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DIFFERENCE IN THE RHIZOSPHERE BACTERIA OF ZINC-DEFICIENCY TOLERANT AND SENSITIVE RICE GENOTYPES AS A CONTRIBUTING FACTOR TO ZINC EFFICIENCY AT THE MICROBIAL LEVEL

GLENDA Z. DOBLAS[1,4], MERLYN S. MENDIORO[3], IDA F. DALMACIO[3], MARIA GENALEEN Q. DIAZ[3], RITA P. LAUDE[3] AND GLENN B. GREGORIO[2]

Corresponding author Email: *gzdoblas@cmu.edu.ph*

Abstract

In an attempt to explain zinc deficiency tolerance at the microbial level, DGGE using 16S rDNA was used to compare the bacterial diversity in the rhizosphere of zinc deficiency tolerant and sensitive rice genotypes. The DNA from the rhizosphere of A69-1 and K. Patong (KP) at seedling stage was extracted and amplified using universal primers for bacteria and archaea. The 16S rDNA PCR amplicons were subjected to DGGE using 35-55% denaturation gradient of urea-formamide. The prominent bands were excised, reamplified and sequenced. A total of 36 bands, each band representing a microbial population, were observed. Only 17 bands were successfully excised and matched with the NCBI database. The sequences have 83 to 96% matches to 11 prokaryotes namely Methylococcus capsulatus, Methylomonas lenta, M. rubra, Methylomagnum ishizawai, Methylocaldum marinum, Methylogaea oryzae, Methylovulum miyakonense, Pseudoalteromonas xiamenensis, Halochromatium roseum, Thermofilum uzonense and Thermogladius calderae. The presence of relatively greater number of microbial population consisting largely of methanotrophs in the rhizosphere of A69-1 might contribute to its tolerance to zinc-deficient soil. Conversely, the sensitivity of KP to zinc-deficiency might be attributed to the relatively reduced microbial population.

Keywords: 16S rDNA, DGGE, rhizosphere, zinc-deficiency

INTRODUCTION

Zinc is an essential plant nutrient being a cofactor of several enzymes such as carbonic anhydrase, dehydrogenase, aldolase, carboxypeptidase, alkaline phosphatase, superoxide dismutase, RNA polymerase, ribulose-bi-phosphate carboxylase, and phospholipase (Alloway, 2008; Hafeez et al., 2013). Low zinc content in soil or failure of the plant to take up sufficient zinc results to zinc deficiency which negatively affects plant functions particularly on carbohydrate metabolism through photosynthesis and sugar transformation, protein metabolism, membrane integrity, auxin metabolism, flowering and seed production (Alloway, 2008; Sharma et al., 2013). These manifest phenotypically as chlorosis, stunted growth, depressed tillering and even plant death resulting in reduced rice production (Yoshida and Tanaka, 1969). Attempts to correct zinc deficiency have been aimed to obtain maximum improvement in yield (Zaman et al., 2018). This includes zinc application in the field to ameliorate zinc deficiency in soil (Cacmak and Kutman, 2018, Humayan et al., 2014). However, this approach has its own drawbacks among which are the unknown long-term effect on the soil nutrient balance, limited availability of the fertilizer, higher cost, limited soil mobility and very low uptake by the plant (Quijano-Guerta et al., Cacmak and Kutman, 2018). The development of cultivars tolerant to zinc deficiency or cultivars that grow well even in zinc-deficient soil, hereafter termed as zinc efficiency, is now targeted as a better alternative to obtain maximum yield despite the stress (Ismail et al., 2007; Gregorio et al, 2002).

The long-term goal of developing zinc efficient rice lines requires understanding of the zinc efficiency mechanisms. Various mechanisms as to how plants counter low available zinc in soil have been proposed based on physiological, biochemical and genetic studies. It has been observed that zinc efficient plants are able to accumulate more zinc compared to the zinc inefficient genotypes as seen in wheat (Rengel and Graham, 1996). Proposed physiological mechanisms include increasing the bioavailability of soil Zn for root uptake, enhancing root uptake and translocation of Zn from the root to the shoot, maintaining Zn in cytoplasm by altering subcellular compartmentation of Zn in shoot cells, and enhancing efficient biochemical utilization of Zn in cells of the shoot

(Hacisalihoglu & Kochian, 2003). Recent studies associated zinc efficiency as a result of maintenance of root growth, increased efflux of Zn ligands, increased uptake of Zn-ligand complexes at low zinc concentration and increased uptake of Na and K (Broadley et al., 2010). Also, a relationship exists between bicarbonate tolerance and the ability of Zn-efficient genotypes to overcome oxidative stress, maintain root membrane integrity and minimize root leakage (Rose et al., 2011).

Zinc becomes more available to plants by increasing the zinc availability in the rhizosphere, a function mastered by several root-associated bacteria known as plant growth promoting rhizobacteria or PGPR (Dotaniya and Meena, 2015). PGPR interacts with plants to improve their growth by solubilizing essential minerals and facilitating its uptake thereby making it more readily available for the plants (Rengel 2015). Several PGPR and other bacteria such as *Gluconacetobacter diazotrophicus*, *Bacillus* and *Pseudomonas* have been found to possess zinc solubilizing potential (Saravanan et al., 2007; Ramesh et al., 2014.). Zinc solubilization is performed with the production of organic and inorganic acid, production of chelating agents and lowering rhizosphere pH (Hacisalihoglu and Kochian, 2003; Ahemad and Kibret, 2014). This potential of PGPRs might be a contributing factor to tolerance of plants to abiotic stresses such as soil zinc deficiency.

Plants differ genotypically in terms of zinc efficiency and may be categorized into two, the zinc efficient and the non-zinc efficient plants. This difference maybe explained at the rhizosphere level. The rhizosphere, defined as the volume of soil shared by the roots and bacteria and by which the roots have influence is a region with intense microbial activities that play an important role in the acquisition of nutrients for the plants (Gregory, 2006). The kind and number of bacteria in the rhizosphere differ from the bacterial community of the bulk soil (Smalla et al., 2001). This difference is influenced not only by the root exudates but also by the microbial activities in the roots (Berg and Smalla, 2009; Zhang et al., 2014). Furthermore, rhizosphere bacteria show a high degree of plant host specifity and the plant species may strongly influence the microbial populations on their roots by determining the kind and number of bacteria in the rhizosphere (Hartmann et al., 2009). Each plant species may select its own specific microbial community. Hence, different plant species growing in the same soil may harbour distinct rhizosphere community. Conversely, same plants in different soil may exhibit similar microbial community (Marschner et al., 2004). Previous study on wheat showed that genotypes tolerant to zinc deficiency exude more phytosiderophore and 2-deoxymuginiec acid than their sensitive counterpart (Rengel, 1997). Similarly, rice genotypes tolerant and sensitive to varying levels of chromium stress also showed differences in the amount of oxalic, malic and citric acid exuded (Zeng et al., 2008). Since the microbial composition is also shaped by the root exudates, it is tempting to suggest that these observations might also be extended to the rhizosphere bacterial composition. However, no direct evidence can answer yet whether stress tolerant and sensitive genotypes of the same plant species will harbor different rhizosphere community. It is important that this area of research will be looked into to determine whether PGPR renders crop to be tolerant to environmental stresses. Hence, this study was conducted to investigate whether zinc deficiency tolerant and sensitive rice genotypes differ in their rhizosphere bacterial profile. This difference might explain one aspect of several possible mechanisms for zinc deficiency tolerance in rice.

Studying the bacterial community structure of the rhizosphere can be done using cultivation-based techniques. However, since only a small proportion of the total bacteria is culturable, only a small proportion can be recovered from the rhizosphere. This limitation is overcome by employing non-cultivation techniques among which are molecular fingerprinting procedures that include denaturing gradient gel electrophoresis (DGGE) (Valásková et al., 2009). The technique amplifies DNA samples extracted from the soil using 16s rDNA and the products subjected to DGGE. Prominent bands are excised and sequenced to determine the identities of the predominantly present bacterial population.

The use of DGGE in rice studies is recently emerging. The impact of organic fertilizers on the bacterial profile in the rhizosphere and the changes in the community structure of methane-oxidizing bacteria at different growth stages of rice were investigated using DGGE technique (Doi et al., 2011). DGGE was also used in investigating the structure of the methanogenic archaea and diazothrophic community in paddy field soil (Wartiainen et al., 2008.) These studies revealed the great potential of DGGE as a technique for differentiating between bacterial profiles of rice rhizosphere subjected to different environments.

It is therefore aimed in this study to determine differences in the bacterial profile of the rhizosphere of zinc deficiency tolerant and sensitive rice genotypes using 16s rDNA DGGE profiling technique which may lead researchers to the understanding of another mechanism for rice tolerance to zinc deficiency.

MATERIAL AND METHODS

Plant Materials: Seeds of A69-1(tolerant genotype) and Kinandang Patong (sensitive genotype; hereafter termed as KP) obtained from IRRI (International Rice Research Institute), Philippines were grown in hydroponics system composed of SNAP solution maintained at pH 5.0 – 5.10. After 14 days, the seedlings were transplanted to zinc-sufficient (hereafter termed as normal) and zinc-

deficient blocks (hereafter termed as stress) located at IRRI, Philippines. Sampling was done at growth stage 2-4 (seedling, tillering, stem elongation) before booting stage.

Rhizosphere Sampling and DNA Extraction: Plants were sampled using the pull and shake method. The roots were shaken gently in the surrounding water and washed with sterile distilled water to remove loosely attached soils. A small portion of the roots were cut off and placed in sterile Falcon tubes containing 20 ml sterile distilled water. Bulk soil were also randomly collected and placed in Falcon tubes. The tubes were temporarily stored in ice bucket while in transport from field to laboratory. The tubes were vortexed horizontally for 30 minutes at 300 rpm to loosen the rhizosphere or the soil immediately attached to the roots. The resulting suspensions were transferred to 2 ml microfuge tubes and centrifuged for 1 min at 600 rpm. DNA was extracted from the rhizosphere and bulk soil samples using the Mo-Bio UltraCleanTM Soil DNA Isolation Kit and quantified in 1% agarose gel stained with SybrSafeR (Invitrogen) at 169V for 45 minutes.

Polymerase Chain Reaction (PCR) Amplification: PCR was performed with G-Storm Thermocycler using the primer pairs 968FGC (5' CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC 3'; AIT Biotech) and 1378R (5'-TACAAGGCCCGGGAACG-3'; Invitrogen) and ARCH 344F (5′-ACGGGGTGCAGGCGCGA-3′) and ARCH 915R (5′-GTGCTCCCCCGCCAATTCCT-3′) . Working PCR cocktail consisted of 5 pmol/ul each primer, 10x buffer (100mM Tris-HCl pH8.3, 500mM KCl, 20mM MgCl2, enhancer solution), 2.5 mM dNTP, 5 U/ul Taq (iTaqTM DNA polymerase), 1ul (50ng/ul) template DNA and water. PCR condition was set at: initial denaturation at 94OC for 5 min; 10 touchdown cycles (-5OC per cycle) that include 1 min at 94 OC, I min at 60 OC and 2 min at 72 OC; 25 elongation cycles that include 1 min at 94 OC, 1 min at 55 OC and 2 min at 72 OC; and final elongation at 72 OC for 30 min. PCR products were verified on 2% agarose gel stained with SybrSafeR DNA gel stain (Invitrogen).

Denaturing Gradient Gel Electrophoresis: PCR products were subjected to denaturing gradient gel electrophoresis (DGGE) using the The DeCodeTM Universal Mutation Detection System (Bio-Rad Laboratories) on 8% polyacrylamide gel with 35-55% denaturation gradient of urea-formamide under a 60V field at 60 OC for 14 hours. The gel was stained with ethidium bromide on some runs and SybrSafeR DNA gel stain (Invitrogen) in other runs. The 16s rDNA profile was verified and photographed with Gel DocTM XR+ System (Bio-Rad Laboratories). DGGE bands were excised from the gels using Safe ImagerTM Transilluminator (Invitrogen) and placed in 2ml microfuge tubes containing sterile distilled water. The tubes were left overnight at -20OC and then incubated at 50OC for 1-2 hours. The excised bands were then mashed using sterile tips.

Reamplification and Sequencing: The excised DGGE bands were re-amplified using the same primers but without the GC clamp and using the same PCR condition as above. PCR products were verified on 2% agarose gel stained with SybrSafeR DNA gel stain (Invitrogen). Bands were excised from the gels using Safe ImagerTM Transilluminator (Invitrogen) and cleaned up using Agarose Gel Extract Mini Kit (5 Prime). The purified DNA samples were sent to Macrogen Korea for sequencing.

Analysis of the DGGE Data: Images of the DGGE gel were analyzed using the PhyElph and GelAnalyzer software (Lazar, I., 2010; Pavel, 2012). Bands were scored manually as 1 (present) or 0 (absent). Each peak represents one bacterial group having 16s rDNA sequences with similar melting behavior. The band intensity was scored as the raw volume generated from the software and represents the density of the population. Data were subjected to ANOVA to determine significant difference in the microbial populations using the Minitab 16 statistical software. Binary logistic regression was performed to investigate the relationship between the microbial population as represented by the DGGE band, to the environment (normal and stress) and the samples (bulk soil, A69-1, KP). Diversity indices such as Shannon's (H') and Simpson's (D) were computed. High values of H' would suggest more diverse communities (Lande, 1996). Typical values are generally between 1.5 and 3.5 in most ecological studies. On the other hand, as D increases, diversity decreases. Coefficients of similarity (Jaccard's index) and dissimilarity (Bray-Curtis) were also computed to compare biodiversity levels across sites (Shi, 1993). In a similarity index, a value of 1 means that the two communities being compared share all their species, while a value of 0 means they share none. In a dissimilarity index, 1 means that the communities are totally different.

DNA sequences were edited for general nucleotide peaks and mis-called nucleotides using BioEdit Sequence Alignment Editor (Hall,1999) and sent to the DNA database for a homology search using BLAST (www.ncbi.nlm.nih.gov/BLAST/). The sequences were deposited at NCBI GenBank. Overall relationship of the bacterial population was determined by constructing a phylogenetic tree based on the sequences of the DGGE bands using Mega7.0.14.

RESULTS

DNA was successfully extracted from the rhizosphere and bulk soil samples, and successfully amplified in one step reaction using the primer pairs 968F(GC) and 1378R optimized with respect to annealing temperatures and template concentration (Fig.1a). The

primer pair gave one single PCR band at its expected size which is approximately 410bp.

Diversity of the bacterial community: The generated PCR products produced a DGGE profile which gave several bands with an almost similar migration pattern (Fig. 1b). In the normal environment, a total of 36 bands were detected in the bulk soil, 30 of which were seen in the A69-1 rhizosphere and 28 in the KP rhizosphere (Table 1, Fig 2). Of the 36 bands, 27 are common to both A69-1 and KP rhizosphere, 3 are unique to A69-1 and 1 band unique to KP. In the stress environment, a total of 31 bands were seen in the bulk soil, 27 of which were observed in A69-1 and 21 in KP. Of the 31 bands, 20 were common to both A69-1 and KP while 7 were seen only in A69-1 and 1 band unique to KP. This shows that a greater number of bands occurred in the normal compared to the stress environment (P=0.009). There was a reduction of bands in the KP genotype when grown in the stress environment. Also, microbial populations differed among the samples/genotypes (P=0.001).

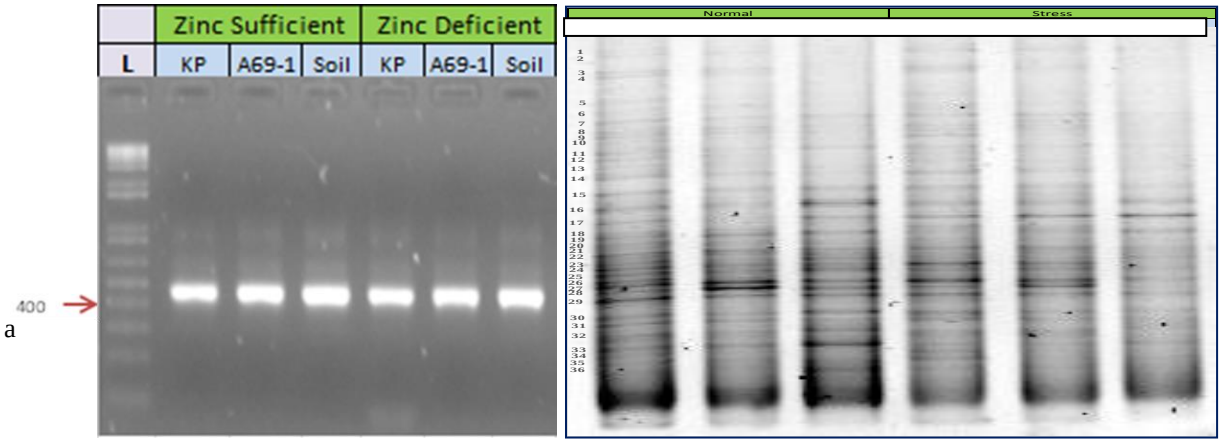

Fig 1. (a) The PCR products (410bp) from the rhizosphere and bulk soil samples using the primers 968F and 1378R as visualized on agarose gel electrophoresis; (b) 16S rDNA PCR-DGGE profiles of bulk soil and rhizosphere bacteria on a polyacrylamide gel with a 35–55% denaturation gradient of urea–formamide.

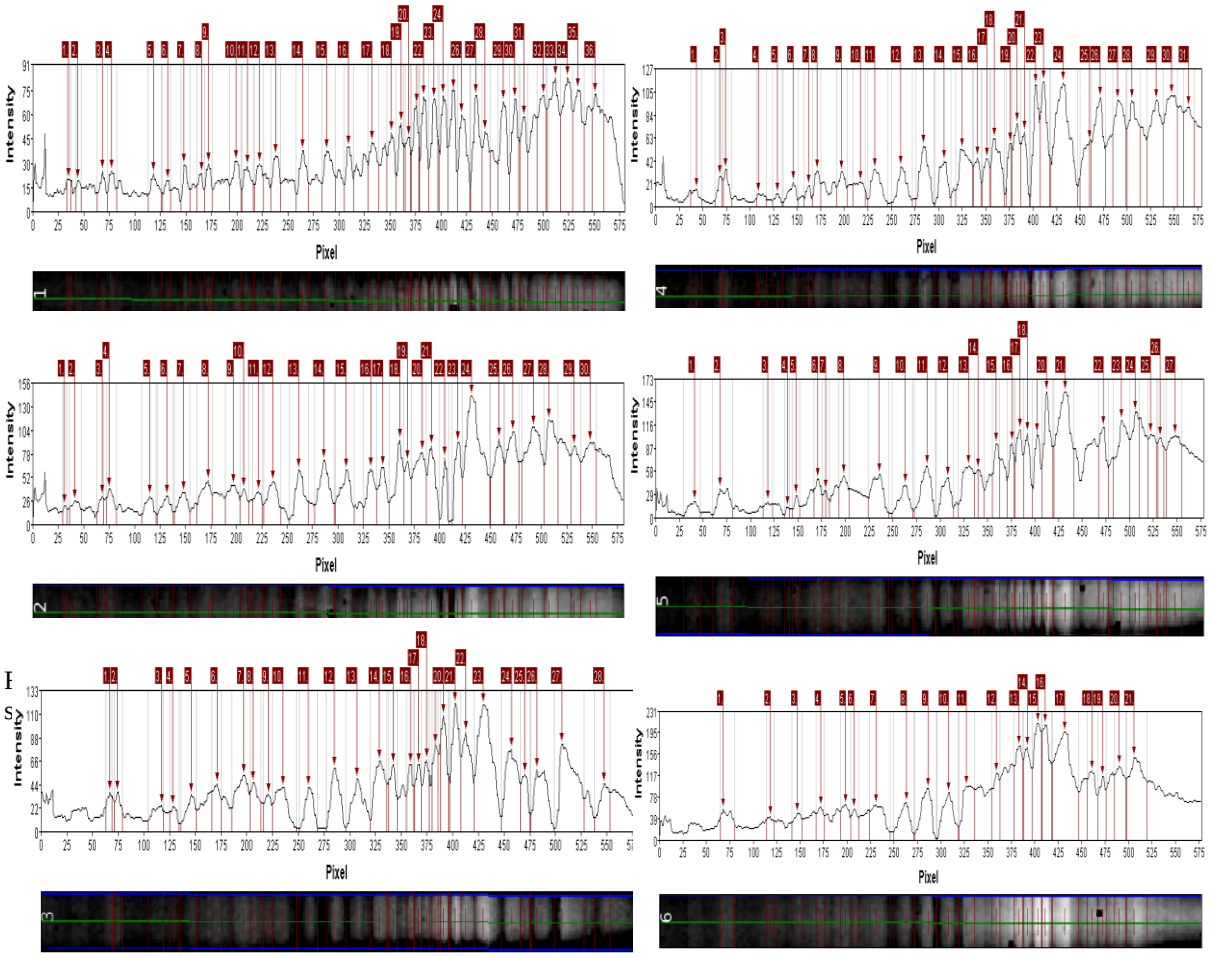

Table 1. Number of DGGE bands in the bulk soil and rhizosphere of A69-1 and KP grown in zinc-sufficient and zinc-deficient environments

Band Number	**Zinc-sufficient**			**Zinc-deficient**		
	Bulk Soil	**A69-1**	**KP**	**Soil**	**A69-1**	**KP**
1	1	1	0	0	0	0
2	1	1	0	1	1	0
3	1	1	1	1	1	1
4	1	1	1	1	0	0
5	1	1	1	1	1	1
6	1	1	1	1	1	0
7	1	1	1	1	1	1
8	1	0	0	1	0	0
9	1	1	1	1	1	1
10	1	1	1	1	1	1
11	1	1	1	0	0	1
12	1	1	1	1	0	0
13	1	1	1	1	1	1
14	1	1	1	1	1	1
15	1	1	1	1	1	1
16	1	1	1	1	1	1
17	1	1	1	1	1	1
18	1	1	1	1	1	0
19	1	0	1	1	1	1
20	1	1	1	1	0	0
21	1	1	1	1	1	0
22	1	1	1	1	1	1
23	1	1	1	1	1	1
24	1	1	1	1	1	1
25	1	0	0	0	0	0
26	1	1	1	1	1	1
27	1	1	1	1	1	1
28	1	0	0	0	0	0
29	1	1	1	1	1	1
30	1	1	1	1	1	1
31	1	1	1	0	0	0
32	1	0	0	1	1	1
33	1	1	1	1	1	1
34	1	0	0	1	1	0
35	1	1	0	1	1	0
36	1	1	1	1	1	0
Total	**36**	**30**	**28**	**31**	**27**	**21**

Table 2 shows the diversity indices. High diversity indices were observed in both environments with Shannon's index H' ranging from 3.0626 – 3.4392. In the normal environment, H' was relatively similar for A69-1 (H'=3.1842) and KP (H'=3.159). However, in the stress environment, Shannon's index H' for KP was low (H'=2.8584) compared to A69-1 (H'=3.0626). Jaccard and Bray-Curtis indices were computed to determine the biodiversity level of microbial population between two sites namely: normal and stress bulk soils, A69-1 in normal and stress environment, KP in normal and stress environment, A69-1 and KP of normal environment, A69-1 and KP of stress environment. Jaccard's indices were 0.86, 0.73, 0.74, 0.87, 0.71, respectively. Bray-Curtis indices were 0.27, 0.34, 0.37, 0.23 and 0.41, respectively. This shows high similarity of the microbial population in the bulk soil regardless of the type of environment (J=0.86). A high similarity of the genotypes A61-1 and KP in normal environment (J=0.87) was also observed but the similarity reduced when the same genotypes were grown in stress environment. Difference in the intensity of the bands were also observed but this difference is not significant (P>0.05). The change in the number of microbial population is significantly associated with stress (P=0000) and the genotype (P=000) as shown by the binary logistic regression (Table 3). The goodness of fit tests are all greater than the significance level of 0.05 indicating that there is not

enough evidence to conclude that the model does not fit the data.

Table 2. Diversity indices of the bulk soil and rhizosphere samples grown in normal and stress environments

Environment	Samples	Number of Bands	Simpson's (D)	Shannon's (H')
Normal (Zinc-sufficient)	Soil	36	0.036	3.4392
	A69-1	30	0.0502	3.1842
	KP	28	0.0504	3.159
Stress (Zinc-deficient)	Soil	31	0.052	3.1528
	A69-1	27	0.0558	3.0626
	KP	21	0.0626	2.8584

Table 3. Jaccard's index to compare biodiversity levels across sites

Compared Communities	Jaccard	Bray Curtis
Between bulk soils	0.86	0.27
A69-1 in both environments	0.73	0.34
KP in both environments	0.74	0.37
A69-1 and KP in zinc-sufficient soil	0.87	0.23
A69-1 and KP in zinc-deficient soil	0.71	0.41

Table 4. Logistic Regression Table performed for binary logistic regression using Minitab 16 to determine the association of the number of microbial population with stress and genotype

					Odds	95% CI	
Predictor	Coef	SE Coef	Z	P	Ratio	Lower	Upper
Constant	3.83847	0.645702	5.94	0.000			
Environment							
2	-0.968496	0.373096	-2.60	0.009	0.38	0.18	0.79
Sample	-0.875615	0.239133	-3.66	0.000	0.42	0.26	0.67

Goodness-of-Fit Tests

Method	Chi-Square	DF	P
Pearson	3.23754	3	0.356
Deviance	4.86118	3	0.182
Hosmer-Lemeshow	3.23754	4	0.519
Brown:			
General Alternative	2.85491	2	0.240
Symmetric Alternative	1.32492	1	0.250

Identity and phylogenetic relationship of the sequences: Of the 36 DGGE bands observed, only 17 were successfully excised, re-amplified, sequenced and sent to BLAST for identity matching. The sequences have 83% - 96% matches to 11 prokaryotes namely *Methylococcus capsulatus, Methylomonas lenta, Methylomonas rubra, Methylomagnum ishizawai, Methylocaldum marinum, Methylogaea oryzae, Methylovulum miyakonense, Pseudoalteromonas xiamenensis, Halochromatium roseum, Thermofilum uzonense* and *Thermogladius calderae*. The latter two are archaeans while all others are bacteria (Table 5). All the bacteria belong to the Phylum Proteobacteria while the archaeans are all members of the Crenarchaeota. Seven of the bacteria belong to the family Methylococcaceae.

Table 5. BLAST identity of the DGGE bands from the bulk soil and rhizosphere of A69-1 and KP grown in normal and stress environments. Legend: BS-bulk soil, A – A69-1, K – Kinandang Patong.

Band	Identity Match	E value	Max Iden	GenBank Accession
7	*Methylococcus capsulatus*	5E-180	95%	MG905449
11	*Methylococcus capsulatus*	7E-166	93%	MG905450
12	*Methylomonas lenta*	6E-73	96%	MG905458
14	*Methylococcus capsulatus*	2E-149	93%	MG905451
15	*Methylomonas rubra strain*	1E-144	93%	MG905452

16	*Methylococcus capsulatus*	2E-65	92%	MG905445
17	*Methylomagnum ishizawai*	1E-62	94%	MG905454
19	*Methylococcus capsulatus*	3E-58	90%	MG905447
25	*Methylocaldum marinum*	3E-52	92%	MG905446
27	*Methylococcus capsulatus*	2E-174	96%	MG905448
28	*Methylogaea oryzae*	2E-78	80%	MG905459
29	*Methylovulum miyakonense*	2E-54	90%	MG905455
30	*Pseudoalteromonas xiamenensis*	4E-63	88%	MG905456
33	*Halochromatium roseum*	1E-158	96%	MG905457
7	*Thermofilum uzonense*	5E-124	83%	MG905460
11	*Thermogladius calderae*	1E-125	83%	MG905461
15	*Thermofilum uzonense*	6E-142	83%	MG905453

The phylogenetic tree showed two major clades. All the bacteria clustered together in one large clade while the archaeans in another clade. Bands 27 and 7 may belong to the samel population, and so with bands 15 and 12, bands 25 and 19, bands 29 and 28, and bands 7 and 11. The tree also shows 10 possible distinct bacterial populations and 2 distinct archaea populations (Fig 3).

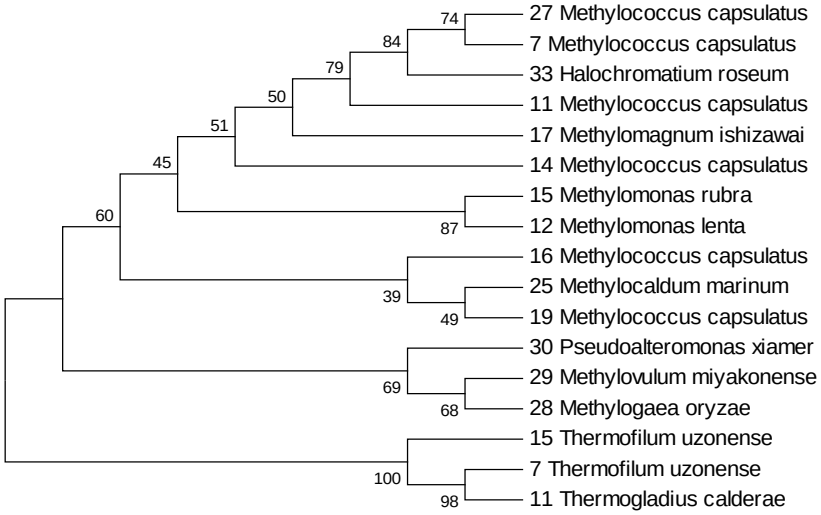

Fig 3. Phylogenetic tree derived from partial 16s rRNA gene inferred using the UPGMA method conducted in MEGA7.

DISCUSSION

Denaturing gradient gel electrophoresis was used to assess the bacterial diversity in the rhizosphere of zinc deficiency tolerant and sensitive genotypes in an attempt to explain zinc deficiency tolerance at the microbial level. This method separates same length DNA fragments of different sequences based on sequence-dependent melting behaviour of the DNA fragments in polyacrylamide gel containing a gradient of denaturant. Each distinct band in the gel represents a unique bacterial species. Using 16s rRNA genes, microbial communities can be dissected at the phylogenetic level (Brons and van Elsas, 2008). This method is particularly useful as an initial step in discriminating among communities (Nakatsu et al., 2000).

The bacteria primers 968F and 1378R amplified a region of the 16S rDNA from bases 968 to 1378 relative to the E. coli rRNA nucleotide sequence. By subjecting 16S rDNA PCR amplicons to DGGE, this study was able to distinguish differences in the microbial profile of A69-1 and K. Patong rhizosphere grown in zinc-sufficient and zinc-deficient soils.

The diversity of bacteria in the soil is an effective and ubiquitous mechanism to solubilize different materials thereby increasing its solubility, mobility and bioavailability (Hayat et al., 2010). This diversity, as supported by the diversity and similarity indices, was observed in the bulk soil of both environments. The number of bands in the rhizosphere is but a subset of the bands in bulk soil as shown in the DGGE profile whereby all bands present in the rhizosphere were also present in the bulk soil. This affirms previous observation that the bulk soil serves as reservoir of microorganisms in the rhizosphere (Berg and Smalla, 2009) and that the microbial populations in the bulk soil determines the rhizosphere community composition (Whalley et al., 2005). Yamamoto et al. (2018) also observed that richness and diversity of bacteria is higher in bulk soil than in the rhizosphere.

Structural and chemical differences were observed between the bulk soil and the rhizosphere soil. Soil in the rhizosphere has increased number of larger pores which may decrease water retention (Whalley et al., 2005). Soil organic matter, available K and P, and pH were observed to be higher in rhizosphere soil (Guo et al., 2015; Yang et al., 2017). This bacterial diversity in the rhizosphere may support the observation of Khoshgoftarmanesh et al. (2018) that plant roots modify the chemical conditions in rhizosphere soil.

This study also shows that not all bacteria in the bulk soil are harbored by the rhizosphere. There seems to be a selection driven by the plant species or genotype itself. Previous studies provide evidence for such selection. The rhizosphere community of chickpea, canola and sudan grass differed, and this difference is also affected by soil type (Marschner et al., 2004). A genotype-specific bacterial population was also shown to exist in soybean plants (Xu et al., 2009). This plant species-specificity shaping the rhizosphere composition may have been brought about by the plant roots exerting selective pressure that influence the microbial composition of the rhizosphere. When artificial root exudates were applied on maize plants, the bacterial density in the rhizosphere increased (Baudoin et al., 2003). Plant roots release exudates that may serve as food for microorganisms.

This relationship between plant species and rhizosphere composition may be extended to the response of plants to stress. As shown in this study, the rhizosphere community of the tolerant rice genotype (A69-1) and the sensitive genotype (KP) is not exactly similar. Several species, as represented by the bands, are common to both genotypes while some are unique to each genotype. This shows that the plant genotype-specificity shaping the rhizosphere is still operative but changes when the plant is under stress. In the absence of stress, the number of species in the tolerant genotype was relatively the same with the sensitive KP. When both genotypes were subjected to stress, the number of microbial population was relatively the same in the tolerant genotype but reduced greatly in the sensitive genotype. The relatively unchanged microbial composition from normal to stress environment may contribute to the zinc efficiency of the tolerant genotype and the reduction of the microbial population in KP maybe a contributing factor to its sensitivity to zinc-deficient soil. The change in the rhizosphere composition during stress maybe explained in terms of root exudates. Previous study on wheat showed that genotypes tolerant to zinc deficiency exudes more phytosiderophore and 2-deoxymuginiec acid than its sensitive counterpart (Rengel et al., 1998). Similarly, rice genotypes tolerant and sensitive to varying levels of chromium stress also showed differences in the amount of oxalic, malic and citric acid exuded (Zeng et al., 2008). The role of root exudates in zinc deficiency tolerance is not related to zinc uptake. Gao et al. (2006) showed that malate exudation of rice genotypes at low zinc does not correlate with zinc uptake. Instead, root exudates in the form of organic acids play a significant role as intermediates for biogeochemical cycling and serve as substrates for microbial metabolism. The amount and composition are specific for each plant species. Moreover, chemicals exuded by the roots also serve as signals that are recognized by the specific bacteria which in turn initiate colonization in the roots (Berg and Smalla, 2009). Since tolerant genotypes release greater amount of root exudates than its sensitive counterpart (Rengel, 1997; Zeng et al., 2008), there would be greater plant-dependent bacterial colonization in the tolerant genotypes.

Plants must sequester zinc as much as it needs in order to adapt to low zinc level. However, only a small fraction of the total zinc in soil is exchangeable or soluble (Barak and Helmke, 1993). Bacteria have been shown to possess zinc solubilization potential either by attacking directly the surface of the mineral or by secreting organic acids, both of which results to solubilization of the zinc salts (Saravanan et al., 2004). Krithica et al (2016) developed zinc solubilizing bacteria inoculants composed of *Enterobacter cloacae* ZSB14 to increase the adsorbed Zn proportions of soil by dissolving the Zn precipitates through organic acid production. Kamran et al (2017) showed the zinc solubilization ability of *Pseudomonas fragi, Pantoea dispersa, Pantoea agglomerans, Enterobacter cloacae,* and *Rhizobium* sp. was investigated. Vaid et al (2014) has shown that rice plants inoculated with suitable combination of Zn solubilizing bacterial strains of *Burkholderia* sp. and *Acinetobacter* sp. were more efficient in acquiring Zn from Zn deficient soil as compared to non-inoculated plants.

Whether the bacteria identified in this study possess zinc solubilizing potential is beyond the scope of this study. However, the role of these bacteria is worth mentioning. *Methylococcus capsulatus, Methylomonas lenta, Methylomonas rubra, Methylomagnum ishizawai, Methylocaldum marinum, Methylogaea oryzae,* and *Methylovulum miyakonense* are methanotrophic bacteria belonging to the family Methylococcaceae. Methane oxidizing bacteria or methanotrophs are capable of utilizing methane as carbon and energy source. Methane is oxidized via methanol to formaldehyde which in turn is assimilated by the cell or further oxidized to formate and CO_2 for further energy production (Ward et al., 2004). The process employs methane monooxygenase which contains three binding sites for copper and zinc (Leiberman and Rosenzweig, 2005). Several methanotrophs are observed in rhizosphere and bulk soil in rice (Lee et al., 2015). *Halochromatium roseum* is a sulphur bacterium capable of reducing sulfate (Kumar et al., 2007). Sulphate reducing bacteria obtain their energy by oxidizing organic compounds or H_2 while reducing sulphate to hydrogen

sulphide. Azabou et al. (2005) demonstrated that sulphate reducing bacteria are capable of removing zinc from phosphogypsum containing zinc and sulphate. Wind et al. (1999) observed several sulphate reducing bacteria in rice roots. *Pseudoalteromonas xiamenensis*, found in this study as one of the rhizosphere bacteria, is a marine bacterium with unknown function yet in plants (Zhao et al, 2014).

The differences in bacterial diversity in the rhizosphere of tolerant and sensitive varieties may have increased the solubility and availability of zinc thereby making it an effective mechanism for stress tolerance

CONCLUSION

The presence of relatively greater number of microbial population consisting largely of methanotrophs in the rhizosphere of A69-1 might contribute to its tolerance to zinc deficiency soil. Conversely, the sensitivity of KP to zinc-deficiency might be attributed to the relatively reduced microbial population.

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