DETERMINATION OF ENDOPHYTIC BACTERIA COMPOSITION OF RICE SEED USING DGGE

C. N. Ngamau[1], M. Hunja[2], A. Tani[3]
[1,2]Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya
[3]Institute of Plant Science and Resources, Okayama University, Kurashiki, Okayama, Japan
E-mail: cngamau@rpe.jkuat.ac.ke

Abstract
Endophytic bacteria have a potential role in promoting plant growth and suppressing disease pathogens in a cost effective and environmentally friendly manner. This study was therefore conducted with the aim of determining the endophytic bacteria composition of rice seeds collected in Kenya in view of their agronomic importance. Different varieties of rice seeds were collected from Bunyala, Hola, Kaloleni, Mwea, Msabweni, and Taveta in Kenya. Rice seeds were surface-sterilized and bacterial DNA isolated. Partial 16SrRNA gene was amplified using Denaturing Gradient Gel Electrophoresis (DGGE) primers. Endophytic bacterial gene fragments from five samples of rice seed varieties were successfully amplified. Amplified DNA was then subjected to DGGE and a total of 41 DNA bands were excised from the gel. The excised DNA bands were then re-amplified and 31 out of the 41 PCR products obtained were sequenced for endophytic bacteria identification. Bacteria strains were identified as *Methylobacteriaceae*, *Sphingomonadaceae*, *Enterobacteriaceae*, *Pseudomonadaceae, Flavobacteriaceae* and *Rhizobiaceae*. *Methylobacterium* species, for example, are very important organisms, which have been shown to stimulate seed germination and plant development, perhaps through production of phytohormones. The next stage of this study will be to isolate such specific bacterial strains and determine their agronomic importance on rice production in Kenya.

Key words: Denaturing Gradient Gel Electrophoresis (DGGE), endophytic bacteria, rice

1.0 **Introduction**

Rice (*Oryza sativa*) is the third most important staple food after maize and wheat in Kenya, forming part of the larger diet for urban population (EPZA, 2005; MoA, 2009). About 80% of the rice in Kenya is grown under irrigation in paddy schemes managed by the National Irrigation Board (NIB) while the remaining 20% is rain fed (MoA, 2009). In 2009, Kenya's rice production was estimated to be in the range of 45,000 and 80,000 tons per annum, against an annual consumption of 300,000 tons. The deficit is met through imports, which were valued at KSH 7 billion in 2008. The annual rice consumption in Kenya is increasing at a rate of 12% as compared to 4% and 1% for wheat and maize respectively. This can be attributed to progressive change in eating habits. With such a rising consumption of rice in Kenya and indeed worldwide, rice yield must be increased. However, increased crop production results to higher production cost and environment degradation because of excessive use of chemical fertilizers and other inputs, which is not sustainable. In Kenya, the high cost of chemical fertilizers is also a challenge for the resource poor farmers. Sustainable production of rice will hence mean increasing the rice yield without the mass use of chemical fertilizers and pesticides. For this reason, scientists are consistently exploring biological alternatives, which are cost effective and environment friendly. Some of these biological alternatives are the endophytic bacteria, which have beneficial characteristics to the cultivation of plants (Long *et al*., 2008). Endophytic Bacteria are present in most plant species, residing latently or actively colonizing plant tissues without causing any apparent disease symptoms (Hallmann *et al*., 1997; Tan and Zou, 2001).

Traditional plating techniques (culture-based methods) usually result in underestimation of bacterial diversity; for example, only less than 10% of the total bacterial community in soil is detected (Torsvik *et al*., 1990). Therefore, molecular fingerprinting techniques independent of culturing and based on small-subunit (SSU) rRNA genes (rDNA) have become popular in addressing the problems of diversity, structural composition and dynamics of microbial communities (Mano and Morisaki, 2008). Denaturing gradient gel electrophoresis (DGGE) is a molecular fingerprinting method that separates polymerase chain reaction (PCR)-generated DNA products (http://www.eeescience.utoledo.edu/faculty/sigler/research/protocols/dgge/dgge.pdf).

The polymerase chain reaction of environmental DNA can generate templates of differing DNA sequence that represent many of the dominant microbial organisms. However, since PCR products from a given reaction are of similar size (bp), conventional separation by agarose gel electrophoresis results only in a single DNA band that is largely non-descriptive. DGGE can overcome this limitation by separating PCR products based on sequence differences that results in differential denaturing characteristics of the DNA. During DGGE, PCR products encounter increasing concentrations of chemical denaturant as they migrate through a polyacrylamide gel. Upon reaching a threshold denaturant concentration, the weaker melting domains of the double-stranded PCR product will begin to denature at which time migration slows dramatically. Differing sequences of DNA (from different bacteria) will denature at different denaturant concentrations resulting in a pattern of bands. Each band theoretically representing a different bacterial population present in the community.

2.0 Materials and Methods

2.1 Bacteria DNA Isolation

Different varieties of rice seeds were collected from various growing regions of Kenya courtesy of the National Irrigation Board and farmers of Kaloleni and Hola. About 10 rice seeds per variety were put into 50 ml tube and washed with sterile MilliQ water (vortex and decantation). The seeds were then surface-sterilized with 5-ml sterilization solution (2.5 ml 10% Sodium hypochlorite and 2.5 µl Tween 20, made up to 50 ml with sterile MilliQ water). This was done twice with gentle vortex for about 1 min each time. The seeds were again washed with sterile MilliQ water (vortex and decantation, 4 times). After that, the seeds were put in sterile Petri dishes and dried in clean bench. Dehusked seeds and husks were put in different tubes. One ml wash solution (1.64 g/L $Na_2HPO_4.7H_2O$, 0.28g/L $NaH_2PO_4.2H_2O$, 8 g/L NaCl, 0.05% Tween 80 for making up to 100 ml) was then added and vortex vigorously for 30 sec. As much as possible, the supernatant (regarded as bacterial suspension) was taken with a pipette into a new tube. The supernatant was then centrifuged at 15,000 rpm at 4°C for 10 min. The supernatant was then discarded and 100 µl of DNA extraction solution (0.2 ml 1 M Tris-HCl (pH8.0), 0.1 ml 500 mM EDTA (pH8.0), 1.33 ml 3 M NaCl, 3.03 ml 10% SDS, 2 mg proteinase K, made up to 10 ml with sterile MilliQ water and filter-sterilized with 0.45-um filter in clean bench) added. This was then followed by incubation at 55°C for one hour.

2.2 16S rRNA Gene Amplification

Partial 16SrRNA gene amplification was done using Denaturing Gradient Gel Electrophoresis (DGGE) primers, namely 16S-DGGE-338-F CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAG and 16S-DGGE-r517-534 ATTACCGCGGCTGCTGG. The PCR mixture was composed of; 25 µl 2 x AmpDirect Plus, 0.25 µl NovaTaq ™ Hot Start DNApolymerase, 2.5 µl of each primer (12.5 pmol/µl), 18.75 µl sterile MilliQ water and 2 µl sample DNA. Gel electrophoresis was done using 2% gel and 0.5 µg/µl GeneRuler 100 bp DNA ladder Plus marker.

2.3 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE gel was made by combing two solutions containing acrylamide (structural material) and differing percentages of denaturants (urea and formamide) to form a gradient of denaturant in which double stranded DNA fragments of differing sequence are denatured during electrophoresis. Lambda EcoT14I and GeneRuler 100 bp DNA ladder Plus were used as markers. After electrophoresis, the gel was soaked with 50 ml of 1xSYBR Green/milliQ water and stained for 30 min. The staining solution was then discarded and gel put onto UV illuminator to reveal the different DNA bands. The bands were then excised from the gel using a sterile blade. Each gel slice was put into a clean tube and washed with sterile MilliQ water. Every gel slice was then stored in 50 µl of TE buffer at -20°C.

2.4 Re-amplification of the Bands

The gel slice was crushed in the tube with a pipette tip. One µl of the solution was then used as PCR template. The reaction mixture for the PCR was as following; 0.1 µl Ex Taq DNA polymerase, 2 µl dNTP, 2.5 µl Ex Taq DNA polymerase buffer, 1 µl F338 and R517 primers (10 pmol/µl) and 17.4 µl sterile MilliQ water. Gel electrophoresis was done using 2% gel and 0.5 µg/µl GeneRuler 100bp DNA ladder Plus marker.

2.5 DNA Sequencing

The ca 200-bp rDNA amplicon were subjected to DNA sequencing using 16S-DGGE-338-F and 16S-DGGE-r517-534 ATTACCGCGGCTGCTGG primers. The reaction mixture composed of 1.5 µl BigDye Ready Reaction Mix, 2.5 µl sequencing buffer (5x), 2 µl of 0.9 pmol/µl DGGE primers (16S-DGGE-338-F and 16S-DGGE-r517-534 [separately]), 3.5 µl sterile MilliQ water and 0.5 µl template DNA (Mag Extractor - purified PCR product).

2.6 Processing and Analyzing of DNA Sequences

Obtained sequences were processed using the Staden Package software and BIOEDIT sequence alignment editor. Assembled sequences were analyzed at the Ribosomal Database Project site (http://rdp.cme.msu.edu/). Phylogenetic analysis was done using the Molecular Evolutionary Genetics Analysis (MEGA) software (http://www.megasoftware.net/).

3.0 Results

3.1 16SrRNA Gene Amplification and Electrophoresis

Endophytic bacterial gene fragments from five samples of rice seed varieties were successfully amplified (Figure 1). Amplified DNA was then subjected to DGGE and a total of 41 DNA bands were excised from the gel (Figure 2).

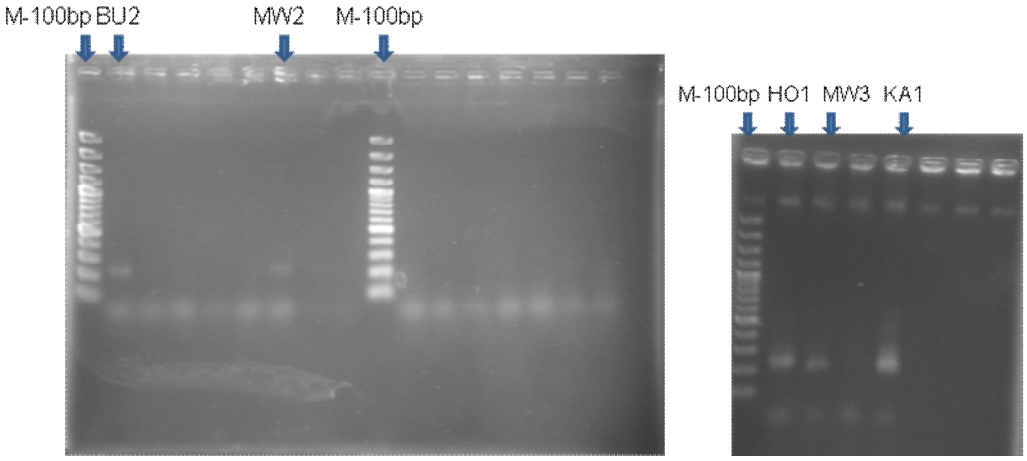

Figure 1: Amplified endophytic bacterial gene fragments from five rice seed varieties. A total of 18 rice seed varieties were processed but only five rice seed varieties yielded visible gene fragments

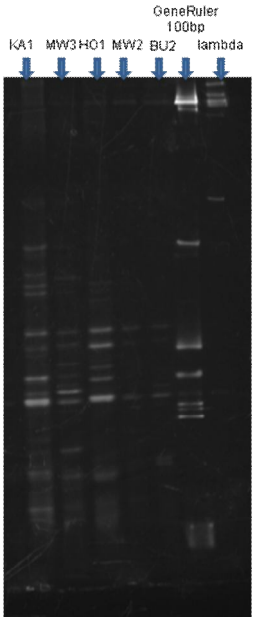

Figure 2: DNA bands of five rice seed varieties after DGGE

3.2 DNA Re-amplification and Sequencing

The excised DNA bands were re-amplified and 31 out of the 41 PCR products obtained were sequenced for endophytic bacteria identification. Table 1 below summarizes the probable endophytic bacteria composition of the five rice seed varieties examined.

Table 1: Probable endophytic bacteria composition of five rice seed varieties determined through DGGE

Variety code	Rice variety	Source	Probable endophytic bacteria composition
KA1	Sigae nyeupe 1	Kaloleni	*Enterobacteriaceae "Enterobacter", Enterobacteriaceae "Pantoea", & Sphingomonadaceae "Sphingomonas".*
MW3	2793 - 80-1	Mwea	*Enterobacteriaceae "Pantoea", Pseudomonadaceae "Pseudomonas", Sphingomonadaceae "Sphingomonas" Rhizobiaceae "Rhizobium" & Flavobacteriaceae "Chryseobacterium".*
HO1	Sindano bahari	Hola	*Enterobacteriace "Enterobacter" & Pseudomonadaceae "Pseudomonas".*
MW2	NERICA 4	Mwea	*Pseudomonadaceae "Pseudomonas".*
BU2	ITA 310	Bunyala	*Methylobacteriaceae "Methylobacterium", Sphingomonadaceae "Sphingomonas" & Pseudomonadaceae "Pseudomonas".*

4.0 Discussion and Conclusions

The putative endophytic bacteria detected in the five rice seed varieties examined (Table 1) match with some of those that other researchers have found inside rice plants (Hironobu and Morisaki, 2008). *Pantoea, Pseudomonas, Enterobacter, Sphingomonas* and *Rhizobium* are among the nitrogen-fixing endophytes that have been isolated from rice plants by use of nitrogen-free medium. On the other hand, *Methylobacterium* have been shown to stimulate seed germination and plant development, perhaps through production of phytohormones and in particular cytokinin (Holland *et al*., 2002). There is therefore

potential in exploration of the above endophytes for use as biological fertilizers in the production of rice in Kenya.

As expected varietal differences exhibited endophytic bacterial diversity with MW3 having the largest diversity and MW2 the least. Both MW3 and MW2 were sourced from the same geographical region indicating that endophytic bacterial diversity is more host dependent than geographical region dependent. The data available is however not sufficient to support this conclusion. Out of 18 rice seed varieties processed for endophytic bacteria DNA isolation and amplification only 5 yielded visible gene fragments. Further research is therefore recommended in the DNA isolation and amplification in order to optimize on gene fragment yield.

In conclusion, the study achieved its overall object of determining endophytic bacteria composition of rice seeds using a culture-indipendent method namely DGGE. The next level of this study will be to isolate specific bacterial strains and determine their agronomic importance on rice production in Kenya.

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