Rhizobial and non-Rhizobial nodulators of *Pueraria phaseoloides*

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Abstract

Pueraria phaseoloides is a widely grown legume cover crop in Sri Lanka. Nitrogen fixation is performed by nodular inhabitants of this cover crop. We endeavored to isolate the whole array of diazotrophs colonizing the root nodules of this economically-significant cover crop. Four isolates were isolated by streaking a macerate of an active nodule from *Pueraria phaseoloides* and the identification of Rhizobial and Non-Rhizobial species was carried out using colony and cell morphology. One isolate from *Pueraria phaseoloides* (Sub 1), a gram negative bacterium contoured by a coccobacillus cell shape, (suggesting a likely non-Rhizobial identity), was a potent nodulator of *Pueraria phaseoloides* seedlings. A further three cultures (Sub 2, Sub 3 and Sub 4) too were able to nodulate *Pueraria phaseoloides* seedlings but were not as effective as Sub 1 in their nodulation potency. All four bacteria secreted to the extra-cellular medium cellulases suggesting their likely involvement in nodule formation and also showed characteristic patterns of motility to the chemoattractant proline. DNA extracted from Sub 1, Sub 3 and Sub 4 gave a PCR amplicon of the anticipated size (360 bp) using universal *nifH* primers, which indicated that the genetic foundation for the production of a unit of the nitrogenase enzyme, was found in the genome of these isolates. In summary, we have unearthed here, a strong, likely non-Rhizobial nodulator, in the legume cover crop *Pueraria phaseoloides*, and three other bacilli bacteria, perhaps Rhizobia, capable of efficient nodulation. Further characterization of these isolates using molecular biology tools is ongoing.

Key Words diazotroph, nitrogenase, cover crop, rubber, nodulation, chemotaxis

Introduction

Nitrogen is an apical nutrient for plant growth. It is essential inside plant cells for the synthesis of enzymes, proteins, chlorophyll, DNA and RNA, and thus forms an important component of plant growth and development (Matiru and Dakora, 2004). Rubber, which is one of the major industrial crops in Sri Lanka, is dependent on proper nitrogen enrichment during growth, leading to optimum latex yields. The nitrogen requirements of young rubber plantations can be supplied, replenished or supplemented with the introduction of leguminous cover crops. Today, the use of leguminous covers such as *Pueraria phaseoloides*, is a standard practice in rubber plantations in Sri Lanka.

Legumes are believed to have emerged 70-130 million years ago early in the Cretaceous period (Balachandar *et al.,* 2007). Legumes possess root nodules which harbor bacteria of the genus *Rhizobium*, which renders a comparative advantage through nitrogen fixation. *Rhizobium* is host specific and therefore the characterization of Rhizobial species specific for *Pueraria phaseoloides* is of strong significance to the rubber industry. Furthermore, in addition to *Rhizobium*, there can be auxiliary inhabitants colonizing root nodules in legumes, which too may possess nodulation and nitrogen fixation capacities.

Pueraria phaseoloides, which is also known by the names, Puero (Australia), tropical kudzu (most of the tropics), centro grande and feuille, is a cover crop that grows profusely in wet land soils and nodulates freely in water-available conditions. One of the unique features of *Pueraria phaseoloides* is its accommodation of a wide range of nitrogen-fixing bacteria inside nodules. This 'promiscuous effective' nature of nodulation makes *Pueraria phaseoloides* an attractive legume to investigate its compliment of both rhizobial and non-rhizobial nitrogen fixers. Therefore we undertook a study to isolate and characterize diazotrophic bacteria from the nodules of *Pueraria. phaseoloides.*

Methods

Isolation of nodule-inhabiting bacteria

Surface sterilized root nodules were crushed by using sterile mortar and pestle while mixing with sterilized distilled water and Yeast Mannitol Agar containing petri plates were streaked with the macerate and incubated at room temperature for 1-2 days.

Optical Microscopy

To study cell morphology, single colonies from agar plates were used to prepare slides and the prepared slides were stained with simple staining and Gram staining techniques and observed under 4×, 10×, 40×, 100× magnifications (Light Microscopy).

Assays for Cellulose, Pectin and Protein Utilization

For Cellulose, Pectin and Protein Utilization, media plates were made with Carboxy Methyl Cellulose, Pectin and Skim Milk and the hydrolysis zones around the bacterial colonies were observed/measured to identify the respective hydrolysis-promoting bacteria.

Chemotaxis Assays with proline

Culture media were prepared with 0.3% of bacto agar, with 10^{-4}M Proline as the chemoattractant and Mannitol as the energy source and the chemotaxis-based migration (swarming patterns) of bacteria were recorded after 4 days of incubation at room temperature.

PCR amplification of nifH gene

Polymerase Chain Reactions were run with the PolF and PolR primers as described in Poly *et al*., (2001).

Results and Discussion

We isolated four independent isolates from the root nodules of *Pueraria phaseoloides,* which were characterized as likely diazotrophs (3/4) by PCR amplification of the *nifH* gene, where amplicons of the expected size (360 bp) were observed by agarose gel electrophoresis (data not shown). We are in the process of sequencing the *nifH* fragments which should allow for the identification of the isolates. The diazotrophic nature is a hallmark of bacteria living inside root nodules, in symbiosis with the legume.

The isolated bacteria were all gram negative and three were rod shaped suggesting a possible Rhizobial identity. This aligned well with the capacity of *Pueraria phaseoloides* to be 'promiscuous effective', accommodating diverse Rhizobial species. One species though was not rod shaped and fitted well with a coccobacillus shape with rounded smooth edges (Table 1). All of the isolates were cellulase-positive and three isolates each were able to secrete pectinases and proteases, suggesting that these extracellular enzymes were of value for colonization and nodulation processes (Table 2). We also checked the capacity of each isolate for chemotactic swarming behavior in response to the chemoattractant proline, widely found in legume root exudates and predicted to be involved in the legume-Rhizobial crosstalk (Webb *et al.,* 2014). Each bacterium showed characteristic patterns of swarming motility, with Sub 3 demonstrating strong chemotactic swarming patterns to the chemoattractant proline (Table 3).

Table 1: Light microscopic observations of bacterial cultures.

Observed Characteristics	Isolated cultures			
	Sub 1	**Sub 2**	**Sub 3**	**Sub 4**
1. Gram staining test	Negative	Negative	Negative	Negative
2. Shape of the cell	Cocobacillus	Bacillus	Bacillus	Bacillus
3. Special features of the cell	Polarized mass was observed	Polarized mass was observed	-	Elongated cells

Table 2: Cellulose, Pectin and Protein utilization by the isolated bacteria.

Biochemical	Cellulose	Pectin	Protein
Sub 1	+	-	+
Sub 2	+	+	+
Sub 3	+	+	+
Sub 4	+	+	-

Table 3: Patterns of chemotaxis of the isolated cultures to the chemoattractant proline.

Bacterial strains	Positive or Negative	Swarming pattern
Sub 1	++	Bull's eye
Sub 2	++	Dendritic
Sub 3	+++++	Featureless
Sub 4	+	Featureless

We also tested the nodulation capacity of each isolate by the reinfection of seedlings grown in a hydroponics system. The hydroponics experiments yielded that it was the likely non-Rhizobial species, Sub 1, which was the primary nodulator, with nodules appearing in seedling roots, 2 weeks after reinfection (Treatment 1, Figure 1). The other bacteria too were unique nodulators but with a longer lag time for nodule formation post-infection (Figure 1). By the 7th week, Sub 1 plants had nearly 12 nodules per plant whereas the other isolates (Sub 2, Sub 3, Sub 4) only contained significantly lesser number of nodules per plant (Figure 1). Surprisingly, seedlings that were reinfected with all four isolates (Treatment 5, Figure 1) formed a significantly lesser number of nodules than three of the four individual treatments.

Several nodulation-competent non-Rhizobial species, belonging to α and β subgroups of Proteobacteria such as *Methylobacterium, Blastobacter, Devosia, Phyllobacterium, Ochrobactrum, Agrobacterium, Cupriavidus, Herbaspirillum and Burkholderia* and some δ-Proteobacteria have been identified this far (Balachandar *et al.,* 2007). Although universally nitrogen fixation and nodulation genes have low divergence propensities, particular attention has been paid on some nodular non-Rhizobial nitrogen-fixers due to significant sequence divergences in the nitrogen fixing (*nifH*) and nodulation (*nodD* or *nodA*) gene sequences (Balachandar *et al.,*

2007). Therefore, there might be unforeseen taxonomical diversity in non-Rhizobial nitrogen fixers such as Sub 1, which requires further investigation to shed light on processes such as nodulation and nitrogen fixation in these emerging group of nodule-forming non-Rhizobial bacteria.

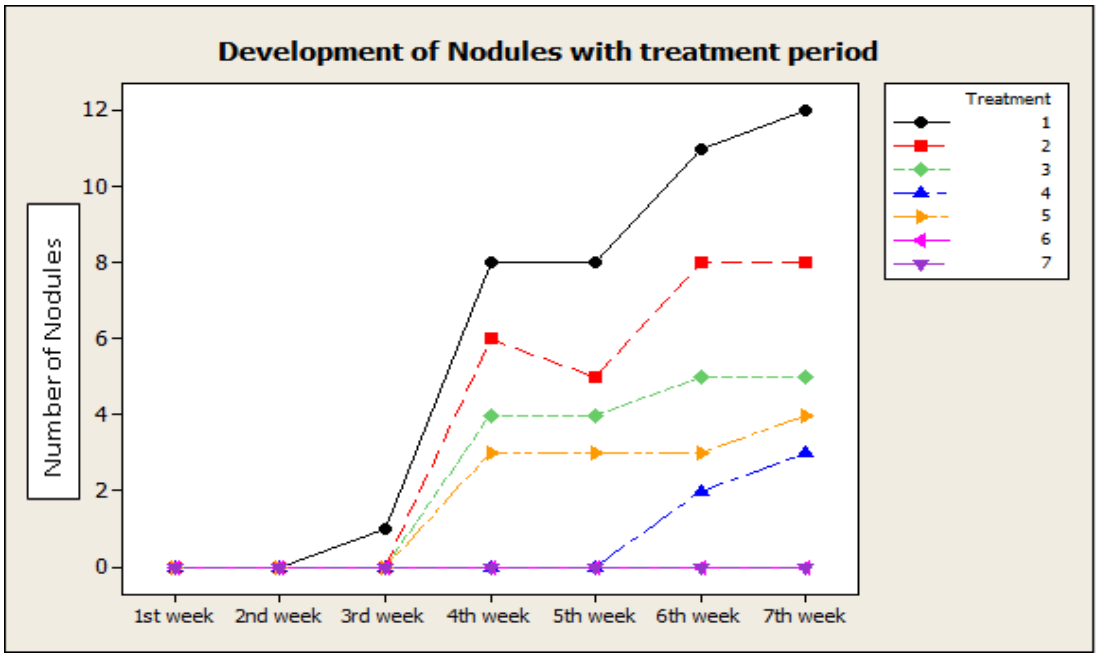

Figure 1: Distribution of nodule number (mean of four replicates) with treatment period. (1- Sub 1, 2- Sub 2, 3- Sub 3, 4- Sub 4, 5- All 4, 6- Sub Ɵ (a control likely-Rhizobial culture from *Mucuna bracteata*), 7- Control)

Conclusions

We have isolated here four nodulation proficient bacteria from the cover crop *Pueraria phaseoloides* and characterized their arsenal of extracellular hydrolytic enzymes and their swarming motility (chemotaxis) to the chemo-attractant proline. The most significant finding of our study is the unearthing of a strong likely-non-Rhizobial nodulator which shows potent nodulation capacity much superior to the three other isolated bacteria in this study. It is significant that nodules arising from three single isolate treatments were superior in number to the conglomerate cultures containing all four isolates. It appears that nodular inhabitants of *Pueraria phaeoloides*, in particular Sub1, are potent nodulators of this cover crop.

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