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Determination of mustard seeds endophytic bacterial population and species composition by development of new method

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Abstract

Endophytic bacteria (EB) population and species diversity in seed of oilseeds crops remain unexplored. This study was conducted to overcome the difficulty imposed in serial dilution method employed for determination of mustard seed EB. The EB population in centrifuged pellet of per gram surface sterilized seeds (SSS) of 4 mustard varieties ranged from 4.11-5.16 log Cfu. Addition of known concentrations (20-100 µl) of surfactant (Tween 20) to crushed SSS and water suspension removed the problem of colony development on nutrient agar and by this method EB population ranged 3.08- 3.37 Cfu/g seed. The method successfully quantified seed EB of peanut (3.60 log Cfu/g) and sunflower (3.93 log Cfu/g). 16S rRNA based identification showed that Bacillus species of phyla Firmicutes were abundant in mustard and will facilitate further studies on seed EB role in mustard crop. Overall, our study provides knowledge and information on bacterial diversity in mature mustard seeds.

Keywords: *Bacteria, diversity, endophytes, mustard, oilseed, 16S rRNA sequencing*

Introduction

Interaction of microorganism in both plant exterior surface (PESM) and interior tissue (PIT) is intimately linked with plant growth, development, defense mechanisms and productivity (Compant *et al*., 2010; Glick, 2014; Jones *et al*., 2019; Li *et al.*, 2012; Lodewyckx *et al*., 2002; Mendes *et al*., 2011; Philippot *et al*., 2013; Shahzad *et al*., 2018; Turner *et al.*, 2013; Whipps, 2001) Depending upon whether it is a cereal, leguminous or oilseed crop, the endophyte species composition and population of individual species in the PIT may differ (Fisher *et al*., 1992; Kandel *et al.*, 2017; Raj *et al.*, 2019; Rana *et al*., 2020). Compared to shoot or root, study on microbiome of seed interior can be more interesting and promising as seeds represent both an endpoint for community assembly within the seed and starting point for community assembly (Nelson, 2018).

While few studies showed culturable bacterial endophytic population (CBEP) in the range of 1.01×10^2 to – 9.54×10^8 CFU/g in surface sterilized seeds (SSSs) of different crops (Compant *et al*., 2011; Raj *et al*., 2019; Rosenblueth *et al*., 2010; Truyens *et al*., 2016) CBEP in matured oilseeds are hard to find in literature. The available data on endophytic bacterial diversity in different tissues including seeds of oilseed crops are generated based on either fatty acid methyl esters (FAME) profiles and gas chromatography (GC) analysis (Germida *et al*., 1998) or Ilumina Miseq amplicon sequencing. Rybakova et al. (2017) showed that amplicon sequence-based seed microbiota of mustard was cultivar specific, and mustard genotype shaped their endophytic bacterial profile, which in turn was a crucial factor for plant health. The only quantitative data on CBEP is available from a study by Graner *et al*. (2003) on pre-germinated seeds of four cultivars of oilseed crop, *B. napus*. Whether the oilseeds were pre-germinated to eliminate the problem of isolation and quantification from normal seeds was not mentioned by the author. During germination, endophytes profiles of seeds may be different than those present at the time of harvesting (Lopez *et al*., 2018; Truyens *et al.*, 2015).

Oilseed crops in the mustard family are commercially important globally (Rahman *et al*., 2018) and our interest was to determine CBEP and their diversity in harvested seeds of different mustard varieties. The initial attempt to isolate and quantify CBEP from SSSs of mustard by serial dilution pour plant method failed. It led us to hypothesize that due to mixing of oil content of seed with water, traces of oil may surround bacterial cells during plating of aliquot from serial dilutions on agar medium, and oil emulsion

effects (oil water immiscibility) may prevent entry of water with dissolved nutrient into bacterial cells, their proliferation and colony formation. None of the earlier studies mentioned the genuine hindrance in determination of CBEP in oilseeds. Mustard seeds contain allyl isothiocyanate (AITC) accounting for up to 71.06% of its seed essential oil content, and AITC exhibits strong antimicrobial activity against both Gram-positive and Gram-negative bacteria(Dai and Lim, 2014; Romanowski and Klenk, 2000). When mustard seeds are crushed/ homogenized in sterile distilled water, the enzyme myrosinase is released and it acts on a glucosinolate known as sinigrin to give allyl isothiocyanate (Dai & Lim, 2014). Therefore, besides oil emulsion effects, AITC may also affect bacterial cell growth. Therefore, it requires an innovative approach to generate CBEP data from seeds of oilseed crop. Surfactants are added to partially or completely immiscible liquids such as oil emulsions to stabilize them, and they are found to have important applications in pharmaceutical, hydraulic fluids and oilfield industry (Mohamed *et al.*, 2017). One such surfactant, Tween 20 has Hydrophilic-Lipophilic system (HLB) value of 16.7, making it a better choice for use as a solubilising agent of oil in an oil-water emulsion. It was also hypothesized that removal of the oil water mixture by centrifugation of serial dilution suspension and plating of the suspended pellets may also eliminate the adverse effect of oil. Overall, the objective of this study was to determine (a) CBEP in serially diluted mustard seed (crushed) suspension by centrifugation and using Tween 20 and (b)the diversity of the EB bacteria based on identification of the colonies obtained from SSSs of four varieties of mustard using 16SrRNA gene sequences.

Materials and Methods

Sample collection and description of oilseeds

Seeds of four different varieties of mustard namely, TS-46, M-27, NRCHB 101 and Binoy (B 9) were collected from Regional Agricultural Research Station (RARS), Shillongani of Assam Agricultural University. TS-46 and M-27 varieties of Toria (*Brassica rapa* ssp. Toria) were of brown colored seed. M-27 variety has been grown by farmers in different agro-climatic zones of Assam for last four decades. TS-46 is a newly recommended variety, grown mostly in Brahmaputra valley zones of Assam. NRCHB 101 is an improved variety of Indian mustard (*Brassica juncea*) whose seeds were brown and Binoy cultivar of *Brassica rapa* spp. Yellow Sarson was of yellow colored, respectively. These varieties attain physiological maturity within 90-100 days from the date of sowing. Seeds of two other oilseed crops namely, peanut (*Arachis hypogaea*) and sunflower var. Sunflower Russian Giant (*Helianthus giganteus L.*) were also used in this study for testing the validity of methods to be developed for isolation of endophytic bacteria from seed interior of mustard seeds. Peanut and sunflower seeds were procured from Plant Seeds Live, seed store, India.

Surface sterilization of seed

Bacteria occur both inside seed and on the seed surface. To isolate bacteria from seed interior (endophyte), first one gram of each of the 4 varieties of seeds were surface sterilized by treating with a mixture of 1% Sodium Hypochlorite (NaOCl) and a sterilising solution comprised of 0.1% Sodium carbonate, 3% Sodium chloride and 0.15% Sodium hydroxide for 1 minute. The seeds were then thoroughly washed with 2% Sodium thiosulphate for 10 minutes to remove any trace of disinfectant on surface coat, followed by washing in sterile distilled water (SDW) thrice. 100µl of water from the last SDW washing step and 7-8 surface sterilized seeds (SSS) of each variety were separately plated on Nutrient Agar (NA) and in Nutrient broth (NB) to check effectiveness of the sterilization process. Plates were incubated at 28° C in an incubator (REMI CI-19 PLUS, India), and the broth tubes were maintained at 28° C/150 rpm in an incubator shaker (Scigenics Biotech, India). The plates and tubes were observed daily for growth of microorganisms for a week.

Sunflower seed (1gm) were first cleaned by washing in three changes of SDW, and then seeds were treated with 1.5% sodium hypochlorite solution for 5 minutes followed by thorough washing in 2% sodium thiosulphate for 15 minutes. The seeds were finally rinsed for three times in sterile distilled water and tested for effectiveness of surface sterilization process by following the steps described above. Similarly, one-gram of peanut seeds was first cleaned by washing in three changes of SDW and then treated with two sterilizing solutions followed by washing in 70% ethanol for 5 minutes and 1% sodium hypochlorite for 5 minutes. This was followed by rinsing thoroughly with 2% sodium thiosulphate for 10 minutes. Subsequently, the seeds were washed thrice with sterile distilled water and effectiveness of the sterilization process was checked following the steps described before.

Visualization of bacterial colonization in seeds by scanning electron microscopy

Surface sterilized mustard seeds var. TS 46 were visualised under a scanning electron microscope (Zeiss- SIGMA VP) to determine the presence, size and shape of bacteria. The samples were treated before visualization. The seeds

were cut into pieces and collected in tubes. 1ml fixative (3% glutaraldehyde in 0.1M Phosphate buffer, pH-7) was added and left for 4 hrs at room temperature. The fixative was decanted and washed with distilled water. To it, 1ml 0.2M Phosphate buffer was added and left for 6 hrs in room temperature. The buffer was decanted and treated with acetone gradient for 15 min each in the sequence (30%, 50%, 70%, 80%, 90%, 95% acetone). Finally, the sample was treated with 100% acetone for 15 min and the acetone was poured off. Dry acetone was added and kept for 15 min. Dry acetone was drained and then drying was done by addition of Tetra methyl Silane (TMS) for 15 min at 4°C and this is a critical drying step required for visualization in SEM. The samples were kept in desiccator overnight. Plasma sputtering with gold was performed on the next day and sample was observed in SEM.

Isolation and enumeration of bacteria from interior of SSS

Excise-paste method

One-gram surface sterilized seeds was homogenized using sterilized mortar and pestle and serially diluted in 9 ml sterile distilled water and 100µl homogenate of each dilution (10^{-1}-10^{-5}) was plated on NA media in triplicates. The plates were observed daily for appearance of colonies up to one week. We failed to observe any bacterial colony in three repeated experiments by this serial dilution method. To ascertain whether our SSSs contain any bacteria, we followed the method described by Sobolev *et al*. (2013). Several SSSs of mustard variety, TS 46 were cut into two transverse section and pasted on petri plate containing NA media. Bacterial growth appeared around the edge of the transverse sections. Bacterial growth was either continuous or sometimes in form of separated colonies. These colonies were isolated on the basis of morphological differences such as size, shape, elevation, margin, pigmentation etc. But this method gave us limited information on seed endophytic bacterial diversity and was not adequate to determine population per unit quantity of seeds.

Centrifugation-based method

Next another method was tried to determine EB quantitatively in the interior of the four varieties of mustard and toria seed. One-gram seed was surface sterilized, then crushed using 9 ml sterile distilled water and the seed suspension was centrifuged at 7000rpm for 15 minutes to obtain two different phases: supernatant and pellet. 100µl each of supernatant was plated in triplicate on separate plates and the pellet was serially diluted and 100µl of each dilution (10^{-1}-10^{-5}) was plated on NA media. The plates were incubated in similar manner as described earlier and observed daily for the appearance of bacterial colonies for 72 hours. The number of colony forming units (CFU) from each replicate was counted and reported in log CFU/g. Based on morphological uniqueness, representative colonies of different morphological groups were randomly selected and streaked onto fresh nutrient media plates for purification and description of their morphology in the sub-culture plates and subsequent identification. The pure colonies were stored in glycerol at 4 °C.

Surfactant-based method

In the second method, surfactants were added to the crushed seed and water suspension instead of centrifugation. The surfactant has been used here as an emulsifier, with a purpose to help the bacterial colonies present in the suspension (emulsion) to come out on the surface and develop colonies on the media. One gram of each of the four mustard seed varieties was surface sterilized and crushed with 9 ml sterile distilled water. Six eppendorf tubes were taken and to each tube, 1ml from 10 ml volume (10^{-1} dilution) of crushed seed and water suspension was added. Surfactant, Tween 20 (Merck) was autoclaved to remove any microbial contamination and then added separately to the five tubes containing 1ml suspension in such a way that each tube contained 20 µl, 40 µl, 60 µl, 80 µl, 100 µl of the surfactant in a total volume of 1.1 ml of suspension and Tween 20. One tube did not receive surfactant and served as control for this experiment. 100 µl suspension from each tube containing specific Tween concentration and seed suspension was then plated on nutrient media plates in triplicates. The plates were incubated at 28ºC and observed daily for 72 hours. The numbers of bacterial colonies on each plate of all the surfactant concentrations were counted for all the varieties of mustard. The above approach was also tested using sunflower and peanut seeds. Similar looking colonies were assumed to belong to one bacterial group. They were isolated based on morphological differences such as colour, shape, size, and margin. Few colonies from each morphological group were randomly selected and streaked individually in fresh NA media plates to obtain pure colonies. These isolates were used for DNA extraction.

DNA extraction from bacterial isolates, PCR amplification and 16S rRNA gene sequencing

Bacterial isolates were grown in Nutrient broth using incubation shaker (Scigenics Biotech, India) for 24 hrs and then transferred separately to 2 ml tubes. Bacterial growth was spun for 15 minutes at 12000 rpm at 4ÚC. Supernatant was discarded. To the pellet, 567µl TE buffer

was added and gently mixed followed by addition of 3µl of 10% SDS, 3µl of 20 mg/ml Proteinase K. Tubes were mixed gently and incubated for 1 hour at 60Ú. 5M NaCl (100µl) was added and mixed gently followed by addition of 80µl of CTAB/NaCl solution (10% CTAB in 0.7M NaCl). The tubes were again gently mixed and incubated at 60ÚC for 15 minutes. RNAse (2µl) was added and incubated for 15 minutes at 60ÚC. The solution volume was extracted with an equal volume of Phenyl Chloroform Isoamyl alcohol and mixed properly and spun for 5 minutes at 12000 rpm. Supernatant was transferred to a fresh tube and extracted again with Chloroform Isoamyl alcohol followed by mixing and spinning for 5 minutes at 12000 rpm. Supernatant was transferred to a fresh tube. DNA was precipitated by adding 36µl of 5M NaCl and 0.6 volume of isopropanol. The tubes containing DNA were incubated for 20 minutes at -80ÚC and then centrifuged for 7 min at 12000rpm. 200µl of chilled 80% ethanol was added to pellet and centrifuged for 5 minutes at 12000rpm followed by removal of supernatant and drying of pellet. Finally, TE buffer (20µl) was added to these tubes. DNA was stored at 4ÚC for further experimentation. The purity and final concentration of DNA were determined by NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA). DNA was checked in 0.8% agarose gel. Amplification of 16S rRNA gene was performed in Eppendorf Master Cycler. Master Mix was comprised of 10X buffer with $MgCl_2$, 10mM NTP's, 10µM each of 27f (5'AGAGTTTGATYMTGGCTCAG) and 1492r (5'-TACCTTGTTAYGACTT) and Taq Polymerase. Water was added along with DNA based on concentration of DNA present in each sample. Annealing Temperature was optimised at 50ÚC. PCR conditions used were 94ÚC (5 mins), 35 cycles of 94ÚC (60 sec), 50ÚC (40 sec) and 72ÚC (60 sec) and finally 72ÚC (10 min). Amplified DNA was run in 1% Agarose gel in 40ml TAE buffer. The amplified DNA was sent to First Base, Malaysia for 16SrRNA gene sequencing and the sequences were obtained.

Phylogenetic analysis

The raw data sequences of the bacterial isolates were aligned in Codon code aligner and the gene sequences were identified by aligning them in NCBI database using the blast nucleotide (blastn) algorithm. The 16S rRNA gene sequences were submitted to NCBI GenBank database and accession numbers were obtained. Phylogenetic tree was constructed in Molecular Evolutionary Genetics Analysis software (MEGA7) (Kumar *et al*., 2016) to determine the taxonomic relationships among them using Maximum Composite Likelihood method (Tamura *et al.*, 2004) with 500 bootstrap replications.

Statistical analysis

EB population were counted in triplicates and the results obtained was presented as mean ± SD. Variations in EB population among the four seed varieties obtained by different methods were examined by one-way ANOVA. The difference in the two methods for each seed variety was examined by t-test, accepted at 1 % ($p<0.001$). Significant variation was measured using SigmaPlot (Systat Software Inc.) statistical software.

Results and Discussion

Visualization, isolation and identification of endophytic bacterial in interior of mustard

Endophytic bacteria isolated from growth around transverse sections of seeds on nutrient agar

Bacterial growth was not observed in either SSS var. TS46, M27, Binay, NRCHB101 placed or in last wash water plated in NA. But the bacterial colony and lawn growth around the transverse sections (TS) of SSS appeared after 48 hours of incubation (Supplementary Fig. 1). Although bacterial cell was not detected on surface of SSS of TS46, bacteria of different size and shape were observed in interior of SSS and SSS originated radicle and plumule by SE microscopy (Fig. 1). Rod and cocci shape bacterial cells were observed frequently in interior of SSS by SEM. All together, eight morphologically distinct bacteria could be separated in pure cultures from these TS derived colonies. These isolates were identified based on their 16S rRNA based sequences when matched to the bacterial strains in NCBI database for sequence similarity (%) and was submitted to NCBI GenBank under accession number MK554481- MK554482, MK554559, MK554561, MK554572, MK554563, MK554520 and MG383559. (Supplementary Fig. 2). However, TS plating method was not useful in getting quantitative data on cfu from unit quantity of seed.

Endophytic bacterial population and diversity in mustard seeds by centrifugation and

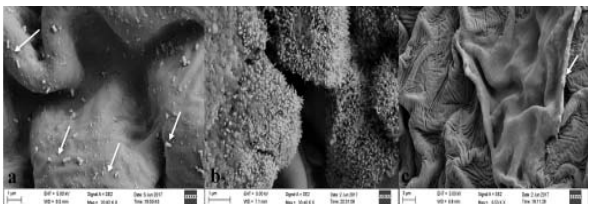

Fig. 1: Scanning electron micrographs of the (a) interior surface sterilized seeds (SSS) of mustard variety (b) cotyledon (c) radicle. Please note the endophytic bacterial cells of different shapes (arrows).

pelleting of serial dilution

No bacterial growth was detected in the NA from the supernatant obtained by centrifugation of 10^{-1} dilution of seeds of 4 mustard varieties (Supplementary Fig. 3). Distinct bacterial colonies appeared on NA on plating and incubation of the serial dilution of pellets of 10^{-1} dilution. The log cfu of endophytic bacteria per gram (CEBPG) seed pellet of TS46, M27, NRCHB101 and Binay were 5.164, 5.143, 4.113, and 4.355 respectively (Table 1). Altogether a total of 13 bacterial isolates were randomly selected and subjected to 16S rRNA sequence-based identification. Both TS46 and M27 contained 4 distinct isolates each, Binay contained 3 isolates and NRCHB101 contained 2 isolates (Fig. 2). The search for the 16S rRNA sequence similarity of these isolates with those in NCBI BLAST showed that they belonged to 5 genera within 3 phyla namely *Firmicutes, Actinobacteria* and *Betaproteobacteria* (Supplementary Table 1). The sequences were submitted to NCBI GenBank under Accession Number (MN704660, MN704765- MN704768,

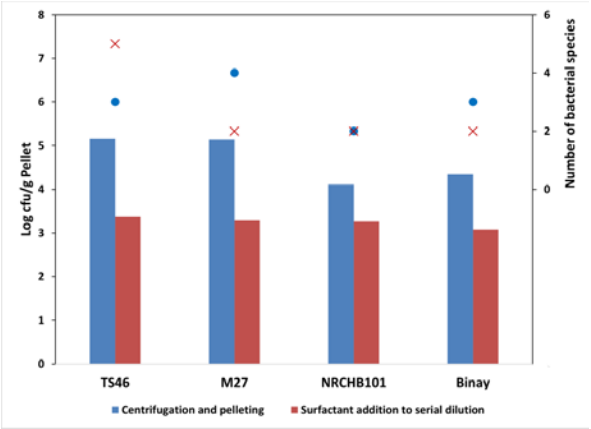

Fig. 2: Culturable bacterial endophytic population and 16S rRNA sequence based EB species obtained from the pellet of SSS of four mustard varieties by centrifugation and pelleting (Blue) and by surfactant addition to serial dilution (Red). Number of bacterial species is shown with cross (surfactant addition to serial dilution) and filled circle (centrifugation and pelleting) markers.

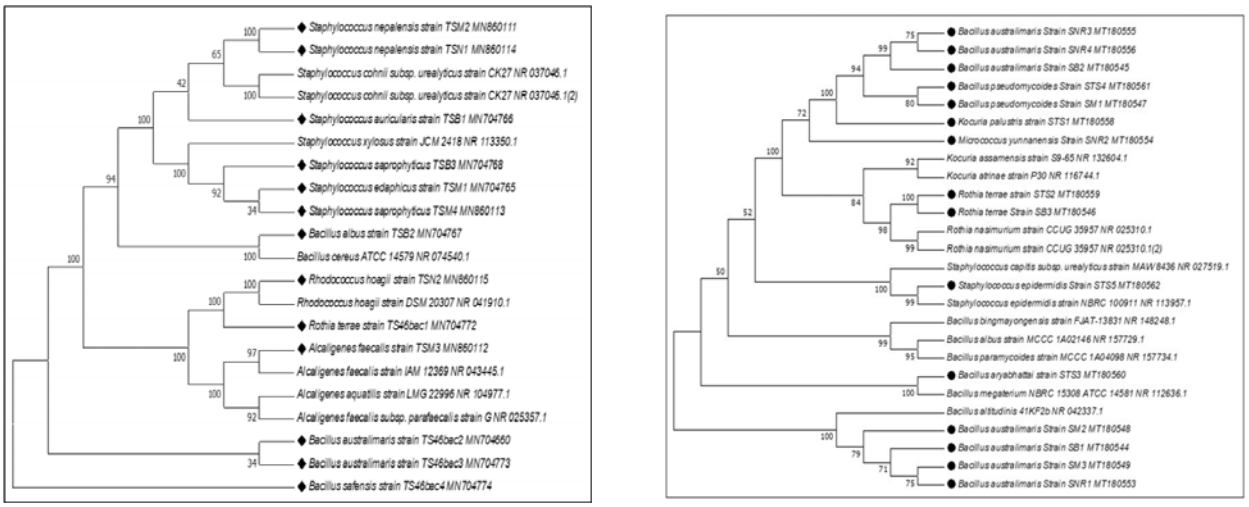

Fig. 3: Phenogram expressing the relationships of bacterial endophytes derived (a) from seed pellet and (b) surfactant added serial dilution to taxonomically similar microorganisms based on the 16S rRNA gene sequences. The tree has been constructed using MEGA7 software. The evolutionary history was inferred using the Neighbour-Joining method. Using the Maximum Composite Likelihood method, the evolutionary distances were computed. The GenBank accession number is given in parentheses for each organism. Taxa marked with markers represent closely similar bacterial species based on NCBI BLAST.

Table 1: EB population and different EB species obtained from pellet of mustard seed varieties

Varieties	Log cfu/g pellet	Bacterial species identified based on 16S rRNA sequence
TS46	5.16 ± 0.12	*Rothia terrae*, *Bacillus australimaris*, *Bacillus safensis*
M27	5.14 ± 0.16	*Staphylococcus edaphicus*, *Staphylococcus nepalensis*, *Alcaligenes faecalis*, *Staphylococcus saprophyticus*
NRCHB 101	4.11 ± 0.27	*Staphylococcus nepalensis*, *Rhodococcus hoagii*
Binoy	4.35 ± 0.46	*Staphylococcus auricularis*, *Bacillus albus*, *Staphylococcus saprophyticus*

MN704772- MN704774, MN860111- MN860115). The phylogenetic tree for these species was constructed in MEGA7 software (Figure 3a) to determine their phylogenetic relationship using Maximum Likelihood method. Among the five genera, *Staphylococcus* (46.2%) was most dominant followed by *Bacillus* (30.8%). Three rare genera namely, *Rothia, Alcaligenes* and *Rhodococcus* of EB were detected in TS 46, M 27 and NRCHB1010 variety seed respectively and comprised of 23.1% of the genus level diversity in the mustard seeds. The frequency of occurrence of the member of EB genus in seed was different depending upon the variety. *Bacillus* was predominant (75.0%) in TS46, while Staphylococcus was predominant (75.0%) in M27 and Binay (66.7%). *Staphylococcus* and *Rhodococcus* genus occurred in equal proportion (50.0%) in NRCHB101. Among the 4 varieties, NRCHB101 variety seed contained least number (2) of species.

Cultural endophytic bacterial population and diversity in surfactant added crushed suspension of crushed SSS

No colony was detected on NA from crushed SSS suspension without Tween 20 surfactant. The CBEP in SSS of four mustard/ toria varieties varied depending upon concentrations of surfactant in the serial dilution (Figure 4). TS46 contained the highest log CBEP population (3.431) at 80 µl and Binay the lowest (3.054) at 60 µl concentration of surfactant (Table 2).

Surfactant addition to serial dilution was tested on two other oilseed crops, groundnut and sunflower. The maximum CBEP of sunflower was found to be log cfu 4.16/g seed at 80 µl concentration of surfactant and of peanut log cfu 3.82/g seed at 40 µl concentration (Fig. 4).

Table 2:. Highest endophytic bacterial population (cfu/g) obtained on NA from crushed SSS serial dilution and the concentration of surfactant at which maximum population was obtained

Crop variety	Endophytic bacteria cfu/g	Surfactant concentration	Bacterial species
TS 46	3.37 ± 0.11	60µl	*Kocuria palustris*, *Rothia terrae*, *Bacillus aryabhattai*, *Bacillus pseudomycoides*, *Staphylococcus epidermidis*
M 27	3.29 ± 0.15	60µl	*Bacillus pseudomycoides*, *Bacillus australimaris*
NRCHB 101	3.26 ± 0.15	60µl and 80µl	*Bacillus australimaris*, *Micrococcus yunnanensis*
Binoy	3.08 ± 0.07	80µl	*Bacillus australimaris*, *Rothia terrae*
Sunflower (Russian Giant)	3.93 ± 0.18	80µl	16S rRNA sequencing not performed
Peanut	3.60 ± 0.23	40µl	16S rRNA sequencing not performed

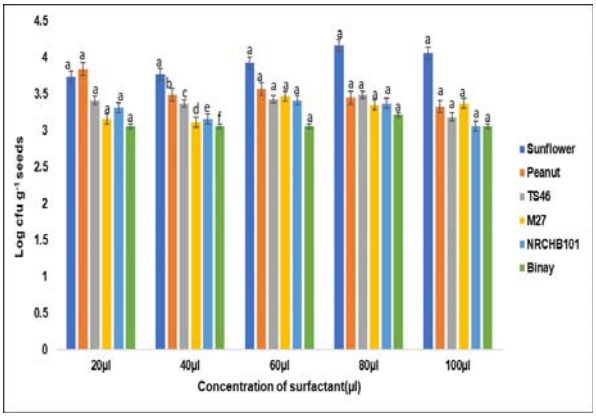

Fig. 4: Log cfu of culturable endophytic bacterial population (CEBP) calculated per gram basis from the dilutions of surface sterilized seeds (SSS)

A total of 15 morphologically distinct endophytic bacterial isolates were obtained from surfactant mixed suspension of crushed SSS four varieties of mustard. The 16S rRNA sequence similarity of these isolates with the closely matched bacterial strains in NCBI database ranged from 82.05 to 100% (Supplementary Table 2). Five of these EB isolates were derived from TS46, 4 from NRCHB101, 3 from M27 and 3 from Binay variety seeds. These EB isolates belonged to 5 genera within 2 phyla namely *Firmicutes* and *Actinobacteria*. The sequences were submitted to NCBI GenBank under Accession Number MT180544-MT180549, MT180553-MT180556, MT180558-MT180562. The phylogenetic tree construct for the isolates using MEGA 7 is shown in Figure 3b. *Bacillus* was the most dominant (66.6%) EB in mustard/ toria seeds. Among the 5 genera, percentage dominance of *Bacillus* was 100%, 75%, 66.6% and 40.00% in M27, NRCHB101, Binay and TS46 variety seeds, respectively (Fig. 5). *Kocuria, Rothia* and *Micrococcus* were 3 rare endophytic bacterial genera detected in the SSS interior.

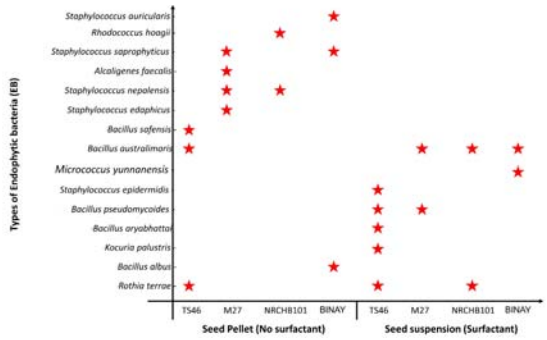

Fig. 5: 16S rRNA sequence-based species diversity in mustard variety seeds obtained by using two methods of isolation of culturable endophytic bacterial population. The common colour indicates that these species were detected by both the method. Please also note some species are common in two or more variety seeds irrespective of methods

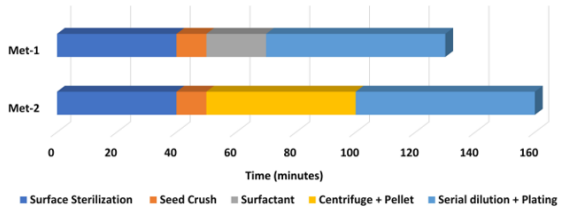

Fig. 6: Time required in min to accomplish different steps of centrifugation and pelleting method (Met-2) and surfactant addition serial dilution (Met-1) for determination of culturable endophytic bacterial population

Comparison among three methods in terms of experiment time and EB diversity

In terms of experiment time, surfactant addition to serial dilution was a better method as it required less time compared to centrifugation and pelleting method to obtain CEBP data (Fig. 6). The CEBP in 4 varieties ranged from 4.11 to 5.16 log cfu/g in case of centrifugation pelleting method and 3.09 to 3.38 log cfu/g in surfactant addition to serial dilution method. Transverse sectioning and placement of SSS on NA cannot give CEBP per unit quantity seed, although this method took least experiment time between surface sterilization of seed and colony appearance. The number of bacterial phyla, genera and species detected based on CEB colonies by the 3 methods are presented in Table 3 along with CEBP in seed. Transverse sectioning method was performed for only TS46 variety and showed EB only in 1 phylum, 1 genera and 3 species. *Bacillus australimaris* and *Rothia terrae* were commonly detected by both centrifugation and surfactant addition method. TS46 seed contained at least 7 species with M27, NRCHB101, and Binoy containing 6, 4 and 5 species respectively. *Bacillus australimaris* was detected as a core species across the four seed varieties (Fig. 5).

To our best knowledge, first time mustard seed varieties growing in Assam have been explored for determining their endophytic bacterial population (EBP). Different methods have been used for the isolation of endophytic bacteria (EB), the centrifugation method of isolation from

Table 3: Comparison of population and diversity of culturable endophytic bacteria (CEB) obtained by three methods on nutrient agar

Method	Variety	CEBP (log cfu/g)	No. within bacterial taxa (4 variety counted)		
			Phylum	Genus	Species
Transverse sectioning and plating	TS46	2.23±0.09	1	1	3
	M27	2.32±0.08			
	NRCHB 101	1.1±0.17			
	Binoy	1.69±0.08			
Centrifugation and pelleting	TS46	5.16±0.12	3	5	13
	M27	5.14±0.16			
	NRCHB 101	4.11±0.27			
	Binoy	4.35±0.46			
Surfactant addition to serial dilution	TS46	3.37±0.11	2	5	15
	M27	3.29±0.15			
	NRCHB 101	3.26±0.15			
	Binoy	3.08±0.07			

supernatant and a new developed method of isolation using surfactant, Tween 20. Using the former method, 13 endophytic bacteria (EB) and using the latter, 15 EB were isolated. Initially 8 EB were isolated from mustard seed using excise paste method. As seeds are the basis to emergence of various parts of the plant, their EBP study is essential and forms the foundation to understand the functioning and colonization of different EB in different parts of plant in addition to factors affecting the EB colonization. In our analysis, therefore only seeds were used to assess the diversity of EB, which reported higher proportions of Firmicutes followed by Actinobaceria and very few Proteobacteria. We assessed the bacterial diversity in seeds of four mustard varieties (TS46, M27, NRCHB101 and Binoy) and maximum endophyte isolation was observed in TS46 var. Though different parts of mustard plant such as leaves, stem and roots have already been explored earlier for endophyte, seeds have not been explored yet thus making them the preferred and essential niche to be considered for EB study. Diverse population of EB has been reported in this study using mustard seeds. *Bacillus* and *Staphylococcus* were dominant in mustard seeds, where *Staphylococcus* was more when isolated from pellet with *Bacillus* emerging as the dominant EB when isolated from suspension with surfactant.

Earlier serial dilution method was used for EB isolation from non-germinated surface sterilized seeds (SSS) of mustard but repeatedly no bacteria were obtained leading to the usage of excise and paste method reported for EB isolation in peanut (Sobolev *et al.*, 2013). Our study involved the investigation of EBP in a certain quantity of mustard seeds and since excise paste method was not suitable for such quantity of seeds, two other methods involving centrifugation of suspension and surfactant addition to suspension were used.

Mustard seed serial dilution is a mixture of seed interior microorganisms, oil, water and other chemical constituents of mustard seed. Due to hydrophobicity nature of oil, when mustard oil in serial dilution drop was spread on NA, it might have hindered water and nutrient absorption by the bacterial cells present in the dilution mixture. Bones and Rossiter (1996) explained the glucosinolate-myrosinase system in mustard and Ratzka *et al.* (2002) reported the process of formation of toxic products when the seeds were damaged or wounded. As reported earlier, mustard oil is also known to have antibacterial and antimicrobial activity (Khan *et al*., 2016). So there might be compounds in the seed that are hindering the growth of bacteria on nutrient media. In Avocado, when its oil was exposed to the environment for six months, no microbial growth was observed due to which it was suggested that the seed oil may contain compounds that inhibit the growth of microbes (Omeje *et al.*, 2018). By removing the supernatant (containing oil) from the suspension mix of SSS of mustard by centrifugation and then plating the suspension of pellets, bacterial colonies were obtained on NA plate. However, this method might underestimate the bacterial population as some cells may escape with the supernatant. Furthermore, centrifugation of a number of serial dilution suspensions is time consuming. Surfactant helps oil water system hydrophobicity to facilitate water and nutrient absorption by bacterial cell. However, at higher concentration of surfactant, bacterial population decreased indicating its toxicity at higher concentration. The effect of higher surfactant concentration was not consistent on the four varieties of mustard. Rauprich *et al*. (2000) suggested that the dose and composition of surfactant influenced the bacterial growth. Thus, the difficulty of quantification of mustard seed interior bacteria can be overcome by using correct combinations and concentrations of surfactant and type of seed.

Bacterial population in the SSS water mix suspension derived pellets was in the range 4.11- 5.16 log cfu g^{-1} and in surfactant used method was 3.08-3.37 log cfu g^{-1}. Data on seed interior bacterial populations of Brassica sp. is scanty (Barret *et al*., 2015). With respect to sunflower and peanut, there is yet no report of EB population in 1 g of seed.

Mustard var. TS46 showed highest EB population followed by M27 and NRCHB 101 showed least population of EB in mustard. In seed interior of the four oil seed crop varieties tested in this study, 15 different bacterial species found belonged to 3 phyla, *Firmicutes, Proteobacteria* and *Actinobacteria*. 7 different genera identified belonged to *Kocuria, Bacillus, Staphylococcus, Rhodococcus, Micrococcus, Alcaligenes* and *Rothia*. 13 isolates belonging to phyla *Firmicutes, Actinobacteria* and *Betaproteobacteria* were identified using the centrifugation and pelleting method. In this centrifugation method, 5 different genera, *Rothia, Staphylococcus, Rhodococcus, Alcaligenes* and *Bacillus* were identified. By surfactant method, *Firmicutes* and *Actinobacteria* were identified. Rare genera, *Kocuria*, *Rothia, Staphylococcus and Micrococcus* were identified for the first time in mustard seeds. *Bacillus* was found to be the most abundant genera obtained by both the methods.

Diversity of EB identified (using surfactant and without it) were found to be different with some similar strains

identified in both isolation methods. Some bacterial strains isolated from pellet of seed suspension such as *B. safensis, B. albus, S. edaphicus, S. nepalensis, A. faecalis, S. saprophyticus, R. hoagii and S. auricularis* were not isolated from surfactant-based method. Similarly, some bacteria obtained from surfactant method such as *K. palustris, B. aryabhattai, B. pseudomycoides, S. epidermidis and M. yunnanensis* were not obtained from pellet. Only 2 bacteria, *Bacillus australimaris* and *Rothia terrae* were similar among these methods. Centrifugation might have killed some of those bacteria or low dose of surfactant might have stimulated some bacteria. Divergence in seed characteristics due to competition for resources among seeds (Fenner, 2004) may lead to two different seeds not being similar in its chemical composition and therefore in 1 gm, the seeds may have different EB composition. Hence, we report surfactant-based approach as the most convenient isolation method in oilseeds.

In comparison of mustard seed EB population and diversity with that of cereals and legume seeds, distinct observations were made. In rice seeds, bacterial population ranged from 3.00-8.98 cfu/g and 16 EB species were identified (Raj *et al.*, 2019) whereas in peanut seeds, 28 bacterial isolates belonging to 8 bacterial species were reported (Sobolev *et al*., 2013). Bodhankar *et al*. (2017) reported 80 EB from 30 maize varieties. *Bacillus* species were more abundant and among them *Bacillus safensis* and *Bacillus pseudomycoides* were found to be similar with mustard. Number of EB in wheat seeds was less than that of mustard as only 6 EB were identified in wheat and most of them belonged to *Paenibacillus* genus (Herrera *et al.*, 2016). *Proteobacteria* were the most abundant in rice whereas *Firmicutes* were the most abundant in peanut seeds. Other crops like tobacco showed *Pseudomonas* as the most abundant genus (Mastretta *et al*., 2009). EB population in mustard was found to be lesser than most of the crops and its EB diversity were found to be different from them. This bacterial diversity differences reveal their role in the development of attributes and mechanism of their host plant such as nutritional benefits, aroma etc.

Oilseeds have quite different EB composition. This EB community may depend on the presence of different content of nutrients such as carbohydrate, protein, fats or oil inside seeds. Unlike cereals and pulses, oilseeds do not contain carbohydrates and have a high content of protein, fibre and fats inside their seeds. The oil is distributed throughout the germ cells inside their seeds, where as in rice and wheat, oil is present only on the sides of endosperm in a germ cell. Depending on their requirements and nutrient availability, microbial community diversity may be different in oilseeds. The role of the endophytes presents in mustard seeds need to be further explored and if beneficial, these microbes can be explored for their biocontrol activity against pathogens. This is the first study showing the diverse EB communities in different mustard varieties grown in Assam. Overall *Bacillus* sp. and *Firmicutes* was the dominant genus and phylum in mustard seeds. Different bacteria especially Bacillus sp. has earlier been reported to produce lipopeptides which exhibit biocontrol properties (Ongena & Jacques, 2008; Zhao et al., 2017). Further, these endophytes may be used to study their role against pathogens affecting mustard cultivation worldwide.

Conclusion

Overall, our study unravels mustard seed microbiome and discusses endophytic bacterial diversity in a certain quantity of seeds. This study reports mustard seed interior bacterial population (3.08 - 5.16 cfu/g) and 16s RNA gene based bacterial composition comprised of representative bacteria of 3 phyla, 7 genera and 15 species. The study also reports two methods of overcoming the mustard seed oil-imposed difficulty of isolation and enumeration which will facilitate further studies on function of EB of seed interior on growth and development of mustard crop varieties and in defence against plant pathogens.

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Availability of data and materials

All sequence data are available in NCBI under:

Accession number: MK554481- MK554482, MK554559, MK554561, MK554572, MK554563, MK554520, MG383559, MN704660, MN704765- MN704768, MN704772- MN704774, MN860111- MN860115, MT180544-MT180549, MT180553-MT180556, MT180558-MT180562

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