THE INTERNATIONAL JOURNAL OF SCIENCE & TECHNOLEDGE

Evaluation of Nitrogen Fixation Ability of Endophytic Bacteria in Kenyan Bananas (*Musa Spp*.) Using Biochemical and Molecular Techniques

Shellemiah Otieno Ouma
M. Sc. Student, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya
Esther Nkirote Magiri
Senior Lecturer, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya
Viviene N. Matiru
Senior Lecturer, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya
Julius Mugweru
Lecturer, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya
Catherine N. Ngamau
Lecturer, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya

Abstract:
Nitrogen is a major nutrient element required by crops and its scarcity in soil adversely affects crop yield. Application of inorganic nitrogenous fertilizers is costly, causes environmental pollution and leads to poor quality and unsustainable banana yield. Bananas are staple food to a substantial population in the world and harbor endophytic bacteria. It is believed that endophytic bacteria, along with rhizospheric bacteria contribute to plant growth. Biochemical characterization the banana endophytic bacteria isolated on a nitrogen free media, quantification of the amount of nitrogen each can fix and molecular analysis of the nifH gene were the objectives of this study, with the view of developing biofertilizers to address the challenges attributed to the use of inorganic fertilizers. Eighteen isolates were obtained from previous research that focused on isolation and molecular identification of the isolates by partial analysis of 16S rRNA gene. The isolates were subjected to morphological tests, biochemical assays for characterization, determination of quantities of nitrogen fixed by the bacteria through Acetylene Reduction Assay (ARA). Bergey's Manual of Determinative Bacteriology was used to identify the isolates based on biochemical assay results. For the molecular analysis, nested PCR approach was adopted to amplify nifH gene. All the isolates showed the ability to fix nitrogen with varying quantities ranging from 0.244µl/ml to 7.03µl/ml. BLAST search yielded high scoring hits of nifH gene sequence in isolate no 5 identified as Raoultella terrigena. However, BLAST could not identify any annotated gene sequences for isolates 4, 15, 21, 27, and 29 of the sequenced isolates. Detection of nifH gene in Raoultella terrigena, quantification of nitrogen fixed by each isolate and the isolates' identities were the key findings this study. These findings are important in banana endophytes research that can lead to their exploitation for sustainable banana production and ecofriendly biofertilizers.

Keywords: *Bananas, Endophytic bacteria, nested PCR, nifH, nitrogenase reductase, biological nitrogen fixation, biofertilizers*

1. Introduction

Bananas are tropical plants of the genus *Musa* and have their origin in South East Asia, specifically in Malaysia Hannelore, (2005). They are high in carbohydrates hence important food crops to the human population in the world. The total nitrogen requirement for banana crop cultivation, just like for any other crop, is high because it is required for the production of chlorophyll and nucleic acid synthesis (Walter, 2003; Peter and Morot-Gaudry, 2001). In Kenya, banana crops are grown in regions with nitrogen deficient soils that are unable to meet the required demand (Esendugue and Chidebelu, 2011) thus requiring supplementation.

Nitrogen fertilization of non-leguminous crops is an expensive input in agriculture especially to poor farmers depending on the cultivation of bananas for subsistence and commercial purposes who cannot afford chemical fertilizers. Besides, excessive and imbalanced use of nitrogenous chemical fertilizer over long periods of time could contribute to adverse effects, such as greenhouse emission of nitrous oxide gas (N_2O), nitrate contamination of underground water and growth of non-target weeds. The possibility of meeting nitrogen requirements through biologically fixed nitrogen provides an ecofriendly and sustainable alternative (Verma *et al*., 2001). This important process is carried out by endophytes.

Endophytes are "microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects" (Bacon and White, 2000). In the 1970's, endophytes were initially considered neutral, not causing benefits nor showing detriment to plants. They may benefit host plants by preventing pathogenic organisms from colonizing their tissues Berg *et al.*, (2005). These organisms are increasingly gaining scientific and commercial interest because of their potential to improve plant quality and growth and their close association with internal tissues of host plant (Carroll, 1992; Schulz *et al.*, 1999). Endophytes can enhance

plant growth in non-leguminous crops and improve their nutrition through nitrogen fixation, phosphate solubilization or iron chelation. These abilities makes endophytes to be of agronomic interest (siderophores production) (Dobereiner and Baldani, 1998; Sturz *et al.,* 2000; Boddey *et al.,* 2003; Iniguez *et al.,* 2004; Ryan *et al.,* 2008; Uribe *et al.,* 2010). These organisms have been exploited for development and production of biofertilizers.

Biofertilizer is a medium containing living microorganisms that can colonize the rhizosphere or the interior of the plant and promote growth when applied to plant surfaces, seed or soil (Vessey, 2003). They increase the supply or availability of primary nutrients to the host plant hence promoting plant growth. Nitrogen fixation, phosphorus solubilization and stimulation of plant growth are natural processes through which biofertilizers add nutrients to plants. These microorganisms restore soils nutrient cycle, build the organic matter, enrich soil fertility and fulfil the plant nutrient requirement by supplying the organic nutrients (Mahdi *et al.,* 2010).

In biogeochemical nitrogen cycle, nitrogen is reduced to yield two ammonia molecules which are then converted into usable forms of nitrogen. Industrial processes for the manufacturer of inorganic nitrogenous fertilizers e.g. Herber Bosch process require metal Iron (Fe) based catalyst, H_2 and high pressures and temperature. Biological systems unlike industrial processes utilizes nitrogenase as the catalyst for nitrogen reduction to ammonia and requires energy input in the form of ATP at room temperature and atmosphere pressure. Nitrogenase is composed of Fe (dinitrogenase) and Mo-Fe protein (dinitrogenase reductase), which is encoded by *nif* gene. The nitrogenase reductase is composed of multiple subunits encoded by the genes *nif*H, *nif*D, and *nif*K. According to Rubio and Ludden 2002, *nif*H is the marker gene for studying the phylogeny, diversity, and abundance of nitrogen-fixing microorganisms.

The iron protein genes *nif*H, anfH and vnfH are highly conserved among diverse microorganism and the *nif*H phylogenetic tree largely resembles the 16S rRNA phylogenetic tree (Hennecke *et al.,* 1985; Normand and Bousquet, 1989; Young, 1992). Thus, many PCR primers have been developed in various studies to target the *nif*H gene (Gaby and Buckley, 2012).

Many studies, in recent years have addressed the importance and contribution of biological nitrogen fixation in ecologically unique terrestrial and aquatic habitats by focusing on the diversity of *nif*H sequences (Zehr *et al.,* 2003). Such studies have provided a rapidly expanding database of *nif*H sequences and revealed a wide diversity of uncultured diazotrophs (Tan *et al.,* 2003). In this study, PCR primers for nitrogen-fixation genes developed by Widmer *et al.,* (1999) were used. The study was aimed at characterizing the Kenyan banana bacterial endophytes using biochemical and molecular techniques with the view of evaluating their potential for development of ecofriendly biofertlizers for sustainable banana production to address challenges of food security in line with Millennium development goals.

2. Materials and Methods

2.1. Study Site

The study was carried out in the Molecular Biology and Microbiology Laboratories at the Departments of Biochemistry and Food Science respectively of Jomo Kenyatta University of Agriculture and Technology.

2.2 . Culturing Banana Endophyte Isolates

A laboratory experimental study was done on eighteen (18) isolates obtained from the previous study by Ngamau *et al.,* (2012). The bacterial isolates were utilized for morphological, biochemical and molecular analysis of *nif*H gene. The 18 endophyte isolates were obtained and cultured on solid N-free medium (0.5g/L K_2PO4, 0.2g/L NaCl, 0.2g/L $MgSO_4$, 6.6g/L $NaMoO_4(H_2O)$, 15mg/L $FeCl_3$, 15g/L agar) in replicate and incubated for 24-48 hours at 30˚C to allow for endophyte growth. Individual colonies were picked and purified by streaking on fresh media to generate pure cultures that were used to perform biochemical tests (Cappuccino and Sherman, 2002) and molecular analysis of *nif*H gene. The isolates were coded 3, 4, 5, 6, 7, 14, 15, 16, 18, 19, 21, 27, 29, 30, 31, 37, 39 and 41 as assigned by Ngamau *et al.,* (2012).

2.3. Morphological and Biochemical Characterization of Endophyte Isolates

Gram staining method was used to determine the morphological characteristics of the bacterial cells as described by Cappuccino and Sherman, (2002). The general biochemical properties of the isolates were determined using the following assays: - Urease test, Nitrate reduction test, Citrate utilization test, Catalase test, Methyl Red-Voges-Proskauer test (MR-VP), Indole production test, Hydrogen sulphide test, Pectinase assay as described by Cappuccino and Sherman, (2002).

2.4. Determination of Nitrogen Fixation Ability of Endophyte Isolates

The nitrogen-fixing ability of each isolate was tested using the Acetylene Reduction Assay (ARA) as described by Eckert *et al.,* (2001). Each bacterial isolate was inoculated into 10 ml vials containing 5 ml nitrogen free media incubated at 28°C in the dark. After 12 h, the air phase was replaced with 12% v/v acetylene and the vials were re-incubated for a total of 36 hours. The amount of ethylene produced was determined as described by Rogel *et al.,* (2001) using a Shimadzu Gas Chromatograph (GC-9A, Japan) equipped with a flame-ionization detector and a packed column of activated alumina (2.0 m×2.0 mm internal diameter, stainless steel, packed with GDX-502). The initial and final column temperatures were both at 120^{o}C. The injection column temperature was at 220^{o}C while the detection temperature was at 240^{o}C. The velocity of flow of N_2, H_2 and dried air were 30, 30 and 300 ml min^{-1} respectively. Nitrogen and hydrogen were the mobile gas and the heating gas respectively. The actual concentration of ethylene was determined based on a standard curve of ethylene concentration (µmol C_2H_2) and the peak area. The nitrogen fixation activity (µl C_2H_2) was defined from the ethylene concentration recorded (µl C_2H_2).

2.5. PCR Analysis of nifH Gene in Banana Endophyte Isolates

Genomic DNA was extracted from 24 hour old bacterial cultures using Bioline ISOLATE II Genomic DNA Kit according to the manufacturer's instructions. The isolated DNA from the eighteen (18) isolates were qualified and quantified using gel electrophoresis and spectrophotometric methods.

A nested PCR approach was carried out to detect the presence of *nif*H gene in the isolated genomic DNA. Universal and nested *nif*H primer sets were used in the first and second PCR respectively. These primer sets were originally designed and used by Widmer *et al.*, (1999). The first PCR was performed with the universal forward primer *nif*H(forA) GCIWTITAYGGNAARGGNGG and Reverse primer *nif*H(rev) GCRTAIABNGCCATCATYTC. Bioline PCR kit was used and their instructions were followed. The Thermocycler condition were as follows: - Initial denaturation at 94^{o}C for 5 minutes, denaturation at 94^{o}C for 1 minute, annealing at 50^{o}C for 1 minute, extension at 72^{o}C for 4 minutes and final extension at 72^{o}C for 10 minutes for 35 cycles.

The second (nested) PCR was performed with the forward primer *nif*H(forB) GGITGTGAYCCNAAVGCNGA and the same reverse primer that was used for the first reaction. Bioline PCR kit and instructions were used to perform nested PCR using 1µl of the first amplicon as template in 25µl volume. Thermocycler conditions for the second (nested) PCR were: - Initial denaturation 94^{o}C for 5 minutes, denaturation at 94^{o}C for 30 seconds, annealing at 56^{o}C for 30 seconds, extension at 72^{o}C for 1 minute, final extension (72^{o}C) for 7 minutes for 35 cycles. PCR products were analyzed on a 1.5% agarose gel at 100V in 1X TAE buffer for 35 minutes. The bands were visualized by Ethidium bromide staining Sambrook *et al*., (1989). The nested PCR products were purified using the Biolone PCR purification Kit protocol according to manufacturer's instructions, separated on a 1.5% (w/v) agarose gel at 100V in 1X TAE buffer for 35 minutes and visualized by Ethidium bromide staining (Sambrook *et al*., 1989). The purified products of thirteen (13) DNA samples were sequenced at Macrogen Inc., Seoul, Korea.

2.6. Sequencing of the Amplified nifH Genes

The purified PCR products of the thirteen (13) DNA samples were sequenced at Macrogen Inc., Seoul, Korea using Sanger method with the BigDye terminator v3.1 sequencing kit and a 3730xl automated sequencer (Applied Biosystem Sanger-s, Foster City, CA). Nucleotide sequences were determined on both strands of PCR amplification.

2.7. Data Analysis

Bergey's Manual of Determinative Bacteriology was used to identify the isolates based on morphology and biochemical assay results. Microsoft Excel was used to plot a standard curve to determine ethylene concentration. Regression line was fixed on the standard curve and used to show the amounts of ethylene gas produced by each isolate. NCBI database BLAST N search was performed to determine the homology of the isolates sequences and to retrieve *nif*H gene sequence available in the database. Pairwise sequence alignment was performed using EMBOSS Matcher, an online bioinformatics tool for the selected *nif*H gene sequence verses the isolate no. 5 sequence.

3. Results

3.1. Morphological and Biochemical Characterization of Bacteria Endophyte Isolates

Morphologically, sixteen isolates were gram negative rods while two (2) were gram positive rods as shown in table 1. All isolates grew on nitrogen free media and tested positive for ARA, catalase and citrate utilization. On the other hand, they all tested negative for hydrogen sulfide production tests. It was further noted that eight (8), ten (10), eight (8), ten (10) and two (2) isolates were positive for Urease, Citrate utilization, Nitrate reduction, Methyl Red (MR), Veges-Proskauer (VP) and Indole production tests respectively. The isolates were identified as shown in table 1.

Isolate	ARA	Nitrogen free media	Catalase test	Gram staining	Morphology	Urease test	Citrate test	Nitrate reduction test	MR-VP		Hydrogen sulfide test	Indole production	Isolate identity
									MR	VP			
3	+	+	+	-	Rods	+	+	-	-	-	-	-	*Pseudomonas spp.*
4	+	+	+	-	Rods	+	+	-	-	-	-	-	*Pseudomonas spp*
5	+	+	+	-	Rods	+	+	-	+	+	-	-	*Raoultella spp.*
6	+	+	+	-	Rods	-	+	+	+	-	-	+	*Enterobacter spp*
7	+	+	+	-	Rods	-	+	+	-	+	-	-	*Rahnella spp.*
14	+	+	+	+	Rods	-	+	+	-	+	-	-	*Bacillus spp.*
15	+	+	+	-	Rods	-	+	+	-	+	-	-	*Serratia spp.*
16	+	+	+	-	Rods	+	+	-	-	-	-	-	*Pseudomonas spp*
18	+	+	+	-	Rods	-	+	+	+	+	-	-	*Serratia spp.*
19	+	+	+	-	Rods	-	+	+	+	+	-	-	*Serratia spp.*
21	+	+	+	-	Rods	+	+	-	-	-	-	-	*Pseudomonas spp*
27	+	+	+	-	Rods	+	+	-	-	-	-	-	*Flavimonas spp.*
29	+	+	+	-	Rods	+	+	-	+	+	-	-	*Raoultella spp.*
30	+	+	+	+	Rods	-	+	+	-	+	-	-	*Bacillus spp.*

31	+	+	+	-	Rods	-	+	+	+	-	-	+	*Enterobacter spp.*
37	+	+	+	-	Rods	+	+	-	-	-	-	-	*Pseudomonas spp*
39	+	+	+	-	Rods	-	+	+	+	+	-	-	*Ewingella spp.*
41	+	+	+	-	Rods	-	+	+	+	+	-	-	*Serratia spp.*

Table 1: Summary of results on morphological, biochemical characterization and identities of the isolates

3.2. Acetylene Reduction Assay Quantification for Nitrogen Fixation

Shimadzu gas chromatography showed a retention time and area under the curve ranging from 1.35-1.38 and 1211-34833 respectively in all the eighteen (18) bacterial isolates as shown in table 2. The observed retention time of 99.5% standard ethylene gas on Shimadzu gas chromatography was 1.34 minutes and the area under the curve was 1586.52. Various concentrations of the standard ethylene gas yielded different areas under the curve as shown in figure 1. The positive results are an indication of the presence of nitrogenase reductase enzyme. The quantities of nitrogen fixed varied from one isolate to the other ranging from 0.244µl/ml to 7.03µl/ml being the lowest and highest respectively. Isolate 31 and 3 identified as *Enterobacter spp.* and Pseudomonas *spp.* produced the lowest and highest concentrations of ethylene respectively. Isolate 5 identified *Raoultella spp.* fixed 0.447336µl/ml of nitrogen. A line of regression fitted in the plot was used to determine the concentrations of ethylene produced from the reduction of acetylene gas ranging from 0.244681-7.037965 µl/ml by all the isolates as shown in table 2.

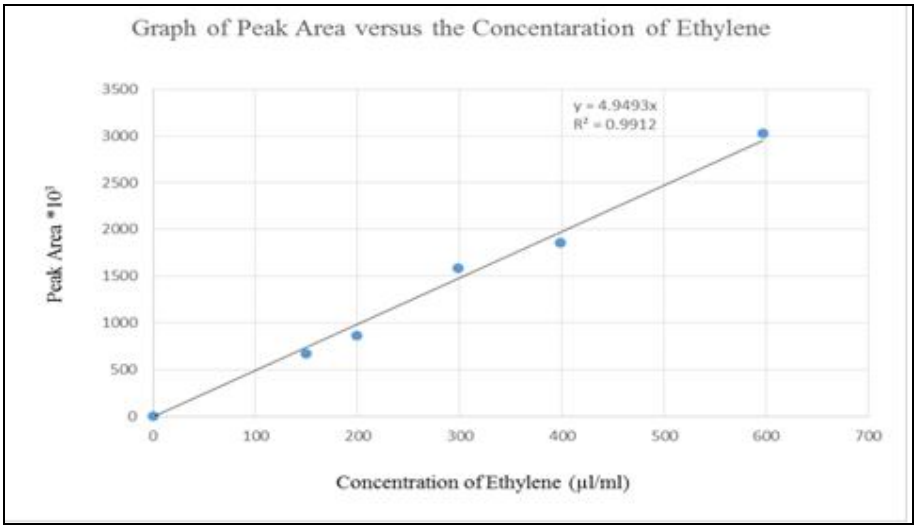

Figure 1: Graph of Peak Area versus the Concentrations of Ethylene standard as output from Shimadzu Gas Chromatograph.

Gas chromatography				
Isolate no.	**Ethylene gas detected**			
	Test Results	**Retention time**	**Area**	**Ethylene produced (µl/ml)**
3	+	1.367	34833	7.037965
4	+	1.357	1777	0.359041
5	+	1.353	2214	0.447336
6	+	1.338	1957	0.395409
7	+	1.365	1551	0.313378
14	+	1.355	1757	0.355
15	+	1.373	1809	0.365506
16	+	1.38	2834	0.572606
18	+	1.372	2538	0.5128
19	+	1.38	2062	0.416625
21	+	1.375	1952	0.394399
27	+	1.38	1400	0.282868
29	+	1.367	2823	0.570384
30	+	1.358	1556	0.314388
31	+	1.365	1211	0.244681
37	+	1.36	2231	0.450771
39	+	1.36	1672	0.337826
41	+	1.367	2458	0.496636

Table 2: Results for Gas Chromatography of the isolate showing ethylene gas detected, retention time, and peak areas under the curves of the ethylene gas produced and volume of ethylene gas produced.

3.3. Molecular Detection of the nifH Gene from the Banana Endophyte Isolates

Ultra-pure agarose gel analysis of the first PCR products showed bands from isolates no. 3, 4, 5, 7, 14, 15, 16, 18, 19, 21, 27, 27, 30, 31, 39 and 41 as shown in figure 2. Some of the bands were clear and distinct. Negative control (water) and isolate no. 37 did not show any bands. DNA ladder approximated the bands to be ranging from 100bp – 900bp as shown in figure 2.

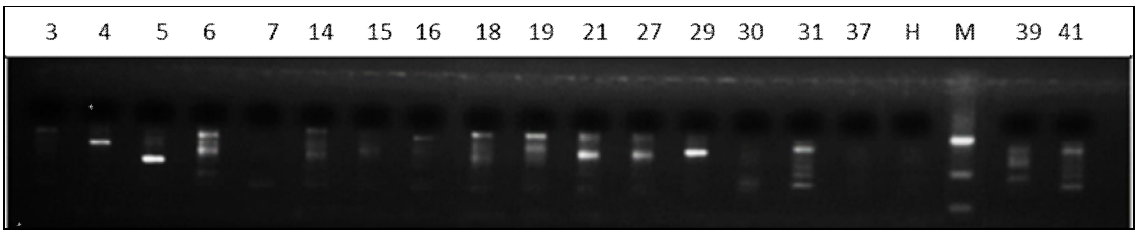

Figure 2: Gel image showing the PCR products from the amplification using universal primers of eighteen (18) isolates 3, 4, 5, 6, 7, 14, 15, 18, 19, 21, 27, 29, 30, 31, 37, 39 and 41, H - negative control (H_2O) and 1000bp marker run in 1.5% (w/v) agarose gel.

Agarose gel analysis of the second (nested) PCR showed distinct and clear bands of approximately 700bp in 6/18 samples. Multiple bands with background smears were observed in some wells while in others bands were absent including the negative control as shown in Figure 3.

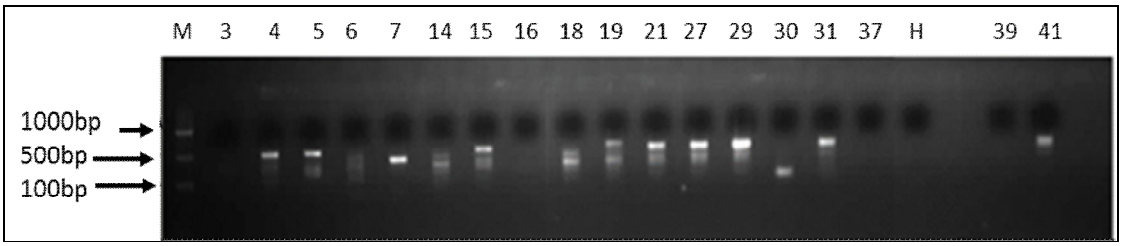

Figure 3: Second (nested) PCR products of eighteen (18) isolates 3, 4, 5, 6, 7, 14, 15, 18, 19, 21, 27, 29, 30, 31, 37, 39 and 41, H - negative control (H_2O) and M - 1000bp marker run in 1.5% (w/v) agarose gel.

Sequences were obtained from six (6) out of the thirteen (13) isolates which showed bands from the second (nested) PCR as shown in figure 2. Isolate no. 7, 14, 18, 19, 30, 31 and 41 could not be sequenced because of the multiplicity of bands.

3.4. Analysis of Sequencing Products

NCBI database BLAST N search showed that isolate no.5 sequence is homologous to *Raoultella terrigena* strain DR-E5 nitrogenase iron protein (*nif*H) gene, partial coding sequence and its sequence is similar to that of *nif*H gene sequence available in the NCBI database with a similarity/identity values of 98.4%. However, it could not identify any annotated *nif*H gene sequences for isolates 4, 15, 21, 27, and 29. Pairwise sequence alignment using EMBOSS Matcher, an online bioinformatics tool also showed a very high percent similarity/identity of 98.4% between the sequence of isolate no. 5 and the *nif*H sequence retrieved from NCBI database. However, four bases showed a mismatch between the two sequences (positions 153, 300, 310, 311 and 313 on JX080198.1 from the NCBI database).

4. Discussion

The Eight (8) isolates 3, 4, 5, 16, 21, 27, 29 and 37 tested positive for urease test. This showed their ability to break down urea, to ammonium (NH_4^+) which can be readily absorbed by the plants to promote growth. This is an important aspect in growth and development of bananas in the case where fertilizers are applied, as the bacteria have shown potential to convert urea to simpler forms. This phenomenon has been observed in studies on rice endophytes such as Tan *et al.,* (2001).

Nitrate reduction test showed the ability of ten (10) banana isolates to reduce nitrates into simpler forms such as nitrogen gas. Eight (8) isolates 3, 4, 5, 16, 21, 27, 29 and 37 tested negative for nitrate reduction. This is a critical feature in banana production as it will give time for the plants to utilize readily available nitrate before it can be converted to free nitrogen gas by other denitrifying bacteria that could be present in the host plant. All the isolates that tested positive for urease test showed negative results for nitrate reduction test and vice versa.

All the isolates were Catalase positive. This is an important aspect required by the bacteria to reproduce avoiding cellular toxicity. Some bacteria contain flavoproteins that reduce oxygen resulting in the production of hydrogen peroxide and superoxide, which are extremely toxic to the cell. This is because superoxides are powerful oxidizing agents and can destroy cellular components very rapidly (Cappuccino and Sherman, 2002). This means they possess the capability to protect themselves from this toxic effect.

Acetylene reduction assay was done in order to find out whether the isolated banana endophytic bacteria have the potential of fixing atmospheric nitrogen. Organisms with this ability possess nitrogenase enzyme that can reduce nitrogen to produce ammonia (Cappucino and Sherman, 2002). Nitrogenase reductase catalyses the reduction of a variety of substrates e.g. nitrogen and acetylene. Acetylene reduction to ethylene has been widely used as a method to of measuring nitrogenase activity in natural samples, isolates and cell free extracts Cappuccino and Sherman, (2002). The activity of nitrogenase was measured *in vivo* using Acetylene Reduction Assay. The isolates showed the ability to grow on nitrogen free media. The assay results showed that isolate

3 was the most efficient nitrogen fixer followed by isolates 16, 29, 18, 41, 37, 5, 19, 6, 21, 15, 4, 14, 39, 30, 7, 27 and 31 respectively. Isolates 3, 16, 29, 37 and 5 ranked as the best nitrogen fixers which tested positive and negative for urease and nitrate reduction tests respectively. This was a clear indication that they had that potential of fixing the soil nitrogen. This is a very important and crucial element for bananas as nitrogen is a limiting factor in the production and growth of bananas.

The Bergey's Manual of Determinative Bacteriology, the main authority in bacterial nomenclature identified the bacteria up to the genus level using the biochemical characteristic as *Pseudomonas spp., Serratia spp., Raoultella spp., Enterobacter spp., Rahnella spp., Bacillus spp., Flavimonas spp and Ewingella spp.* These biochemical identification are in concurrence with the 16S rRNA gene sequencing for molecular identification of banana endophytic by Ngamau *et al.,* (2012).

Nested PCR was adopted to enhance specificity and sensitivity. Universal and nested primers designed by Widmer *et al.,* (1999) were used. The first amplification consistently yielded multiple product bands and some less resolved background smearing from some isolates while in some, the bands were distinct. Most of the bands from the second amplification were clear and distinct with. This observation is because the phylogenetic differences among nitrogen fixers are very diverse and the *nif*H gene sequences have diverged considerably Widmer *et al.,* (1999). Due to codon redundancy for most amino acids, the sequences of DNA encoding the conserved regions may differ considerably Zehr *et al.,* (1989). Universal primers design of *nif*H requires a high degree of DNA sequence degeneracy which may result in reduced specificity during amplification. Isolate no. 5 showed a band size of 642bp (Figure 3). Previously published studies of *nif*H gene sequences show that the amplification range of many primer sets is either lower or higher than the theoretically expected Poly *et al.,* (2001). Widmer *et al.,* (1999) obtained a band size of approximately 371bp. Gyaneshwar *et al.,* (2001) obtained *nif*H fragment of 390bp from *Azotobacter vinelandii*. Singh *et al.,* (2010) used the primers originally developed by Widmer *et al.,* (1999) to analyze *nif*H gene from bacteria extracted from soil samples and reported a band size of approximately 460bp. This may be attributed to reduced amplification efficiency of certain primer combinations of certain primer set used. In this study, 642bp band size of *nif* H gene obtained is longer than the previously published band sizes. This may be due to either weak nonspecific amplification which resulted in co-amplification of non-target templates or the relative abundance of different *nif*H target sequences in the genomic DNA extract which may vary over several orders of magnitude Bürgmann *et al*., (2004).

NCBI database BLAST search was performed to in order to identify the relatedness of the obtained sequences to the *nif* H gene sequences deposited in the NCBI database. It showed that isolate no.5 sequence is homologous to *Raoultella terrigena* strain DR-E5 nitrogenase iron protein (*nif*H) gene, partial coding sequence. However, it could not identify any annotated gene sequences for isolates 4, 15, 21, 27, and 29. A very high percent similarity/identity between the sequence of isolate 5 to the *nif*H sequence identified using BLAST N was obtained (98.4%) by aligning the two sequences using EMBOSS Matcher. However, four bases showed a mismatch between the two sequences (positions 153, 300, 310, 311 and 313 on JX080198.1 from the NCBI database). This could suggest a possible gene-environment interaction or multisite mutation. This identity of isolate no. 5 as *Raoultella terrigena* is in tandem with the finding on molecular identification of banana endophytic bacteria isolate by Ngamau *et al.,* (2012). In addition to testing positive and negative for urease and nitrate reduction tests respectively, isolate 5 is an efficient nitrogen fixer. The presence of *nif*H gene in its genome confirmed these findings.

5. Conclusion

The findings of this study show that banana endophytes are of diverse genus, have different morphology and biochemical properties and can fix varying quantities of nitrogen as shown by the Acetylene Reduction Assay. Isolate 3 (*Pseudomonas spp.*) was the most efficient nitrogen fixer among the banana bacterial endophytes. The study identified isolate 5 as *Raoultella terrigena* and showed the presence of *nif*H gene sequence in its genome. Therefore, it can be concluded that *Raoultella terrigena* is a potential biofertilizer because of *nif*H gene responsible for the fixation of nitrogen. However, the study cannot authoritatively conclude on the presence or absence of *nif*H gene in isolates 4, 15, 21, 27, and 29 because the primers were designed specific for *nif*H gene amplification.

6. Acknowledgement

I would like to first, thank the Almighty God for the wonderful gift of life He has bestowed in me and for being the source of inner strength, inspiration and the strongest pillar in my entire life. My sincere and heartfelt gratitude to the Research, Production and Extension Division of JKUAT for the generous financial support. To the Departments of Biochemistry and Food Science of JKUAT, thank you for enabling me to explore the world of genes and microbes respectively in your laboratories with the aid of your staff, Dr. Ger, Njuguna, Rotich, Karanja, Jenifer, Cecilia, Rasugu, Jesca and Waswa. My utmost appreciation goes to my dear parents Mr. Samuel Ouma Omoro and Mrs. Eucabeth Awuor Ouma for giving me the most precious gift, Education. To my brothers Petro and Elly and my only sister, Merab thank you for your motivation and prayers. To Beatrice, your unending love and support gave me strength to persevere during the lonely and difficult times and to live my life to the fullest. Finally, to Andrea Sawega, the General Manager Comet Healthcare Limited your motivation to pursue further education has enabled me to achieve the content of this document. May God bless you all. TO GOD I DO SUBMIT.

7. References

1. Bacon, C.W. and White, J.F. (2000). Microbial endophytes, Marcel Dekker, New York
2. Berg, G., Eberl, L., Hartmann, A. (2005). The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. Environmental Microbiology. 7, pp 1673–1685.
3. Bergey, D.H., Breed, R.S. (1957). Bergey's Manual of Determinative Bacteriology. Baltimore, Williams & Wilkins Co. 7th Edition. pp 247-267

4. Boddey, R.M., Urquiaga, S., Alves, B.J.R., Reis, V. (2003). Endophytic nitrogen fixation in sugarcane: present knowledge and future applications. Plant Soil. 252, pp 139-149.
5. Bürgmann, H., Widmer F., Von, S.W., Zeyer, J. (2004). New molecular screening tools for analysis of free-living diazotrophs in soil. Applied Environmental Microbiology. 70, pp 240 - 247.
6. Cappuccino, J.G. and Sherman, N. (2002). Microbiology: A laboratory manual. 6th edition. Pearson education Inc. San Francisco, California. pp 215 - 224.
7. Carlson, C.A. and Ingraham, J.L. (1983). Comparison of denitrification by Pseudomonas stutzeri, Pseudomonas aeruginosa, and Paracoccus denitrificans. Applied Environvironmental Microbiology, 45, pp 1247 - 1253.
8. Carroll, C.G. (1992). Fungal mutualism. In: Carroll C.G., Wicklow D.T., (Eds.) Fungal mutualism, Dekker, New York. pp 327 - 254.
9. Chowdhury, S.P., Schmid M., Hartmann, A., Tripathi, A.K. (2009). Diversity of 16S-rRNA and nifH genes derived from rhizosphere soil and roots of an endemic drought tolerant grass, Lasiurus sindicus. European Journal of Soil Biology. 45, pp 114–122.
10. Dobereiner, J. and Baldani, V. L. D. (1998). Biological nitrogen fixation by endophytic diazotrophs in non-leguminous crops in the tropics. Developments in Plant and Soil Sciences. 79, pp 3-7.
11. Eckert, B., Weber, O.B., Kirchhof, G., Hilbritter, A., Stoffels, M. and Hartmann, A. (2001). Azospirillum doebereinerea species, a nitrogen- fixing bacterium associated with the C4-grass Miscanthus. International Journal for Systemic Evolution Microbiology, 51: 17-26.
12. Esendugue, G.F. and Chidebelu, S.A. (2011). Nitrogen, Economics of Banana Production and Marketing in the Tropics, African Books Collective Publication. pp 32-38.
13. Gaby, J.C. and Buckley, D.H. (2012). A Comprehensive Evaluation of PCR Primers to Amplify the nifH Gene of Nitrogenase. PLoS ONE. 7(7).
14. Gyaneshwar, P., Euan, K.J., Natajan, M., Pallavolu, M.R., Reinhold-Hurek, B., Jagdish, K.L. (2001). Endophytic colonization of rice by Diazotrophic strain of Serratia marcescenes. Journal of bacteriology. 183, pp 2634 – 2645
15. Hannelore, S. (2005). Optimization of in vitro Multiple Meristem Cultures and Embryonic Cell Suspensions in Banana (Musa spp), Infomusa, Biodiversity International Publication. 14, pp 43-46
16. Hennecke, H., Kaluza, K., Thöny, B., Fuhrmann, M., Ludwig, W., Stackebrandt, E. (1985). Concurrent evolution of Nitrogenase genes and 16S rRNA in Rhizobium species and other nitrogen fixing bacteria. Archives of Microbiology. 142(4), pp 342-348.
17. Iniguez, A.L., Yuemei, D., Eric, W.T. (2004). Nitrogen Fixation in Wheat Provided by Klebsiella pneumonia 342. APS Journals. 17(10), pp 1078-1085.
18. James and William, (2008). Forms of Nitrogen in Soil, Nitrogen in Agricultural Systems, ASA-CSSA Publishers. pp 31-35
19. John, C. and Victor, G. (2010). Origins and Early Distribution (of Bananas), Bananas and Plantains, CABI International Publishers. pp 1-9
20. Mahdi, S.S., Hassan, G.I., Samoon, S.A., Rather, H.A., Dar, S.A. and Zehra, B. (2010). Bio-fertilizers in organic agriculture. Journal of Phytology, 2 (10), pp 42-54.
21. Michael, P. and Abdou, T. (2011). Genetic Resources for Banana Improvement, Banana Breeding: Progress and Challenges, CRC Press. pp 43-47
22. Ngamau, C.N., Matiru, V.N., Tani A., Muthuri, C.W. (2012). Isolation and identification of endophytic bacteria of bananas (Musa spp.) in Kenya and their potential as biofertilizers for sustainable banana production. African Journal of Microbiology Research. 6(34), pp 6414-6422.
23. Normand, P. and Bousquet, J. (1989). Phylogeny of nitrogenase sequences in Frankia and other nitrogen-fixing microorganisms. Journal of Molecular Evolution. 29, pp 436–447.
24. Peter and Morot-Gaudry, 2001, Remobilization of Nitrogen from Senescing Leaves, Plant Nitrogen. Springer Publishers. pp 282-284.
25. Poly, F., Monrozier, L.J., Bally R. (2001). Improvement in the RFLP procedure for studying the diversity of nifH genes in communities of nitrogen fixers in soil. Res. Microbiol. 152, pp 95–103.
26. Robert, E.W. (2000). Nitrogen: Uptake and Translocation, Plant-Environment Interactions, CRC Press. pp 66-70
27. Rogel, M.A., Hernandez-Lucas I., Kuykendall L.D., Balkwill D.L. and Martinez-Romerol, E. (2001). Nitrogen-fixing nodules with Ensifer adhaerens harboring Rhizobium tropici symbiotic plasmids. Applied and Environmental Microbiology, 67, pp 3264 – 3268.
28. Rolf, N. and Dinesh, K.B, (2008). Carbon and Nitrogen Pools in Terrestrial Ecosystems, Carbon and Nitrogen in Terrestrial Environment. Springer Publication. pp 5-11
29. Rosado, A.S., Duarte, G.F. Seldin, L., Van Elsas, (1998). Genetic diversity of nifH gene sequences in Paenibacillus azotofixans strains and soil samples analyzed by denaturing gradient gel electrophoresis of PCR amplified gene fragments. Applied environmental microbiology. 64, 2770-2779
30. Rubio, L.M. and Ludden, P.W. (2002). The gene products of the nif regulation. Nitrogen fixation at the millennium. Elsevier Science B.V. pp 101–136.
31. Ryan, R.P., Germaine, K., Franks, A., Ryan, D., Dowling, D. (2008). Bacterial endophytes: recent developments and applications. FEMS Microbiology Letters. 27, pp 1-9.
32. Rösch, C., Mergel A., Bothe, H. (2002). Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil. Applied Environmental Microbiology. 68, pp 3818–3829.

33. Sambrook, J., Fritsch, E., Maniatis, T. (1989). Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory Press. New York.
34. Sanginga, N. and Woomer, P. (2009). Agro-minerals in Integrated Soil Fertility Management, Integrated Soil Fertility Management in Africa: Principles, Practices and Developmental Process. CIAT Publishers. pp 31-43
35. Schulz, B., Rommert, A.K., Dammann, U., Aust, H.J. (1999). The endophyte-host interaction: a balanced antagonism? Mycological Research. 103(10), 1275-1283.
36. Singh, C., Soni, R., Jain, S., Roy, S., Goel, R. (2010). Diversification of nitrogen fixing bacterial community using nifH gene as biomarker in different geographical soils of Western Indian Himalayas. Journal of Environmental Biology. 31(5), pp 553-556
37. Sturz, A.V., Christie, B.R., Nowak, J. (2000). Bacterial endophytes: potential role in developing sustainable systems of crop production. Critical Reviews in Plant Sciences. 19(1), pp 1-30.
38. Tan, Z.Y., Hurek, T., Vinuesa, P., Muller, P., Ladha, J.K., Reinhold, H.B. (2001). Specific detection of Bradyrhizobium and Rhizobium strains colonizing rice (Oryza sativa) roots by 16S−23S ribosomal DNA intergenic spacer-targeted PCR. Applied Environmental Microbiology. 67, pp 3655–3664.
39. Tan, Z., Thomas, H., Barbara, R.H. (2003). Effect of N-fertilization, plant genotype and environmental conditions on nifH gene pools in roots of rice. Environmental Microbiology. 5(10), pp 1009–1015.
40. Tisdale, S.L., Nelson, W.L. and Beaton, J.D. (1985). Soil fertility and fertilizers. Macmillan, New York, 2, pp 161–168.
41. Ueda, T., Suga, Y., Yahiro, N., Matsuguchi, T. (1995). Remarkable N_2-fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of nifH gene sequences. Journal of bacteriology. 177, pp 1414–1417.
42. Uribe, D., Jimena, S.N., Javier, V. (2010). Role of Microbial Biofertilizers in the Development of a Sustainable Agriculture in the Tropics. Soil Biology. 21, pp 235-250.
43. Verma, S.C., Ladha, J.K., Tripathi, A.K. (2001). Evaluation of plant growth promoting and colonization ability of endophytic diazotrophs from deep water rice. Journal of Biotechnology. 91, pp 127–141.
44. Vessey, J.K. (2003). Plant growth promoting rhizobacteria as biofertilizers. Plant and Soil, 255, pp 571-586.
45. Walter, L. (2003). CO_2 Gas Exchange and Mineral Nutrition, Physiological Plant ecology: Ecophysiology and Stress Physiology of Functional Groups. Springer Publishers. pp 134-141
46. Wang, S., Chen, J., Johnson, J.L. (1988). The presence of five nifH-like sequences in Clostridium pasteurianum: sequence divergence and transcription properties. Nucleic acids research. 16, pp 439–454.
47. Widmer, F., Shaffer, B.T., Porteous L. A., Seidler, R.J. (1999). Analysis of nifH gene pool complexity in soil and litter at a Douglas fir forest site in the Oregon Cascade Mountain Range. Applied Environmental Microbiology. 65, pp 374–380.
48. Young, J. P. W. (1992). Phylogenetic classification of nitrogen fixing organisms. Biological nitrogen fixation. pp 43-46.
49. Zehr, J.P., and McReynolds, (1989). Use of degenerate oligonucleotides for amplification of the nifH gene from the marine cyanobacterium Trichodesmium thiebautii. Applied environmental microbiology. 55, pp 2522-2526
50. Zehr, J.P., Jenkins, B.D., Short, S.M., Steward, G.F. (2003). Nitrogenase gene diversity and microbial community structure: a cross-system comparison. Environmental microbiology. 5, pp 539–554.