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Exploiting the Genetic Diversity of *Rhizobia* to Produce Universal Inoculants with High Quality

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ABSTRACT

This study was done to study the possibility of producing high quality, universal or regional commercial inoculants. Sequences of 16SrRNA, glnII and recA genes of rhizobia isolated from different sites in Gadarif State, Sudan were aligned using A plasmid Editor (APE) software to choose the most genetically related strains. The result showed that *Rhizobium sp*. Haw1 and *Rhizobium sp*. G6-11 are identical according to the 16SrRNA and glnII genes sequences. They are also almost identical in their recA sequences because they were found differ in one position only. At the same time, these two strains were found differ at 9 positions when compared with *Rhizobium sp*. UoG27 also isolated from groundnut grown in another location. On the other hand, the sequences alignment of *Rhizobium sp*. Sab13 (isolated from nodules of Bambara groundnut) and *Rhizobium sp*. Umk34 (isolated from nodules of cowpea) also isolated from different locations showed that there are one and five positions of differences in the sequences of 16SrRNA and glnII, respectively. The study concluded that the relationship between rhizobia strains isolated from different sites and different related legumes can be exploited to produce universal or regional high quality inoculants

KEY WORDS: LEGUMES, STRAINS, ISOLATES, GROUNDNUT, BAMBARA GROUNDNUT, BACTERIOPHAGE.

INTRODUCTION

Variations in bacterial genes occur by deletion or addition of element like plasmids and through bacterial viruses which called bacterio-phages. This variation leads to genetic diversity due to differences in gene content and nucleotide variation in or between structural genes (Ochman et al. 2000). Variation among allels arises from nucleotide mutation, horizontal gene transfer, and intragenic recombination events (Reid et al., 2001).There are two types of substitutions occur in protein encoding genes; synonymous and non-synonymous single nucleotide polymorphisms (SNPs). Non synonymous SNPs (nsSNPs) result in amino acid replacements and hence provide substrate for evolutionary selection. While synonymous SNPs (sSNPs) do not alter the structure of proteins and are evolutionarily neutral (Kimura 1983; Schork et al. 2000; Gut 2001).

This type provides useful targets for large-scale molecular population genetic studies examining evolutionary relationships among bacterial strains. It is also permits

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all closely related strains to be assigned to lineages that are identical or related by descent and removes a critical barrier to population-based studies of the relationships between strain genotypes (Gutacker et al., 2002) and the host.

Rhizobial inoculants have been used successfully in world agriculture for about 100 years. The area inoculated is about 20 million ha in the world each year. This area can increase if inoculants with high-quality were available to all farmers. Therefore the future of the inoculant industry, and its potential benefits for world agriculture, depends on improving the quality inoculants (Herridge et al., 2002) and producing inoculants valid to be used for the related legumes and in a wide range in the world or at least suitable to group of countries in one geographical region or environmental zone. However, after over a century of rhizobial inoculation, most of the inoculants produced in the world are still of relatively poor quality (Lupwayi et al., 2000; Stephens and Rask, 2000, Santos et al., 2019).

In many countries in Africa biological nitrogen fixation is critical to the agricultural sustainability, but is often constrained by the absence of efficient and competitive rhizobia in the soil which need to improve its quality for every cropped legume (Hungria et al., 2005). So the poor inoculant quality, in those areas of the world where inoculation is ineffective or little used needs to be addressed (Hall and Clark, 1995; Marufu et al., 1995 Santos et al., 2019).

Agricultural Microbial Genetic Resources (AMiGR) usage in developing countries is often limited by lack of manufacturing capacity and quality control which also needs to be addressed (Howieson and Committee, 2007). It was stated that "the whole question of inoculants and their use starts with quality. If the quality is poor, then everything else is irrelevant" (Herridge et al., 2002). More than that, there is need to develop rapid methods to identify genetic relationships among all strains. However, it depends on the availability of partial and whole genomes sequencing of the different strains of rhizobia. Therefore this study was conducted to study the possibility to produce high quality, universal or regional commercial inoculants by exploiting the genetic diversity of different strains.

MATERIAL AND METHODS

Bacterial strains: The isolation of the strains used in this study and the amplification of the genes were described before elsewhere (Idris et al., 2012).

Nucleotide accession numbers: The accession numbers of the different strains genes used in this study were as follow: MN211542, MN211544, MN211545, MN211546 and MN211548 for 16SrRNA. The accession numbers for glnII were: MN218340, MN218342, MN218343, MN218344 and MN218346 and for recA the accession numbers were: MN218348, MN218350, MN218351, MN218352 and MN218354 for the different genes isolated from *Rhizobium sp*. Haw1, *Rhizobium sp*. G6-11, *Rhizobium sp*. UoG27, *Rhizobium sp*. Sab13 and *Rhizobium sp* Umk34, respectively.

Data analysis:Each two sequences of 16SrRNA, glnII and recA genes of *Rhizobium sp*. Haw1, *Rhizobium sp*. G6-11, *Rhizobium sp*. UoG27, *Rhizobium sp*. Sab13 and *Rhizobium sp* Umk34 were aligned using APE (A plasmid Editor) version 4.0.49.0 soft ware to detect the nucleotide variations.

RESULTS AND DISCUSSION

In this study sequences of the most related rhizobia isolates were chose and aligned to detect the polymorphism. The alignment of the 16SrRNA gene sequences of *Rhizobium sp*. Haw1 and *Rhizobium sp*. UoG27 (both isolated from groundnut) revealed that they are differ at 9 positions. The same result was obtained when *Rhizobium sp*. UoG27and *Rhizobium sp*. G6-11 sequences were aligned. However, *Rhizobium sp*. Haw1 and *Rhizobium sp*. G6-11 alignment showed that they are identical although they were isolated from groundnut grown in different sites (western and eastern parts of Gadarif state, Sudan). While the alignment of the same gene of *Rhizobium sp*. Sab13 isolated from Bambara groundnut and *Rhizobium sp*. Umk34 isolated from cowpea resulted in difference in one position only although they were isolated from different hosts and different locations (Tables 1 and 2).

Table 1. 16srRNA gene single nucleotides polymorphisms between *Rhizobium sp*. Haw1 and *Rhizobium sp*. UoG27

Positions	Isolates/Nucleotides	
	Rhizobium sp Haw1	*Rhizobium sp* UoG27
64	C	T
68	A	G
111	C	T
116	T	C
120	G	A
136	A	C
881	C	T
914	T	G
936	C	G

Table 2. 16srRNA gene single nucleotides polymorphisms between *Rhizobium sp* Sab13 and *Rhizobium sp* Umkh34

Positions	Isolates/Nucleotides	
	Rhizobium sp. Sab13	*Rhizobium sp*. Umk34
1028	G	A

The sequences alignment of glnII of *Rhizobium sp*. Sab13 and *Rhizobium sp*. Umk34 showed that there are five positions of differences, although 16SrRNA sequences

alignment showed that there is difference in one position only. This difference in one position clustered these two isolates in one group in the phylogeny tree (data not shown). The clustering in one group in the phylogeny tree is an indication that these isolates are at least of the same ancestor (Table 3). This difference in the five positions resulted in changes in five positions in the amino acids composition of glutamine encoded and synthesized by glnII gene (Figures 1 and 2). Despite this, we detected that *Rhizobium sp*. Haw1 and Rhizobium sp. G6-11 are identical in their glnII sequences. Accordingly, the glutamine encoded and synthesized was identical.

Table 3. GlnII gene single nucleotides polymorphism between *Rhizobium sp*. Sab13 and *Rhizobium sp*. Umk34

Positions	Isolates/Nucleotides	
	Rhizobium sp. Sab13	*Rhizobium sp.* Umk34
224	C	T
254	C	T
365	C	A
398	G	A
422	C	A

Although *Rhizobium sp*. Haw1 and *Rhizobium sp*. G6-11 were typically the same in their 16SrRNA and glnII genes, they were found differ in one position in recA sequences alignment (Table 4). This difference in one position in recA resulted in addition of one amino acid at the end of the recombinaze synthesized by *Rhizobium sp*. G6-11. This addition modified the synthesized protein structure and may modify its function. So the recombinaze synthesized by *Rhizobium sp*. G6-11 differed from that synthesized by Rhizobium sp. Haw1 (Figures 3 and 4).

In this study we aligned sequences of different genes isolated from different legumes grown in different locations to detect the genetic variation which may be useful in inoculants production. DNA sequencing is a viable method for assigning alleles of polymorphisms (Gut, 2001). The sequences alignment in this study showed that the most related isolates were found identical in some genes sequences and differ in others as in the case of Rhizobium sp. Haw1 and *Rhizobium sp*. G6-11 in which single nucleotide polymorphism was detected in recA sequences in spite of the identity of 16SrRNA and glnII sequences. This indicates that recA is characterized by "good capability to identify and classify strains" (Martens et al., 2008).

Figure 1: Glutamine II synthesized by *Rhizobium sp*. Sab13

Arg-Asn-Arg-Ala-Thr-Arg-Leu-Arg-Arg-Val-Leu-Thr-Thr-Arg-Ala-Ser-Ala-Ser-Arg-Thr-Ser-Val-Arg-Leu-Pro-Ala-Lys-Ser-Leu-Lys-Ser-Thr-Leu-Ile-Ser-Ala-Ser-Lys-Pro-Ala-Ser-Thr-Thr-Lys-Ala-Ser-Thr-Pro-Lys-Trp-Pro-Arg-Ala-Ser-Gly-Asn-Ser-Arg-Phe-Ser-Ala-Arg-Ala-Pro-Ser-Ala-Pro-Pro-Thr-Arg-Ser-Gly-Ser-Leu-**Ala75**-Thr-Cys-Cys-Cys-Val-Phe-Ala-Asn-Ser-**Thr85**-Ala-Ser-Thr-Ser-Asn-Ser-Ile-Ala-Ser-Arg-Ser-Ala-Thr-Pro-Thr-Gly-Thr-Val-Arg-Ala-Cys-Thr-Ala-Thr-Ser-Pro-Pro-Ser-Thr-Cys-Val-Lys-Leu-Ala-Ala-Arg-**Thr122**-Ile-Ser-Lys-Pro-Ser-Trp-Pro-Leu-Leu-Pro-**Arg133**-Thr-Gly-Lys-Ser-Thr-Ser-Thr-**Phe141**-Thr-Val-Arg-Thr-Thr-Thr-Phe-Ala-End

Figure 2: Glutamine II synthesized by *Rhizobium sp*. Umk34

Arg-Asn-Arg-Ala-Thr-Arg-Leu-Arg-Arg-Val-Leu-Thr-Thr-Arg-Ala-Ser-Ala-Ser-Arg-Thr-Ser-Val-Arg-Leu-Pro-Ala-Lys-Ser-Leu-Lys-Ser-Thr-Leu-Ile-Ser-Ala-Ser-Lys-Pro-Ala-Ser-Thr-Thr-Lys-Ala-Ser-Thr-Pro-Lys-Trp-Pro-Arg-Ala-Ser-Gly-Asn-Ser-Arg-Phe-Ser-Ala-Arg-Ala-Pro-Ser-Ala-Pro-Pro-Thr-Arg-Ser-Gly-Ser-Leu-**Val**-Thr-Cys-Cys-Cys-Val-Phe-Ala-Asn-Ser-**Met**-Ala-Ser-Thr-Ser-Asn-Ser-Ile-Ala-Ser-Arg-Ser-Ala-Thr-Pro-Thr-Gly-Thr-Val-Arg-Ala-Cys-Thr-Ala-Thr-Ser-Pro-Pro-Ser-Thr-Cys-Val-Lys-Leu-Ala-Ala-Arg-**Asn**-Ile-Ser-Lys-Pro-Ser-Trp-Pro-Leu-Leu-Pro-**Lys**-Thr-Gly-Lys-Ser-Thr-Ser-Thr-**Tyr**-Thr-Val-Arg-Thr-Thr-Thr-Phe-Ala-End

In addition, this verifies that identification of SNPs as a molecular marker is extremely valuable in phylogenetic studies (Flores et al., 2005). Also the wide range of inconsistency of the different genes may act as an indication of genetic instability which is considered as important characteristic in strain selection for inoculants production (FAO, 1991).

Table 4. recA gene single nucleotides polymorphism between *Rhizobium sp.* Haw1 and Rhizobium sp. G6-11

Positions	Isolates/Nucleotides	
	Rhizobium sp. Haw1	*Rhizobium sp.* G6-11
438	G	C

Figure 3: Recombinaze synthesized by *Rhizobium sp.* Haw1

Pro-Thr-Thr-Ser-Ser-Arg-Ser-Leu-Thr-Glu-Pro-Met-Arg-Arg-Met-Ser-Arg-Arg-Thr-Glu-Ala-End-Asn-Phe-Ser-Ala-Leu-Pro-Pro-Val-Val-Val-Ser-Gly-Glu-Pro-Asn-Met-Thr-Pro-Ile-Phe-Met-Arg-Ile-Trp-Leu-Met-Asn-Ile-Thr-Met-Gln-Phe-Asp-Leu-Glu-Ile-Glu-Ala-Val-Ser-Leu-Arg-Ser-Ala-Trp-Leu-Ile-Ser-Arg-Ala-Cys-Met-Pro-Gly-Arg-Leu-Ser-Pro-Ile-Ser-Pro-Ser-Ile-Ser-Ala-Arg-Gly-Val-Ser-Ala-Ala-Thr-Glu-Ser-Thr-Thr-Arg-Thr-Ser-Thr-Ala-Pro-Glu-Arg-Thr-Ser-Val-Ser-Val-Ile-Ser-Ser-Ala-Cys-Ser-Pro-Val-Ser-Gly-Cys-Glu-Ile-Arg-Arg-Phe-Cys-Arg-Ser-Thr-Pro-Ser-Leu-Arg-Ala-End-Thr-Gly-Ser-Ser-Ala-Cys-Ser-Ala-Ser-Thr-Lys-Ala-Gln-Met-Pro-Pro-Phe-Phe-Cys-Asp-Ser-Ala-Met-Val-Cys-Ser-Ala-Ser-Val-Val-Leu-Pro-Glu-Leu-Ser-Gly-Pro-End-Ile-Ser-Met-Ile-Arg-Pro-Phe-Gly-Arg-Pro-Pro-Met-Pro-Ser-Ala-Ile-Ser-Arg-Pro-Ser-Glu-Pro-Val-Glu-Thr-Val-Ser-Ile-Ser-Thr-Thr-Phe-Ser-Leu-Glu-Pro-Ser-Phe-Met-Ile-Glu-Pro-Leu-Pro-Asn-Asp-Arg-Ser-Ile-Cys-Glu-Ser-Ala-Ala-Ser

Figure 4: Recombinaze synthesized by *Rhizobium sp.* G6-11 (150)

Pro-Thr-Thr-Ser-Ser-Arg-Ser-Leu-Thr-Glu-Pro-Met-Arg-Arg-Met-Ser-Arg-Arg-Thr-Glu-Ala-End-Asn-Phe-Ser-Ala-Leu-Pro-Pro-Val-Val-Val-Ser-Gly-Glu-Pro-Asn-Met-Thr-Pro-Ile-Phe-Met-Arg-Ile-Trp-Leu-Met-Asn-Ile-Thr-Met-Gln-Phe-Asp-Leu-Glu-Ile-Glu-Ala-Val-Ser-Leu-Arg-Ser-Ala-Trp-Leu-Ile-Ser-Arg-Ala-Cys-Met-Pro-Gly-Arg-Leu-Ser-Pro-Ile-Ser-Pro-Ser-Ile-Ser-Ala-Arg-Gly-Val-Ser-Ala-Ala-Thr-Glu-Ser-Thr-Thr-Arg-Thr-Ser-Thr-Ala-Pro-Glu-Arg-Thr-Ser-Val-Ser-Val-Ile-Ser-Ser-Ala-

Other important of using single nucleotide polymorphism in inoculants production is that it is delineates relationships among closely related strains and allows construction of genetic frameworks for examining the distribution of host range and provides new insight into genetic relationships (Gutacker et al., 2002). More than that single nucleotide polymorphism of single-strain inoculants will act as facility in quality control (Thompson, 1980), it also enhance "the tendency to use single-strain inoculants in countries with strong inoculant quality-control programs as well as in those with a tendency to recommend specific strains for each ecosystem" (Date, 2001). In addition, now the issue of inoculants quality has centered on some problems one of them associated with genetic instability of rhizobial strains (Herridge et al., 2002). Single nucleotide polymorphism can plays crucial role to detect this genetic instability.

Some previous studies addressed inculant quality control as final step after "choosing and processing the carrier, culture maintenance and growth at increasing scales of production, in addition to aseptic injection of broth culture into the peat, proper maturation, and adequate packing" (Hungria et al., 2005). This quality control regulated in most countries in which mainly concentrates in some criteria one of them is the quality of the strain (Herridge et al., 2002). Isolating of different strains from different sources and comparing the different genes of the inoculants to determine the degree of the differences at single nucleotide polymorphism level will contributes in the quality of the inoculants strains by selecting strains related genetically, effective, capable to adapt to different environments, with wide host range, and therefore it will be suitable to be used as inoculants in different regions over the world. This will help to overcome the problem of searching for effective rhizobial strains for each legume which was described as "a labor- and time-consuming process" (Binde et al., 2009).

The importance of this study is that it can be applied to produce a universal inoculants or at least effective inoculants to be used in different regions, for example inoculants suitable for the tropical regions, others for the temperate or cold regions and so on. This can be done by surveying in the gene bank data base and search for the most related isolates from different sources over the world and select the most related strains to be used as inoculants. Before inoculants production, different genes especially symbiotic genes of the selected strains should be aligned to detect the degree of the relationship; in addition they should be tested in different sites in the world to authenticate their tendency to adapt to the different and adverse environmental conditions.

The model of *Rhizobium sp*. Haw1 and Rhizobium sp. G6-11 in this study supports the idea of the possibility of producing universal or regional inoculants because as mentioned that the isolates were obtained from groundnut grown in different locations far away from each other, despite this they were found identical in their nucleotide sequence of some genes. Like that, the model of Rhizobium sp. Sab13 and Rhizobium sp. Umk34 alignment is in this study also supports the above mentioned idea and indicates to the possibility to produce inoculants from the most related rhizobia strains which can establish symbiotic relationship among the most related hosts.

These latter strains were isolated from legumes belong to the same host (Vigna) but different species. So for example in the first model mentioned above, one of the strains can be used to manufacture inoculant suitable to be used in the two sites from which the isolates were obtained instate of manufacturing two inoculants, the same is true for the second model in which inoculant produced of one isolate will be more general because it can be used in different sites and different legume

species. However, the non synonymous (SNP) found in the glnII and recA sequences which resulted in change in amino acid composition of the glutamine and recominase mentioned in the result section leads to change in the synthesized protein structure and function which may reduce the chance of these isolates to replace each other as inoculants. The assumption of manufacturing inoculants suitable to different places and hosts depends on the degree of the relationship in the symbiotic genes and the ability of the strains to tolerate the different environmental conditions. This assumption may be logic because it was stated before that the genomic diversification is a result of recombination, transposition and horizontal transfer of genes between the most related strains (Flores et al., 2005).

The process of producing universal or regional inoculants with high quality requires isolation of rhizobia from different countries over the world, amplify different genes with concentration in symbiotic genes, aligning different genes to select the most related strains and test their ability to tolerate different and adverse environmental conditions. To do this a network and collaboration between researchers over the world should be conducted. Finally, improving inoculants quality will guarantee brilliant future for inoculant industry and its potential benefits for world agriculture as it was stated by Herridge et al. (2002).

CONCLUSION

There is strong genetic relationship between rhizobia strains isolated from different sites and different related legumes. This relationship makes producing universal or regional high quality inoculants possible if the different strains have the susceptibility to achieve effectively in the different environmental conditions. However, more studies required to collect different genes sequences of different effective rhizobia strains over the world and study their relationship with concentration in the symbiotic genes.

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