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ORIGINAL RESEARCH

Cultivable bacterial diversity and early plant growth promotion by the traditional organic formulations prepared using organic waste materials

Rangasamy Anandham[1,2] · Nagaiah Premalatha[1] · Hyeong Jin Jee[2] · Hang Yeon Weon[3] · Soon Wo Kwon[3] · Ramasamy Krishnamoorthy[1] · Pandiyan Indira Gandhi[4] · Yong Ki Kim[2] · Nellaiappan Olaganathan Gopal[1]

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Abstract
Purpose Traditional organic formulations are widely used as a plant growth promoters; however, the knowledge on the microbial aspect of traditional organic formulations is still limited. The aim of this study was to illustrate the cultivable bacterial diversity of various traditional organic formulations and their potential for early plant growth promotion.
Methods Five different traditional organic formulations such as 100 % *panchagavya*, 33 % *panchagavya*, plant extract with native microorganisms, commercial organic fertilizer extract with two percent leaf soil extract and commercial organic fertilizer extract with 2 % yogurt were prepared and used in this study. The liquid fraction of these traditional organic formulations were used to analyze the beneficial effect on plant growth by seed treatment and foliar applications.
Results Bacterial 16SrDNA analysis revealed that the isolates fell into forty-three different genera, which can be grouped into seven different classes, such as *Alphaproteobacteria*, *Actinobacteria*, *Bacilli* (or *Firmibacteria*), *Betaproteobacteria*, *Cytophagia*, *Flavobacteriia* and *Gammaproteobacteria*. Higher bacterial diversity was observed in cow dung followed by 33 and 100 % *panchagavya*. Radish and Chinese cabbage seed germination and growth were significantly improved by traditional organic formulations compared to control.
Conclusion The results of this study showed that the bacterial diversity changes depend on the type and concentration of ingredients used in traditional organic formulations. Substantial increase in plant growth by the traditional organic formulations indicates the suitability of using these organic preparations in eco-friendly agriculture.

Keywords Eubacterial diversity · Organic farming · Organic formulations · *Panchagavya* · Plant growth-promoting bacteria

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✉ Rangasamy Anandham
anandhamranga@gmail.com

[1] Department of Agricultural Microbiology, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai 625 104, Tamil Nadu, India

[2] Organic Agriculture Division, National Academy of Agricultural Science, Rural Development Administration, Suwon 441-707, Republic of Korea

[3] Korean Agricultural Culture Collection, National Agro Biodiversity Center, National Academy of Agricultural Science, Rural Development Administration, Suwon 441-707, Republic of Korea

[4] Regional Research Station, Tamil Nadu Agricultural University, Vridhachalam 606 001, Tamil Nadu, India

Introduction

Intensive agriculture depends on expensive chemical inputs from off farm, which generates lot of pollutants and affects environment (Horrigan et al. 2002). Due to the negative impact of chemical inputs, organic farming gained more attention from last few decades (Badgley et al. 2007). The Food and Agricultural Organization of the United Nations (FAO) estimated that the area under organic agriculture was 37.2 million ha in 2011, which was three times higher than that of 1999 (FAO 2013). In organic farming, synthetic pesticides, mineral fertilizers, genetically modified

organisms and sewage sludge applications are strictly excluded. Nonetheless, biofertilizers or traditional organic formulations prepared by using organic materials/wastes are encouraged to use as a plant nutrition (Badgley et al. 2007). Numerous studies stated that the yield and quality of plant products produced by organic farming are considerably better than that of conventional farming (Chemura 2014; Oliveira et al. 2013; Luthria et al. 2010). Organic farming not only improves the quality of the food products, but it also improves the soil fertility. In a long-term study in Switzerland, organic farming with composted animal manure improved soil quality significantly as compared to conventional farming with mineral fertilizer (Maeder et al. 2002).

Traditional organic formulations may contain numerous plant growth-promoting bacteria (PGPB), which may enhance plant growth by nitrogen fixation, growth hormone production and control phytopathogens (Amalraj et al. 2013; Naik and Sreenivasa 2009). In many Asian countries, farmers formulate their own organic formulations by combining different organic materials and treating them by fermentation or composting. For example, in India, *panchagavya* (PG) is one of the widely used traditional organic formulations, which is mostly prepared by farmers themselves. *Panchagavya* is a fermented product made from five ingredients obtained from cow, such as milk, urine, dung, curd and clarified butter (Amalraj et al. 2013). South Korean farmers use wild weed wineberry (*Rubus phoenicolasius*) extract with native microorganisms, which is prepared by mixing wild weed extract, salt, molasses and decomposed leaf soil extract. Thailand farmers use fermented plant extracts or fermented plant juices, which can promote plant growth and act as a biocontrol agent (Kantachote et al. 2009). *Bokashi* is a Japanese traditional organic formulation preparation which contains effective microorganisms (EM). Japanese first developed the EM concept during 1980s, and this preparation contains various PGPB and yeast (Yamada and Xu 2001). These traditional organic formulations have not been well characterized microbiologically.

In recent past, several culture-independent methods have been developed to illustrate higher percent of bacterial population than usually found with cultural-dependent methods (Videira et al. 2013). These culture-independent methods may give in-depth knowledge about bacterial diversity, but it is impossible to use this information to identify the physiology of particular bacterial strain, interaction between microorganisms and response of bacterial strains to different environmental conditions (Stewart 2012). However, the culture-dependent method can be more useful in studying bacterial physiology and their role in plant growth promotion (Palleroni 1997). In addition, a cultural-dependent method may be helpful in industrial applications such as developments of antibiotics or biofertilizers. Only a few reports elucidate the microbial population in different organic formulations prepared by farmers (Radha and Rao 2014; Amalraj et al. 2013; Girija et al. 2013). Recently, higher number of cultivable bacterial genera was obtained from the organic formulation prepared using fermented cow manure (Giannattasio et al. 2013). In addition, few novel and plant growth-promoting bacteria such as *Larkinella bovis* sp. nov. and *Microbacterium suwonense* sp. nov. were isolated from traditional organic formulations and tested for their plant growth promotion (Anandham et al. 2011a, b; Sreenivasa et al. 2009).

In this study, culture-dependent method was used to illustrate the bacterial diversity in various traditional organic formulations made from organic materials and evaluated the effect of organic formulations on early growth of radish and Chinese cabbage. Successfully cultivated bacterial isolates may be used to develop a microbial consortium to support sustainable agricultural practice in future.

Materials and methods

Traditional organic formulations preparations

Organic formulations such as 100 % PG, 33 % PG, plant extract with native microorganism (PNM), commercial organic fertilizer extract with 2 % leaf soil extract (OLE) and commercial organic fertilizer extract with 2 % yogurt (OY) were prepared and used in this study. Ingredients and their concentration of different traditional organic formulations are given in Table 1.

Commercially available pelleted organic fertilizer (OF) extract was used to prepare OLE and OY. Pelleted OF contains 55 % caster bean waste, 40 % rice bran and 5 % palm waste with the nutrient content of 45 % N, 1.5 % P and 1.0 % K. For preparing an organic fertilizer extract, forty gram OF was boiled in 400 ml of water (sterile distilled water) for 30 min and then the filtrate was collected using the filter paper (Whatman No. 2–90 mm). Leaf soil extract was prepared by mixing 20 g of soil [containing decomposed leaf, collected from the hilly region Gangwon Province of South Korea (37°49′22.64″N; 128°09′23.00″E)] with 120 ml of water (sterile distilled water) and shaken for 30 min, then filtered through filter paper (Whatman No. 2–90 mm). The ingredients of the each traditional organic formulation were mixed in a flask and allowed for fermentation for 5 days in shaking condition (120 rpm) at 30 °C. During fermentation, microorganisms can degrade the organic materials and enhance the production of organic formulations. All five organic

Table 1 Ingredients and their concentrations of different organic formulations

100 % PG	33 % PG	PNM	OLE	OY
Cow dung (1.5 g)	Cow dung (0.5 g)	0.2 % wild weed wineberry extracts, 0.1 % sodium chloride	40 % OF extract, 2 % molasses	2 % yogurt
Cow urine (4.5 ml)	Cow urine (1.49 ml)	0.5 % molasses and make up to 1 l with water	0.2 % sodium chloride	40 % OF extract
Cow milk (3.0 ml)	Cow milk (0.99 ml)		2 % leaf soil extract and make up to 1 l with water	2 % molasses and make up to 1 l with water
Yogurt (3.0 g)	Yogurt (0.99 g)			
Molasses (4.5 g)	Molasses (4.5 g)			
Potato (3.0 g) and make up to 1 l with water	Potato (0.99 g) and make up to 1 l with water			

formulations were separately filtered through a cloth filter (performed under aseptic condition to avoid contamination), and then, the liquid fraction was used for further experiments.

Cultivable bacterial diversity analysis

Bacteria were isolated from the liquid fraction of various traditional organic formulations using trypticase soybean agar (TSA; Difco), Lysogeny agar (LA; Difco) and R_2A agar (Difco). In addition, PG ingredients (cow urine, cow dung and cow milk) were also used for bacterial isolation (by serial dilution and plating) and diversity analysis. Isolated bacterial cultures (169 isolates) were grouped into different genera based on their short-length sequencing of 16S rDNA with 895F (CRCCTGGGGAGTRCRG), 47F (GCGCCAGGGAATTCTAAAAC) and 783R (ACCMGG GTATCTAATCCKG) primers. Only 96 bacterial isolates were selected (by avoiding duplicate within the treatments), and nearly full-length sequence of 16S rDNA was sequenced for species-level identification. Genomic DNA of 96 bacterial isolates was extracted using Ultra Clean DNA isolation kit (MO BioLaboratories Inc., Carlsbad, CA, USA) and amplified using universal 16S rDNA primers (27F and 1492R). The 16S rDNA nucleotide sequences were identified by PCR direct sequencing by fluorescent dye terminator method (ABI Prism™ Bigdye™ Terminator cycle sequencing ready reaction kit v.3.1; Applied Biosystems, Foster, CA, USA), and the products were purified by Millipore-Montage dye removal kit (Beckman Coulter Inc., Fullerton, CA, USA). Finally, the products were run into an ABI3730XL capillary DNA sequencer (50 cm capillary). 16S rDNA sequences from the automatic sequencer were aligned, and bacterial identities deduced by using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/) to ascertain their closest relatives. The sequences obtained in this study were submitted in GenBank, with the accession numbers from GQ246652 to GQ246748. 16S rDNA sequences were aligned using the CLUSTALW program, and the phylogenetic tree was constructed using MEGA version 6.0 (Tamura et al. 2013). Neighbor-joining tree was constructed with the bootstrap values of 1000.

Pathogen detection in organic formulations preparations and their ingredients

Five traditional organic formulations and PG ingredients were tested for the presence of pathogens such as *Staphylococci* and *Enterobacteriaceae* using the STAPH PLUS (Thermo Fisher Scientific Inc., Waltham, MA, USA) and RapID One (Thermo Fisher Scientific Inc., Waltham, MA, USA), respectively, according to the manufacturers instruction.

Plant growth experiments

Radish (*Raphanus sativus* L. cultivar Hong Feng No. 1) and Chinese cabbage (*Brassica rapa* subsp. *pekinensis* cultivar Norangchusurk) seeds were surface sterilized and imbibed in 5 ml liquid fraction of five different organic formulations with 0, 50 and 100 times dilutions and incubated at 25 ± 2 °C for 72 h for seed germination. The seeds were taken as germinated if the radicle emerges from the seeds. Three-day-old germinated seeds were transplanted into 40-L pot containing 20-kg pot mixture (pH 7.0; EC—1.2; NH_4^+N—170 mg kg^{-1}; NO_3^-N—110 mg kg^{-1}; available P_2O_5—300 mg kg^{-1}; K_2O—340 mg kg^{-1}; CEC—10 $cmol_c$ kg^{-1}) and maintained for 30 days. Pot experiment was performed with sixteen treatments (five organic fertilizers with three dilutions and one control). The plants were irrigated once in 2 days until the pot mixture reached 70 % of its water-holding capacity. Additional to seed treatment, 25 ml of respective traditional organic formulations was sprayed on the plants until runoff using the handheld pneumatic sprayer, on seventh day after transplantation. In the case of control, sterile distilled water was sprayed to maintain the same conditions as other treatments. There were three replications for each treatment and arranged in a completely randomized block

design in the greenhouse. Root length, shoot length and dry weight of the plants were measured 30 days after transplantation (DAT).

Statistical analysis

Data on microbial count and plant parameters were subjected to analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) using the SAS package, version 9.1.3 (SAS Institute Inc., Cary, NC, USA) and tested significant differences at 0.05. Data on plant growth of individual traditional organic formulation treatment and their dilutions were compared with control plants in order to find out the effect of traditional organic formulations on radish and Chinese cabbage growth. Microbial count data were log10-transformed before statistical analysis. Heat map was constructed using Microsoft Excel conditional formatting.

Results

Bacterial count and cultivable bacterial diversity of various traditional organic formulations and PG ingredients

LA and TSA were found to support a higher number of bacterial group than that of R2A medium (Online Resource 1). No significant differences in bacterial count were observed among different organic formulations prepared and used in this study. Short-length sequencing of 169 bacterial isolates obtained from different organic formulations and PG ingredients (cow urine, cow dung and cow milk) revealed that the isolates were belonged to 43 different genera (Fig. 1). These 43 genera can be grouped into seven different classes, such as *Alphaproteobacteria*, *Actinobacteria*, *Bacilli* (or *Firmibacteria*), *Betaproteobacteria*, *Cytophagia*, *Flavobacteriia* and *Gammaproteobacteria* (Fig. 2). Fifty-four percentage of the cultivable bacterial population in 100 % PG preparation was *Bacilli* (or *Firmibacteria*). In particular, *Bacillus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Lysinibacillus* genera were dominated. The higher proportion of *Alphaproteobacteria* was observed in 33 % PG followed by *Firmibacteria* and *Betaproteobacteria*. Other organic formulations such as PNM, OLE, OY and PG ingredients were found to be dominated by *Firmibacteria* and *Alphaproteobacteria* or *Actinobacteria* (Fig. 2). Higher number of cultivable bacterial species were observed in cow dung (54 species) followed by 33 % (37 species) and 100 % PG (28 species).

Out of 169 bacterial isolates, 96 were carefully selected for full-length sequencing by avoiding duplicates within the treatments. All sequenced isolates with closest neighbor and their accession numbers are given in Online Resource 2. Our sequencing results revealed that PG preparation had thirteen unique bacterial genera, which were belong to various PGPB and fermentative groups of bacteria. *Azospirillum*, *Brevundimonas*, *Lactobacillus*, *Xenophilius*, *Xylophilus*, *Comamonas* and *Acetobacter* are the representatives of PGPB genera found in PG preparation (Fig. 3).

Pathogen detection in traditional organic formulations and their ingredients

RapID STAPH PLUS system and RapID One analysis revealed the presence of *Staphylococcus aureus* and *Escherichia coli* in cow dung which was one of the ingredients in PG preparation, but those pathogens were not observed in PG organic formulations. In addition, 16S rDNA amplification confirmed the presence of foodborne pathogens (*Shigella flexneri*, *Bergyella zoohelcum* and *Enterococcus durans*) in cow dung and cow milk (Online Resource 2). However, these pathogens were not observed in 33 and 100 % PG organic formulations.

Germination assay and plant responses to different organic formulations and their dilution

Germination percentage of radish and Chinese cabbage treated with different organic formulations is presented in Fig. 4. Dilution of 33 % PG, PNM, OLE and OY fertilizers showed a significant increase in radish seed germination. On the other hand, traditional organic formulations did not show any significant increase in Chinese cabbage seed germination (Fig. 4).

Organic formulation-treated plants showed higher growth compared to control plants (except in 100 % PG treatment). Zero times dilution of 100 % PG had negative impact on plant growth, which registered the lower root length, shoot length and dry weight of radish than that of diluted treatments (Fig. 5; Online Resource 3, 4). Higher root length of radish (25.6 cm) and Chinese cabbage (33.33 cm) was observed in OY (50 times diluted) and 33 % PG (zero times diluted) treatment, respectively (Online Resource 3). Radish and Chinese cabbage shoots length was found to be higher in 33 % PG and OY (zero times diluted) treatment, respectively (Online Resource 4). Higher dry matter accumulation was found in OLE-treated (100 times diluted) radish and Chinese cabbage plants (Fig. 5). Dry weight of radish was significantly improved by 100 % PG, 33 % PG, PNM and OLE treatments than that of control plants. Traditional organic formulations have no significant impact on Chinese cabbage dry weight.

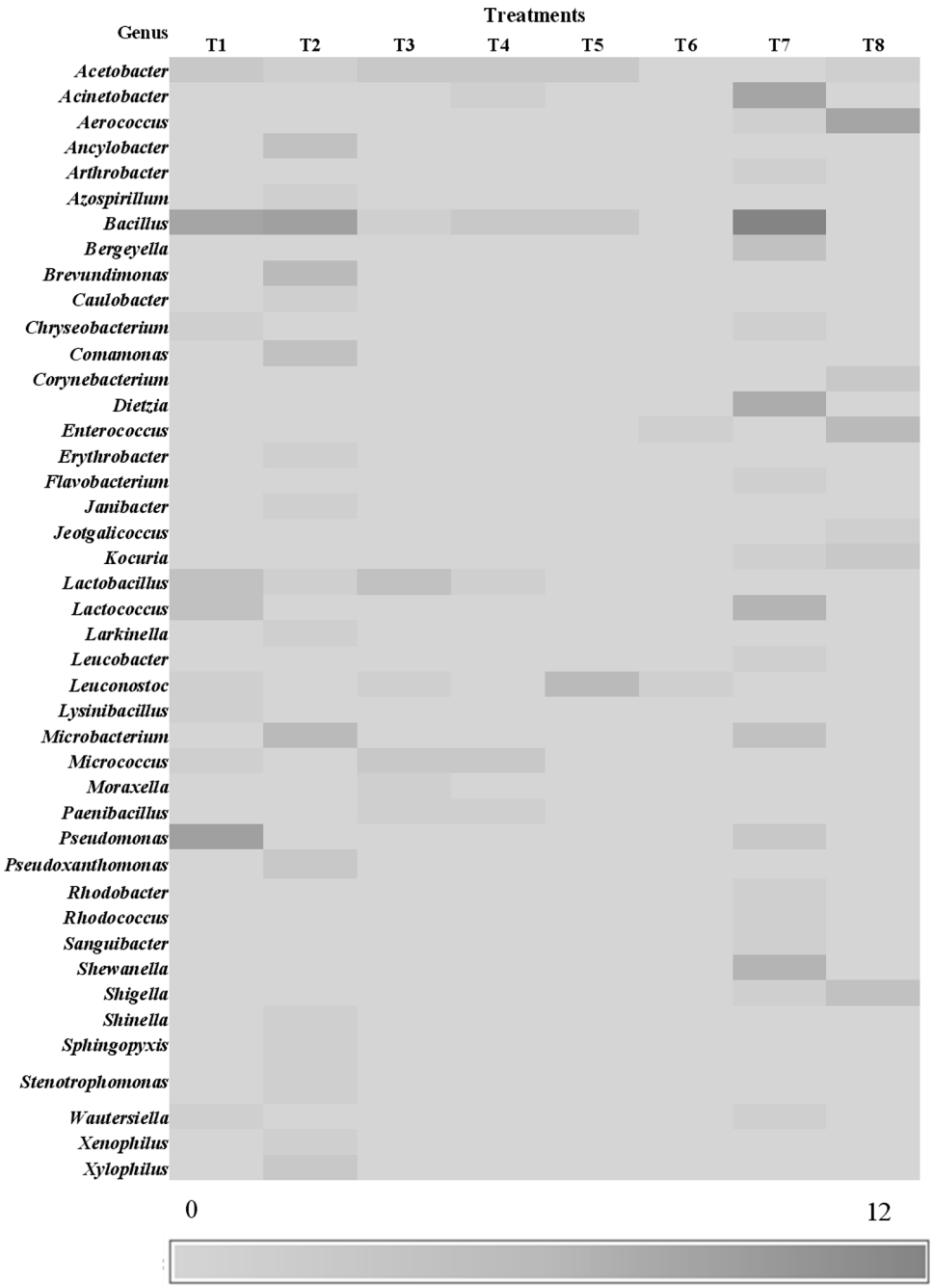

Fig. 1 Heat map shows the number of bacterial species isolated from different organic formulations and their ingredients. *T1* 100 % *panchagavya*; *T2* 33 % *panchagavya*; *T3* plant extract with native microorganisms; *T4* commercial organic fertilizer extract with 2 % leaf soil extract; *T5* commercial organic fertilizer extract with 2 % yogurt; *T6* cow urine; *T7* cow dung; *T8* cow milk

Discussion

Farmer utilizes locally available organic materials for preparing organic formulations, and there are many well-developed traditional organic formulations which are available worldwide, but most of them are unpopular. Chemical and microbiological aspect of many traditional organic formulations is not well studied. Recently, nuclear magnetic resonance analysis of organic formulations revealed the presence of various bioactive compounds, which helps in plant growth improvement (Spaccini et al. 2012). In this work, we have chosen five different traditional organic formulations such as 100 % PG, 33 % PG, PNM, OLE and OY to analyze microbiological aspects and the effect of organic formulations on early plant growth was also illustrated.

Three diverse microbiological media have been used to obtain higher number of cultivable bacterial population from different organic formulations. This cultivation-dependent approach exhibited the presence of novel bacterial isolates such as *L. bovis* sp. nov. and *M. suwonense* sp. nov. in organic formulations and PG ingredients, respectively

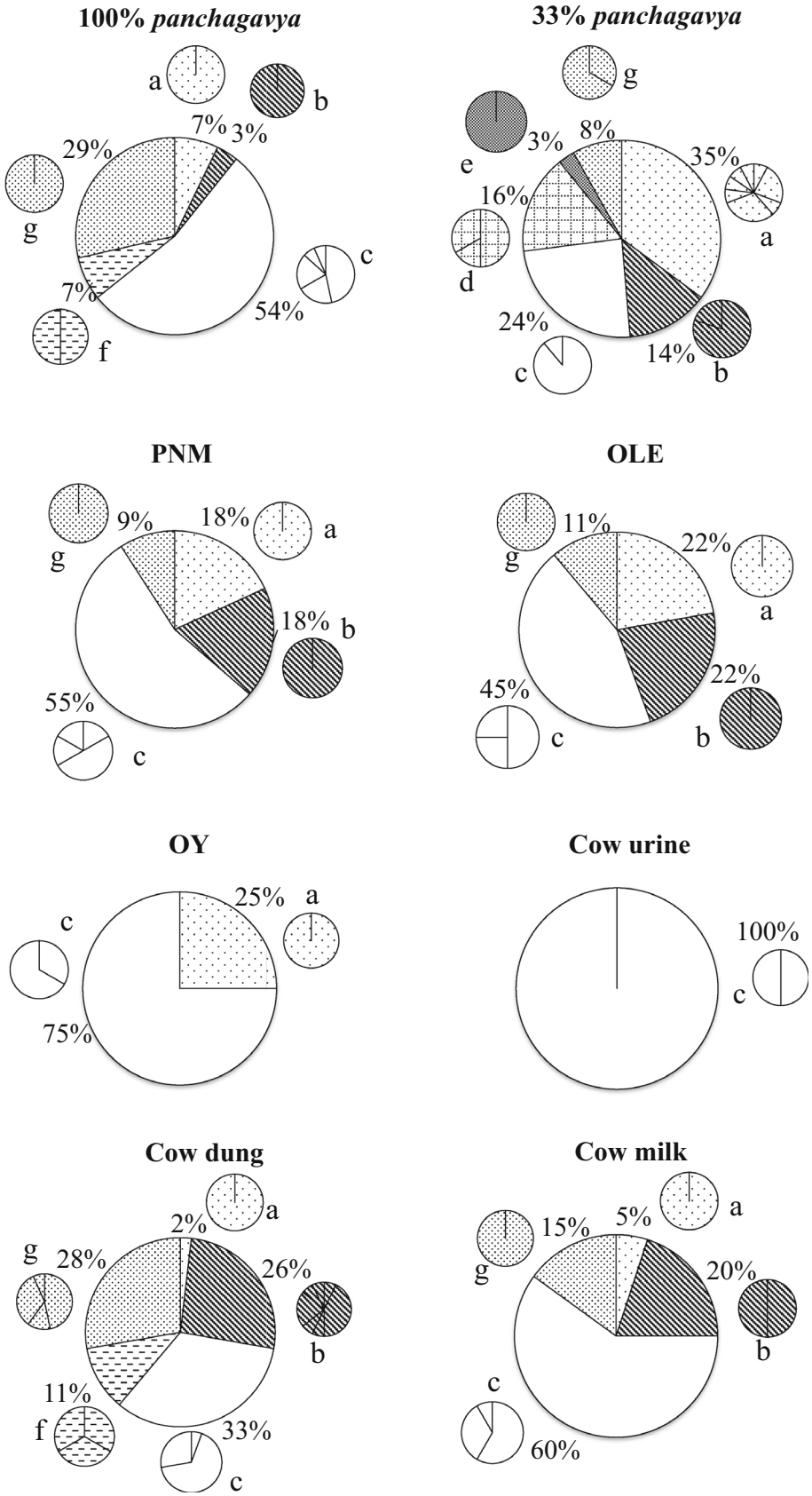

Fig. 2 Class and genus-level distribution of bacteria in different organic formulations and their ingredients. **a** *Alphaproteobacteria*; **b** *Actinobacteria*; **c** *Bacilli* (or *Firmibacteria*); **d** *Betaproteobacteria*; **e** *Cytophagia*; **f** *Flavobacteriia*; **g** *Gammaproteobacteria*. *Larger circle* represents percentages of different classes of bacteria present in organic formulations and ingredients. Partitioning of *small circle* represents number of genera present in a particular class

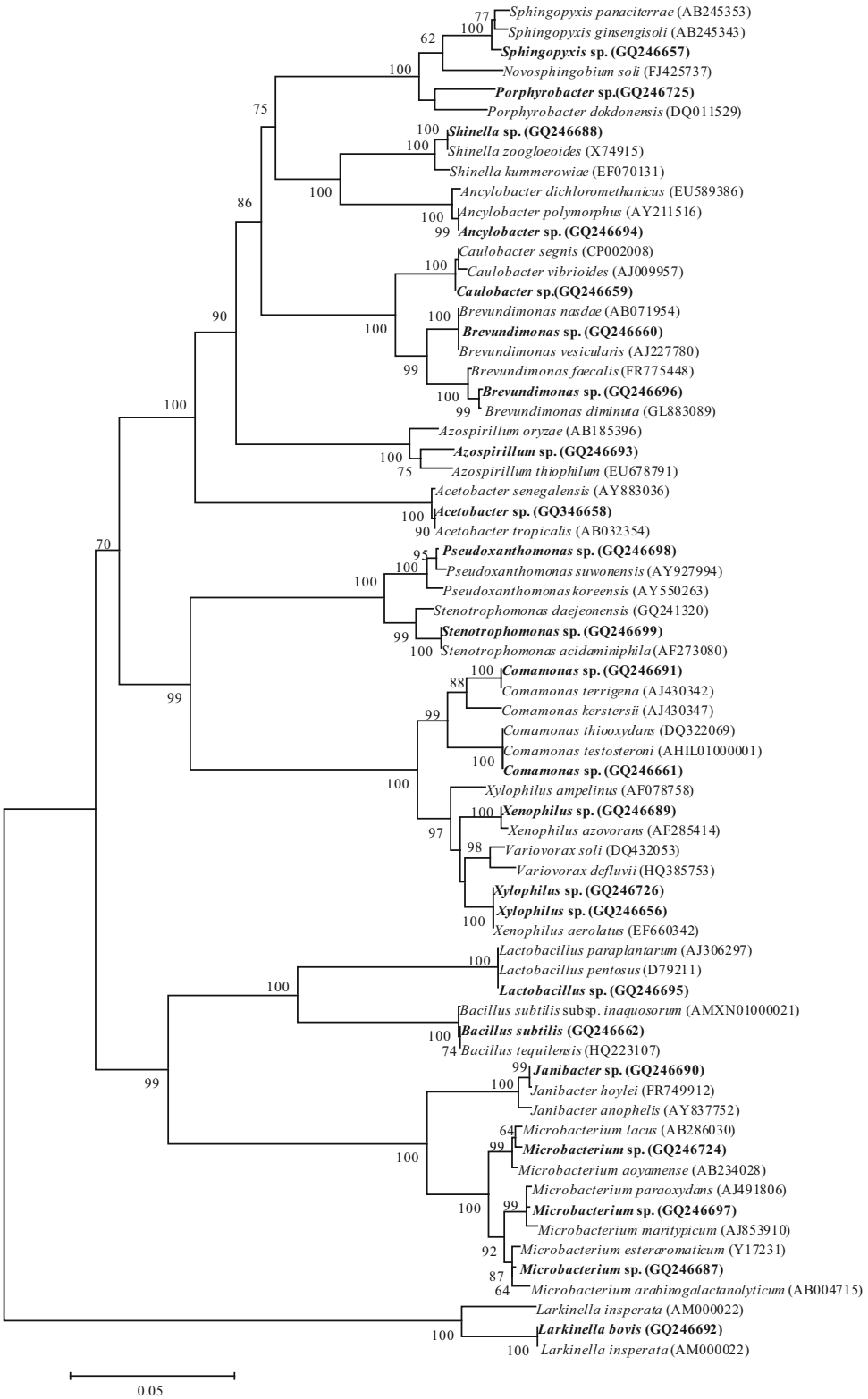

Fig. 3 Neighbor-joining phylogenetic tree shows the relationship among the bacterial species isolated from 33 % *panchagavya* preparations (highlighted in *bold*) and their closest neighbors. Tree was constructed using near-full-length sequences of bacterial 16S rDNA sequences with 1000 bootstraps values

(Anandham et al. 2011a, b). Fermentation process during the preparation of traditional organic formulations encourages different group of microorganisms to develop (Yan et al. 2013). These microorganisms may be grouped into three different groups such as plant growth promoters, fermentative microorganisms and antibiotic producers based on their functions. Fermentative group of microorganisms break down complex nutrients into simple

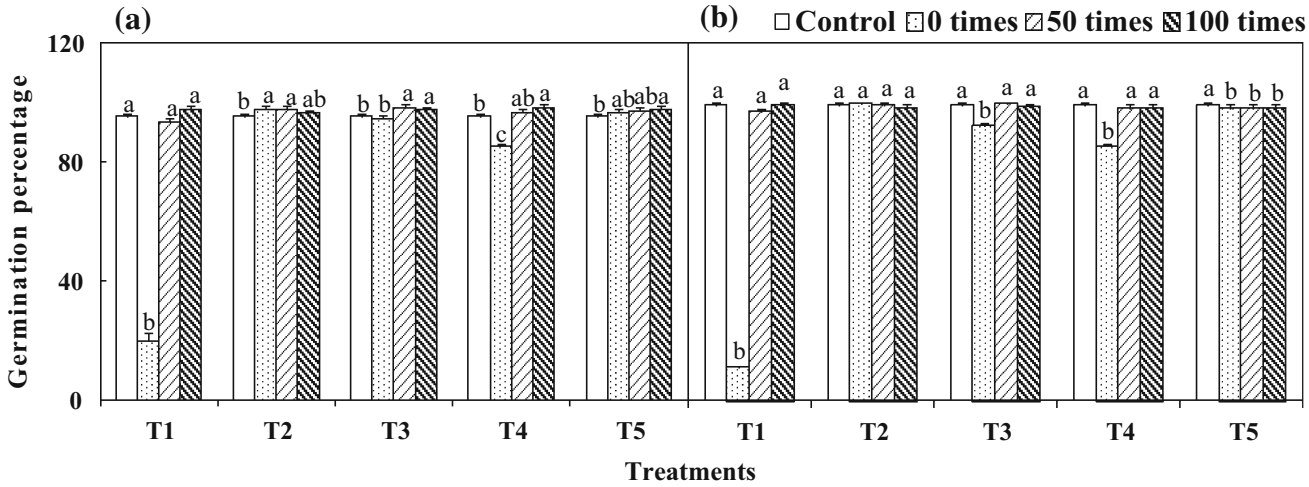

Fig. 4 Effect of seed treatment of different organic formulations on radish (**a**) and Chinese cabbage (**b**) seed germination percentage under in vitro condition. *T1* 100 % *panchagavya*; *T2* 33 % *panchagavya*; *T3* plant extract with native microorganisms; *T4* commercial organic fertilizer extract with 2 % leaf soil extract; *T5* commercial organic fertilizer extract with 2 % yogurt. Control, 0, 50 and 100 times dilution of organic formulations are represented in different shades. Values are mean ± standard error (SE) of three replications, and *same letters* in each treatment are not significantly different from each other at $P < 0.05$ according to Duncan's multiple range test (DMRT)

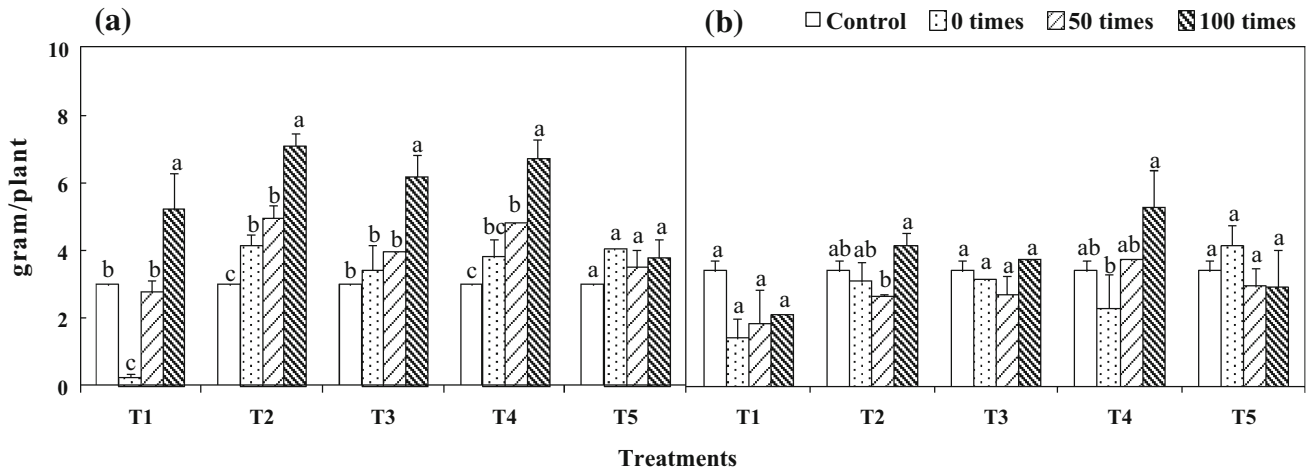

Fig. 5 Effect of different organic formulations on dry weight of radish (**a**) and Chinese cabbage (**b**) under pot culture condition. *T1* 100 % *panchagavya*; *T2* 33 % *panchagavya*; *T3* plant extract with native microorganisms; *T4* commercial organic fertilizer extract with 2 % leaf soil extract; *T5* commercial organic fertilizer extract with 2 % yogurt. Control, 0, 50 and 100 times of dilution organic formulations are represented in different shades. Values are mean ± SE of three replications, and *same letters* in each treatment are not significantly different from each other at $P < 0.05$ according to DMRT

nutrients, and antibiotic producers inhibit pathogenic bacterial growth (Yadav et al. 2013; Gea et al. 2009). On the other hand, PGPB harbored in the organic formulations improves plant growth by various mechanisms (Naik and Sreenivasa 2009). PGPB are the group of bacteria found in the rhizosphere and/or phyllosphere region of plants, which can enhance plant growth by direct and indirect mechanisms (Vacheron et al. 2013). Presence of various PGPB and slow release of nutrients are the two main reasons for plant growth promotion and improving soil health by organic formulations (Amalraj et al. 2013).

In our study, total bacterial populations in raw and processed organic formulations were not significantly changed. However, bacterial genus/species between organic formulations and their raw ingredients were changed. This clearly indicates the occurrence of bacterial succession during fermentation process. In recent past, use of pyrosequencing technique revealed the presence of 142 bacterial genera in

cow dung, which includes cultivable and non-cultivable bacteria (Dowd et al. 2008). However, only 19 cultivable bacterial genera from the cow dung were able to cultivate under laboratory condition. Less number of cultivable bacterial genera obtained in this study proven the uncultivable nature of numerous bacterial genera in common laboratory media (Joseph et al. 2003). Other than 169 bacterial isolates, we were also obtained some yeast strains, which might also played an important role in the breakdown of complex sugars into simple components in the fermentation process during organic formulations preparation (Kantachote et al. 2009).

One of the PG ingredients (cow dung) was considered to be an carrier for foodborne pathogens like *S. aureus*, *E. coli*, *Salmonella* sp. and *Campylobacter* sp. (Simujide et al. 2013). In line with this, our results also showed the presence of *S. aureus* and *E. coli* pathogens in cow dung. However, these pathogens were eliminated by fermentation process during organic formulations preparation. Similar to our results, El Fels et al. (2015) showed the elimination of coliforms during the composting of organic waste material. Particularly, antibiotic-producing microorganisms in organic formulations might have played an important role in eliminating foodborne pathogens and phytopathogens (Qiu et al. 2012). Similar to this, composting of cow dung was also reported to be effective in removing foodborne pathogens such as *S. aureus* and *E. coli* (Simujide et al. 2013). Recently, Liu et al. (2013) reported that the inoculation of antagonistic bacteria with organic formulations was found to be effective in phytopathogen control.

Dominance nature of *Firmibacteria* in organic formulations used in this study might be due to the use of soil and plant extracts as one of the ingredients. Recently, Chen et al. (2013) reported the dominance of *Firmibacteria* in plant and soil ecosystem. Similarly, *Firmibacteria* was also dominated in compost prepared using agricultural waste materials (Chandna et al. 2013). PGPB such as *Acetobacter* sp. (Sevilla et al. 2001), *Azospirillum* sp. (Fages and Arsac 1991) and *Brevundimonas* sp. (Kumar and Gera 2014) found in 33 % PG were belong to *Alphaproteobacteria*. In addition, *Firmibacteria* class PGPB like *Bacillus subtilis* (Mohamed and Gomaa 2012) and *Lactobacillus* sp. (Limanska et al. 2013) were also observed (Fig. 3). Soil bacteria like *Micrococcus* sp., *Paenibacillus* sp. and *Acetobacter* sp. were dominated in PNM and OLE preparations. This may be due to the use of soil as one of the ingredient in these organic formulations preparations. Whereas *Leuconostoc mesenteroides* was found to be dominant in treatment containing OFE with 2 % yogurt, this bacterial species was mainly involved in milk fermentation process (Kihal et al. 2007).

Traditional organic formulations exhibited a significant positive impact on radish growth except 100 % PG. In line with this, many authors reported the beneficial effect of organic formulations on plant growth improvement (Naik and Sreenivasa 2009; Amalraj et al. 2013). Similarly, Pigeon pea treated with PG, vermicompost and farm yard manure improved plant growth compared to control, but the results were not significantly different among various organic formulations used (Gupta et al. 2014; Amalraj et al. 2013). Organic compost application significantly improved the dry biomass accumulation in wheat (Carpenter-Boggs et al. 2000). In agreement with this, our study also showed significantly higher radish dry biomass on organic formulations application. One hundred percent PG-treated plants showed significantly lower radish root length, shoot length, dry weight and root length of Chinese cabbage compared to that of diluted treatments and control. This might be due to the presence of plant growth inhibitors, acidic pH (5.6) and nutrients in undiluted 100 % PG. In line with this, Kadam and Pathade (2014) also showed the negative effect of higher concentration of vermicompost application on French bean growth compared to lower concentration of compost application. There were no consistent positive results for plant growth improvement on dilution of original organic formulations, except for 100 % PG. Similarly, different rate of compost application to vegetable crops showed no significant differences in plant growth between high and low concentrations (Chang et al. 2007).

Conclusion

This study illustrated the cultivable bacterial diversity of organic formulations traditionally prepared and used by farmers. The results of this study revealed that the bacterial diversity changes depend on the type and concentration of ingredients used in organic formulations. Significant increases in plant growth by organic formulations indicate the appropriateness of substituting organic formulations in the place of chemical fertilizers to enhance eco-friendly agriculture. Future study focusing on the plant growth-promoting characters of the isolates and symbiotic association among the isolates might help in developing microbial consortium for sustainable agriculture.

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