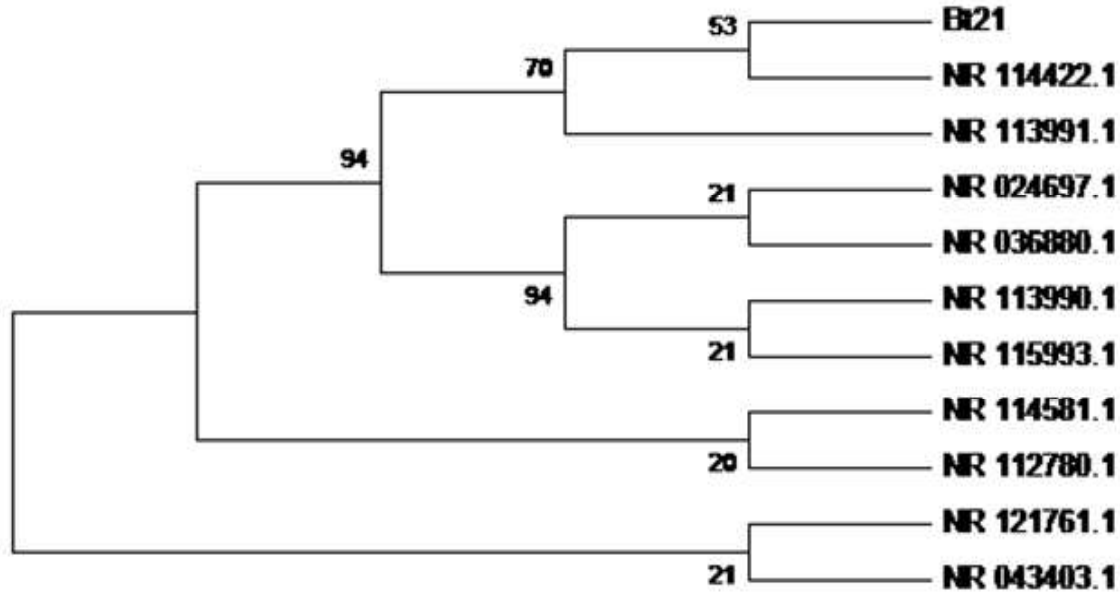

Fig. 2. Phylogram of Bt 21 based on 16S rDNA homology. NR114422.1 - *Bacillus pseudomycoides*, NR113991.1 - *Bacillus pseudomycoides* Strain NBRC 101232, NR024697.1- *Bacillus weihenstephanensis* strain DSM-11821, NR036880.1- *Bacillus mycoides* strain 273, NR113990.1- *Bacillus mycoides* strain NBRC 101228, NR115993.1- *Bacillus mycoides* strain ATCC 6462, NR114581.1- *Bacillus thuringiensis* strain ATCC 10792, NR112780.1- *Bacillus thuringiensis* strain NBRC 101235, NR121761.1- *Bacillus toyonensis* strain BCT-7112, NR043403.1- *Bacillus thuringiensis* strain IAM12077

but none produced IAA and HCN (Tables 7, 13) which proved polyvalent PGP functions of different endo-Bt of OB and suggests that they would support OB not only for insect control but promote growth also (Raddadi et al., 2008, 2009; Naik et al., 2009; Francis et al., 2010; Lacava and Azevedo 2014). The results suggest that alike environmental counterparts, endo-Bt too has polyvalent PGP functions like nutrient supplement, control of insects by exo-/endotoxins, as well as, pathogens by pathogenicity related enzyme and antimetabolites/inhibitors (Raddadi et al., 2008, 2009; Zhou et al., 2008; Roy et al., 2013; Lacava and Azevedo, 2014).

The investigation reveals that the wild rice *O. brachyantha* harbors diverse endo-microbial guilds which sustain endospheric functionalities and microbial activities are more pronounced in roots. Phenotypic, genetic, *cry*, serological (phenotype based) identity and PGP traits data proved structural and functional diversity of the endospheric SCF bacteria in *O. brachyantha* and indicated that they would be effective against different lepidopteran insects and enhance plant growth. Besides, the endo-microbiome would induce intrinsic resistance/tolerance against pests, diseases and ease dependency on nutrients.

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