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Phylogenetic grouping by pcr analyses of *Sinorhizobium meliloti* strains isolated from eutrophic soil

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ABSTRACT

This study evaluated the ability of *Sinorhizobium meliloti* strains (SEMIA-116, SEMIA-134 and SEMIA-135) to persist in eutrophic soils after three complete growing cycles of alfalfa. PCR fingerprinting using primers for the nifH, Eric, Rep, RP genes and P25 RAPD-PCR performed the phylogenetic grouping of the three commercial strains and 39 field isolates. These analyzes were sufficiently sensitive to discriminate and group the strains and isolate. Our preliminary studies were based upon Eric primers and RP01 RAPD-PCR.

KEYWORDS: Alfalfa, Phylogenetic grouping, *Sinorhizobium meliloti*

INTRODUCTION

The rate of the biological nitrogen fixation through the interaction alfalfa (*Medicago sativa* L.) /*Sinorhizobium meliloti* is variable and depends upon the environmental conditions as well as the appropriate strains to observe an efficient symbiotic association. Under optimal conditions the alfalfa/*Sinorhizobium* association can fix up to 450 kg/ha of nitrogen that is converted into high quality plant material. In Brazil, the production of commercial inoculum for alfalfa follows the recommendation given by the Brazilian Department of Agriculture, which requires three strains of *Sinorhizobium meliloti* (SEMIA-116, SEMIA-134, and SEMIA-135). However, the survivability and the capacity of each strain to nodulate alfalfa vary. In the production of alfalfa hay in the field at ESALQ/USP, Piracicaba, SP, Brazil, the successive planting of alfalfa in the same plot over the past nine years resulted in the establishment of a bacterial population capable of nodulating non-inoculated plants. The identification of these isolates may help to define a more specific recommendation for the production of commercial alfalfa inocula. Standard microbiological procedures did not show significant differentiation of isolates when compared with the commercial strains (Oliveira *et al.*, 1999). The objective of this study was to continue the identification of the soil isolates through DNA genomic analyzes and obtain the phylogenetic relationships among these isolates in comparison to the commercial strains.

MATERIAL AND METHODS

Twenty-five alfalfa plants were harvested randomly within the hay production field. From each one of these plants forty intact nodules were selected. The bacteria were isolated according to Somasegaram and Hoben (1994) by culturing them on YMA medium to obtain pure cultures. *Sinorhizobium* genomic DNA extraction and PCR reactions were conducted based upon Kay *et al.* (1994), Perret and Broughton (1998), Machado (1998) using the oligonucleotides for Eric, *nif*H, RP and P25 RAPD-PCR. Table 1 illustrates the oligonucleotide sequences and the PCR and RAPD amplification reactions that will be used in this study. The amplification products resulted from Eric and P25 primers were separated on a 1.4-% agarose gel. The banding patterns were visualized with ethidium bromide and documented with the "Fluor-S MultiImager-PC" from BIORAD. The data analyses and the phylogenetic classification of the isolates and wild type were performed using the NTSYS program version 1.70.

Table 1 - Oligonucleotide sequences and the PCR and RAPD amplification reactions that will be used in this study.

Primer	Sequence
P25	5' TATTTATTCCGGCCAATCATCCGC 3'
*nif*H1	5' CGTTTTACGGCAAGGGCGGTATCGGCA 3'
*nif*H2	5' TCCTCCAGCTCCTCCATGGTGATCGG. 3'
Eric1	5' ATGTAAGCTCCTGGGGATTCAC. 3'
Eric2	5' AAGTAAGTGACTGGGGTGAGCG. 3'
RP01	5' ATTTTCAAGCGTCGTGCCA. 3'
Rep1	5'. NNNNCGCGNCATCNGGT. 3'
Rep2	5'. NCGNCTTATCNGGCCTAC. 3'

Cycle		P25 RAPD	*nif*H PCR	Eric PCR	RP PCR	Rep PCR
	1°Denaturation	30 s at 94°C	30 s at 94°C	420 s at 95°C	60 s at 94°C	420 s at 98°C
5 Cycles	Denaturation	30 s at 94°C	30 s at 94°C		60 s at 94°C	
	AnnealingAT1	45 s at 60°C	30 s at 55°C		60 s at 50°C	
	Polymerization	120 s at 72°C	60 s at 72°C		120 s at 72°C	
30 Cycles	Denaturation	30 s at 94°C	30 s at 94°C	60 s at 94°C	60 s at 94°C	60 s at 94°C
	AnnelingAT2	45 s at 60°C	30 s at 65°C	60 s at 52°C	60 s at 50°C	60 s at 40°C
	Polymerization	120 s at 72°C	60 s at 72°C	480 s at 65°C	120 s at 72°C	480 s at 65°C
	Final step	300 s at 72°C	300 s at 72°C	420 s at 65°C	300 s at 72°C	420 s at 65°C

RESULTS AND DISCUSSION

The banding patterns, which resulted from the Eric as well as RP01 primer amplifications, revealed in agarose gels common fragments among the isolates and commercial strains. Molecular techniques used to obtain phylogenetic relationship have produced precise and accurate results. The identification of polymorphic molecular markers among isolates from the same species allows the differentiation and clustering among known strains. According to Perret and Broughton (1998), the choice of primers with highly conserved sequence of interest, such as *nif*H, Eric, Rep, P25 and RP01 have demonstrated to be some of the most flexible and adequate techniques for phylogenetic studies of prokaryotes.

Our preliminary results obtained with the primers RP01 RAPD-PCR and Eric are illustrated in Figure 1. Studies with *nif*H, P25 and Rep are been conducted now to confirm the data. According to the amplified genomic regions there is 65% of similarity between the commercial strains SEMIA-116 and SEMIA-134, 57% between SEMIA-116 and SEMIA-135 and 95% between SEMIA-134 and SEMIA-135. There is a variable distribution, from 65 to 89% of similarity, among the isolates of interest and the commercial strains with the exception of isolate 31, which showed only 57% of similarity when compared with the commercial strains. These data will be confirmed with *nif*H, P25 and Rep primers and should also provide better differentiation among the isolates of interest and the commercial strains. Moreover, through these data will be possible to confirm which commercial strains persisting in the eutrophic soil.

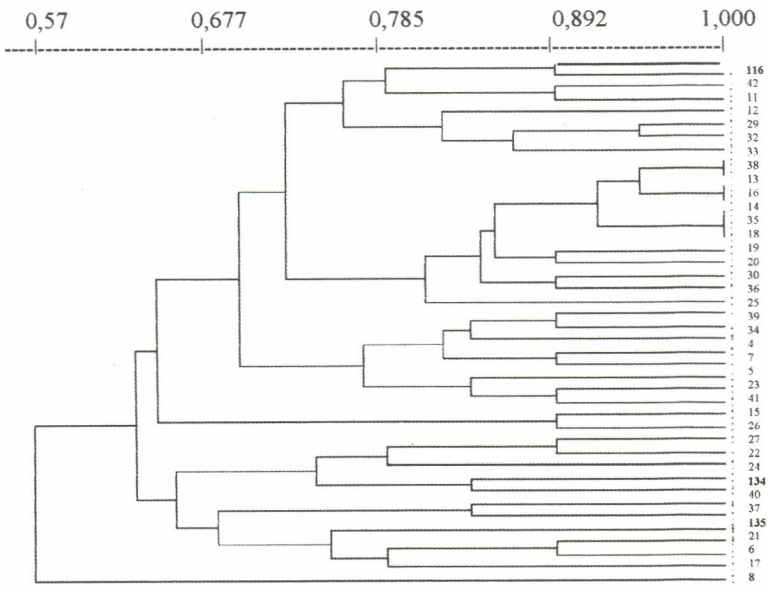

Figure 1 – Phylogenetic relationships among commercial strains of *Sinorhizobium meliloti* (SEMIA-116, SEMIA-134, SEMIA-135) and field isolates of *Sinorhizobium meliloti* from alfalfa nodules (4 to 42).

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