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Short Communication

Isolation and characterization of plant growth promoting non-rhizobial Root nodule bacteria of major legumes in Malawi

Samuel Mwafulirwa[1], Shanmuga Priya Samson[2], Emanuel Mpumulo Nkasala[1] and Fortune Kanyada[3]
[1]Ministry of Agriculture, Chitedze Agricultural Research Station box 158, Lilongwe, Malawi
[2]Malawi University of Science and Technology, P.O BOX 5196, Limbe, Malawi
[3]Department of Forest, Kasungu District Office, P.O BOX 314 Kasungu, Malawi
samuelmwafulirwa54@gmail.com

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Abstract

Plant Growth Promoting Rhizobacteria (PGPR) are important microorganisms inoculated into agricultural land and act positively to crop production to achieve sustainable agriculture. Rhizobium inoculation is the most commonly used PGPR for production of legumes. However, isolation, reproducibility, competitive survivability and efficacy of rhizobial inoculation need understanding of their compatibility in natural state. To address the problem research has shown that inoculation of nonrhizobial nodule-associated bacteria acts positively on plant growth and nodulation when coinoculated with rhizobia. This study investigated nonrhizobial nodule-associated bacteria for legume crops grown in Malawi. Microbes were isolated from root nodules using yeast extract mannitol agar supplemented with congo red. Biochemical test and genetic characterization using 16S rDNA gene were used for strain identification which was supplemented by testing for the presence of Plant Growth Promoting Traits (PGPT). Results showed diversity of gram-negative nonrhizobial nodule-associated bacteria in the genus of Klebsiella, Leclercia, Enterobacter, Pseudomonas and Enterococcus. Isolated microbes were not host specific and have PGPT. The study puts assumption that these nonrhizobial nodule-associated bacteria isolates are not crop specific but site specific and are responsible for increase in yield, yield components and nitrogen fixation in legume production.

Keywords: Nodule, plant growth-promoting rhizobacteria, plant growth promoting traits, soil microbes, biofertiliser, Malawi.

Introduction

Leguminous plants are symbiotically associated with rhizobium which is one of Plant growth promoting rhizobacteria (PGPR) which causes nodulation and increase in yield and yield components[1]. PGPR which are involved in nitrogen fixating, phosphate solubilizing, potassium solubilizing bacteria, phytohormone production etc. constitute 2 to 5% of the total rhizobacteria community[2]. These microbes promote plant growth through direct mechanism by regulating nutrient uptake and production of plant growth regulators or indirect mechanisms through production of metabolites like lytic enzymes, siderophores, antibiotics, and hydrogen cyanide which supresses plant pathogens and deleterious microbes[3].

Some PGPR associated with nodule have direct and indirect benefit through growth and nodulation and other studies have found co-inoculation of rhizobia with compatible rhizospheric bacteria increase nodulation and yield legumes[4].

More studies have put recommendation of co-inoculation to Enhance nitrogen fixation with PGPR for sustainable agriculture production systems[5-8]. PGPR are either free-living rhizobacteria or endophytic. Endophytic microbes may reside either intercellular or intracellular of the host plant cell and this minimises inter specific competition between bacteria and free from environmental stress compared to free-living bacteria[9,10].

Several studies have reported the colonisation of rhizobia and other microorganisms in the genera of *Erwinia, Agrobacterium, Sphingomonas Aerobacter, Pseudomonas, Curtobacterium, Chryseomonas, Enterobacte, Flavimonas,* and *Bacillus* in the root nodules of the Leguminosae. Coinoculation in biofertilisation is dependent on compatibility of these bacteria and the rhizobia hence the need to isolate nonrhizobial Nodule-Associated Bacteria (NAB) with PGPT in different legumes[8].

The present work deals with isolation and characterisation of nonrhizobial nodule-associated bacteria for different legumes and from different agro ecological zones as a potential sustainable approach to co inoculation biofertilisation.

Materials and methods

Soybean groundnuts and pigeon peas nodule recovery and preservation: Nodules were systematically sampled from un-

inoculated farmer-managed soybean plots with no known history of inoculation to maximize the chance that only indigenous nonrhizobial bacteria are sampled. The farmer-managed soybean farms were sampled in a judgemental non-probability sampling procedure based on areas of importance in soybean cultivation and no history of rhizobia inoculation with scientific backing by measuring chlorophyll levels. Plants with Chlorophyll of greater than 20 µM/g leaf fresh weight were sampled. Chlorophyll Estimation was done by crushing one gram of Midrib of the leaf in acetone (80%). Chlorophyll concentration was measured using spectrophotometer with absorption coefficients of 664nm for chlorophyll and 647 nm for chlorophyllb according to Rajendran et al.[2]. Nodule recovery was done by using procedures for nodule recovery and preservation as described in Rajendran et al.[2] with slight modifications where by soil with nodules was carefully removed from the roots while avoiding detaching any. The nodules were preserved by desiccation in vials of silica gel in readiness for laboratory processing.

Isolation and characterization: Nonrhizobial NAB were isolated from nodules of groundnuts, soybean and pigeon peas from different agro ecological zones. Nodules from healthy plants with Chlorophyll of greater than 20 µM/g leaf fresh weight [2] were washed with sterilised water to remove the soil and mud. Pink to red nodules were chosen for isolation of nonrhizobial NAB. These Nodules were subjected to 95% ethanol to break their surface tension. Thereafter each nodule was soaked for 2-4 min in a 3% (v/v) solution of sodium hypochlorite, followed by rinsing in sterilised distilled water 5 times using sterile forceps. Thereafter nodules were crushed in the biosafety cabinet using sterile glass rod and the resultant suspensions of all effective (brown colour inside) nodules were streaked on YEMA following a procedure of Stajković et al.[11]. The cultures were incubated at 28°C for 8 days and observed every day for colony growth and emergence of contaminants. BTB and CR were used as indicators to detect the acid/base forming properties of the isolates and ascertain contamination or lack thereof respectively[12,2]. This presumptive test was used because rhizobia do not generally absorb CR in a dark incubation environment while most other bacteria inside the nodule absorb CR in the dark. A blue colour on BTB is for slow-growing Bradyrhizobium spp while yellow color (acid) reaction is for fast-growing Rhizobium species and other bacteria[2].

Morphological characterisation: All the nonrhizobial Nodule-Associated isolated strains were inoculated on YEMA and observed for their colony morphology, motility and Gram's nature[13].

Determination of the plant growth promoting traits: Isolates were observed for their ability to solubilise phosphorous, nitrogen fixation and also the production of Siderophores, catalase, ammonium and Indole Acetic Acid, by method described by Mwafulirwa et al.[14].

Organic acid production: Isolated bacteria were inoculated on MM9 agar medium[11,15] and checked for change in pH using an indicator. Positive test for production of organic acid was confirmed by development of pink colour.

Chemotaxis assays and utilization cellulose, pectin and protein: Culture medium was prepared with 0.3% of bacto agar, with 10^{-4} M Proline as the chemoattractant in which Mannitol served as the energy source as describe by Wedage and Gunawardana[13]. Swarming patterns (chemotaxis-based migration) of isolates were observed 4 days after room temperature incubation. Isolates were tested for utilisation of Pectin, Cellulose and Protein by modifying culture media with Pectin, Carboxy Methyl Cellulose, and Skim Milk respectively. hydrolysis-promoting bacteria showed hydrolysis zone around colonies[13].

Identification of the Microbes by genetic analysis: Characterizations of isolates were done by sequencing 16S rDNA gene. Genomic DNA was extracted and purified using the ZR-kit following manufacturer's manual and amplified using Polymerase Chain Reaction (PCR) of the 16S rDNA supplied by Inqaba http://www.inqababiotec.co.za. The primers were 907R (5'- CCGTCAATTCMTTTRAGTTT-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') and Sequencing of the isolates 16S rDNA was done using Sanger sequencing. A consensus sequence of two overlapping PCR products of 16S rDNA sequence data was done using BioEdit software. The consensus sequence obtained in BioEdit was analysed by BLAST algorithm for comparison of a nucleotide query sequence against public nucleotide sequence database to find the closely related strains**.** The nucleotide sequences of the 16S rDNA were subjected to BLAST analysis with the National Centre for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi, Sequences with high similarity scores were downloaded from the NCBI database. Based on maximum identity score first sequence was selected and aligned with isolate sequences using multiple alignment software program MUSCLE Distance matrix was generated using RDP database. First step the Neighbour Joining method was used for defining dataset because it establishes relationships between sequences according to their genetic distance (a phenetic criterion) alone, without taking into account an evolutionary model. Later Maximum Likelihood was used to because investigates the space of all possible phylogenetic trees, trying to identify those that are best its consideration of all possible trees to identify the best ones (i.e., exploring the space of all possible trees). Phylogenetic tree was constructed using Sea view. All sequences were deposited in the GenBank sequence database, and the accession numbers were obtained.

Results and discussion

Nonrhizobacteria NAB strains were isolated from root nodules of groundnuts, soybean and pigeon peas from different agro ecological zones on the YEMA. Colonies of isolates having different morphology from rhizobia on the media plates were

the candidates for further characterized. A total of 10 strains from genera *Klebsiella, Leclercia, Enterobacter, Pseudomonas* and *Enterococcus* were isolated from different agro ecological regions and from different crops as shown in Table-1. These isolates showed PGPT and have synergistic effect when co-inoculated with specific rhizobia[16]. All isolate were found to fix nitrogen on nitrogen free media. Nitrogen fixing ability by non-rhizobia nodule bacteria like Klebsiella pneumonia have been reported to contain Nif gene and that Lateral genes transfer is responsible for nodulation to non-rhizobia[17]. All isolates were motile, gram negative as shown in the data on Table-2 which concur with other studies.

Isolates investigated showed phosphate solubilisation as shown in Table-2. Phosphate solubilizing bacteria improve the conversion of insoluble forms of P to soluble (plant-available forms) by secretion of organic acids[18] and phosphatases which can be beneficial in co-inoculation[15]. All the isolates produced IAA, one of important phytohormones, mostly associated with rhizobacterial which stimulate plant growth by controlling cell division and root elongation[19]. Roots release extrudes that have tryptophan a precursor for IAA production in soil microorganisms. Production of IAA by PGPR increase root length and growth, enabling the crop to have large surface area for nutrients absorption. Many PGPR isolates have multiple mechanisms for enhancing plant growth, and that plant growth stimulation is a synchronised net of those traits result[20].

Siderophore production is another paramount PGP trait possessed by majority of isolates for iron uptake which was also found by other researchers[19]. Siderophores bind to the available form of Fe^{3+} thus making it not available to phytopathogens microbes.

Several studies put preposition that effect of rhizobacteria on plant growth depends on the bacterial strain and plant species. *Pseudomanas sp* and *Klebsiella pneumonia* have already been reported to significantly increase shoot dry weight and nutrient uptake in several crops[20]. Some strains of isolatedrhizo bacterial have already been reported to significantly increase yield in oat and barley and also have direct link to sustainable agricultural practices.

All of the isolates were cellulose positive and were able to secrete proteases and pectinases, concluding that isolates were able to colonise and help in nodulation process (Table-2) which contradicts with other studies which suggest that colonisation of nodule non rhizobia bacteria is though synergistic effect of co-inoculation[21]. Chemotactic swarming behaviour in response to the chemoattractant proline, by isolates which is widely found in legume root exudates, predicted the involvement of legume-Rhizobial crosstalk[13].

A synergetic effect shown when isolated microbes were combined with rhizobium comparable to results from either single inoculation was also observed in Sudan at EL–Hudeiba Research Station farm[22] and other places[11]. The study shows that biofertilisers with compatible effective strains can replace inorganic fertilizers to reduce production cost and prevent pollution of environment[23]. This set of experiment was done to assess synergistic effects of co-inoculation because its known fact that soluble phosphorous availability is one of determining factor for the uptake of nitrogen and its utilisation by crops[24]. Therefore the co-inoculation of compatible effective strains could be considered as an appropriate substitute for all inorganic fertiliser and sustainable agricultural systems.

These microbes were isolated from different crops as shown in Table-1 and some have been isolated from other plants by other researchers which mean that these microbes are not plant or nodule specific but site specific e.g. *Klebsiella pneumoniae* was isolated in soybean and ground nuts while other studies isolated them from grass, wheat and maize[25–27].

Table-1: Plant Growth Promoting Non-Rhizobial Root Nodule Bacteria of Major Legumes from selected districts of Malawi.

Isolate	Microorganism	Host	Location	Chlorophyll
1001	*Klebsiella pneumoniae*	Soybean	Mchinji	25 µM/g
10009B	*Klebsiella pneumoniae*	Groundnut	Mchinji	35 µM/g
1009A	*Enterobacter sp*	Groundnut	Mchinji	22 µM/g
1010	*Leclercia adecarboxyylata*	Soybean	Dowa	38 µM/g
1008	*Enterobacter cloacae*	Groundnut	Karonga	29 µM/g
1007	*Klebsiella pneumoniae*	Pigeon peas	Karonga	25 µM/g
1005	*Enterobacter cloacae*	Soybean	Lilongwe	26 µM/g
1004	*Pseudomonas spp*	Soybean	Dowa	25 µM/g
1003	*Enterococcus gallinarum*	Soybean	Dedza	25 µM/g
1002	*Klebsiella variicola*	Soybean	Kasungu	25 µM/g

Table-2: Cellulose, Pectin and Protein utilization and Plant growth promoting traits of isolates.

Isolate	Chemotaxis	Protein	Cellulose	Pectin	Solubilisation index	nitrogen fixation	Siderophores	Organic acid	Indole Acetic Acid (IAA)
1001	+	+	+	+	1.6	+	+	+	+
1002	+	+	+	+	1.6	+	+	+	+
1003	+	+	+	+	1.5	+	+	+	+
1004	+	+	+	+	1.7	+	+	+	+
1005	+	+	+	+	+	+	+	+	+
1007	+	+	+	+	1.6	+	+	+	+
1008	+	+	+	+	+	+	-	-	+
1009a	+	+	+	+	-	+	+	-	+
1009b	+	+	+	+	+	+	+	+	+
1010	+	+	+	+	+	+	+	+	+

Table-3: Isolates and their blast related species and GenBank deposit accession numbers.

Isolate	Microorganism	Location	Accession Numbers
10009B	Klebsiella pneumoniae	Mchinji	MG877667
1009A	Enterobacter sp	Mchinji	MG877668
1010	Leclercia adecarboxyylata	Dowa	MG881790
1008	Enterobacter cloacae	Karonga	MG881789
1007	Klebsiella pneumoniae	Karonga	MG877669
1005	Enterobacter cloacae	Lilongwe	MG877666
1004	Pseudomonas spp	Dowa	MG877670
1003	Enterococcus gallinarum	Dedza	MG877671
1002	Klebsiella variicola	Kasungu	MG877672

Phylogenetic tree of Plant Growth Promoting Non-Rhizobial Root Nodule Bacteria of Major Legumes from selected districts of Malawi revealed diversity of isolates forming unique clades. Almost all isolate formed its unique clades not based on agro ecological zone indicating that they have distant relationship.

Conclusion

The experiment has led to isolation of non rhizobacteria nodule microbes of major legumes in Malawi. This is most significant finding that will enable future co-inoculation studies of non rhizobacteria and rhizobacteria nodule bacteria.

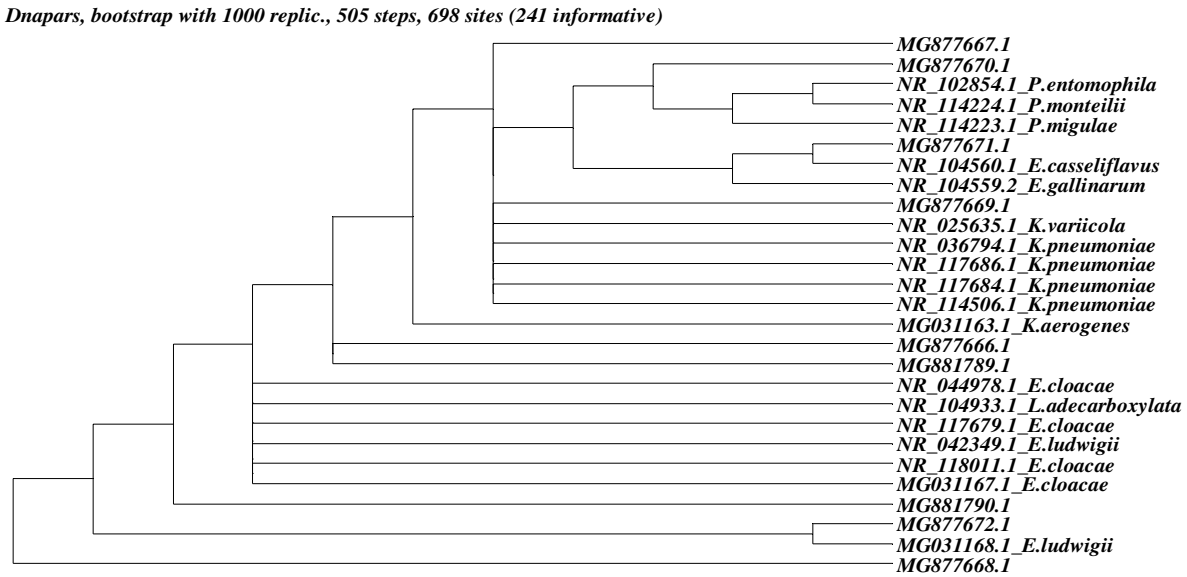

Figure-1: Phylogenetic tree based on 16S rDNA gene sequence depicting the position of Plant Growth Promoting Non-Rhizobial Root Nodule Bacteria of Major Legumes from selected districts of Malawi and those of NCBI.

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