

VARIABILITY OF ISOLATED COLONIES IN BEAN NODULATING *RHIZOBIUM* STRAINS BEFORE AND AFTER EXPOSURE TO HIGH TEMPERATURE

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ABSTRACT

Irregular response to bean plants to *Rhizobium* inoculation has been attributed to among other factors, low competitive ability, low N₂ fixation efficiency and genetic instability of the symbiont. This genetic instability caused by high rates of genomic rearrangements and/or plasmid deletions can be accentuated by high temperatures. This fact may limit the utilization of these strains as inoculants, especially in tropical soils. In this study, the variability of isolated colonies derived from effective *R. leguminosarum* bv. *phaseoli* (SLP1.3 and BR 10.026) and *R. tropici* (SLA2.2 and BR322) strains was evaluated before and after exposure to high temperatures (four consecutive thermal shocks at 45°C). This evaluation involved plant dry matter analysis of inoculated plants and genotypic (plasmid profile and genomic patterns via RAPD) analysis of the *Rhizobium* strains. The results evidenced that high temperature improve the natural performance variability especially between isolated colonies from *R. leguminosarum* bv. *phaseoli* strains. The plasmid profile of isolated colonies from *R. tropici* strains were identical regardless of temperature treatment whereas isolated colonies from *R. leguminosarum* bv. *phaseoli* alterations were detected especially after the thermal treatment. The genomic patterns generated by AP-PCR showed more alterations and genetic variation in isolated colonies from *R. leguminosarum* bv. *phaseoli* strains indicating that *R. tropici* strains are more stable and lower affected by high temperature.

Key words: *Rhizobium tropici*, *Rhizobium leguminosarum* bv. *phaseoli*, bean plants, high temperature, cerrado soils

INTRODUCTION

Irregular response of bean plants to *Rhizobium* inoculation has been attributed to, among other factors, low competitive ability, low N₂ fixation efficiency and genetic instability of the symbiont. Several research laboratories have reported genetic instability of *Rhizobium* strains as one of the causes that compromise relevant characteristics of these organisms in the nitrogen fixation, both in wild-type strains obtained by conventional selection procedures (13,14,17,25,26) and in genetically improved strains (3,29). *Rhizobium* strains capable

of nodulating bean display particular genetic characteristics that favor such instability. The genes responsible for nodulation and N₂ fixation in these *Rhizobium* strains are localized on a single replicon, the symbiotic plasmid (pSym). The genome of *Rhizobium* is complex and contains several reiterated DNA sequences (6,9,12). The reiterated copies are usually necessary for the expression and effectiveness of the N₂ fixation (5,9,12); they also provide sites for recombination that can give rise to genomic rearrangements at high rates (12,20), resulting in alterations on the original characteristics of the cells (5). High temperatures can accentuate this instability enabling genomic

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rearrangements and plasmid deletions (9,22), thus jeopardizing the utilization of these strains as inoculants, especially in tropical soils. In this study, isolated colonies derived from effective *R. leguminosarum* bv. *phaseoli* (SLP 1.3 and BR 10.026) and *R. tropici* (SLA 2.2 and BR 322) strains were evaluated before and after exposure to high temperatures. This evaluation involved both dry matter analysis of inoculated plants and genotypic (plasmid profile and genomic patterns via RAPD) analysis of the *Rhizobium* strains.

MATERIALS AND METHODS

1. Obtention of isolated colonies from original *Rhizobium leguminosarum* bv. *phaseoli* (SLP 1.3 and BR 10.026) and *Rhizobium tropici* (SLA 2.2 and BR 322) cultures before and after exposure to a high temperature

Ten colonies from each original strain were selected randomly in YMA (24). Then, standard liquid cultures (YM, 21) of each colony (10^8 cells/ml) were inoculated in sterilized soil (0.7 ml of inoculum/g soil). In the first experiment the inoculated soil was incubated at 45°C for 4 hours. This procedure was repeated four times within intervals of 48 hours and at the end of the fourth shock the inoculated soil was poured into plates containing YMA medium. The second experiment was done with soil inoculated with the same colonies, not subjected to high temperature. The experiments were performed in 4 repetitions. The colonies from both experiments were grown and stored at 4°C for further utilization in plant tests and genetic characterization studies. BR 10.026 and BR 322 are commercial strains and SLP 1.3 and SLA 2.2 are native strains from Cerrado soil.

2. Bean plant inoculation tests

The colonies isolated previously from each of the original strains, submitted or not to high temperature, were grown in YM medium for bean plant inoculation. They were standardized for approximately 10^8 cells/ml and were inoculated in “Leonard jars” containing aseptically cultivated beans (cv. aporé). The experiments were carried out using four replicates of a completely randomized block design. The Leonard jars were kept in a green house in a modified N-free nutrition solution (11) for 30 days, after which the plants were collected and dried to evaluate the dry matter, a parameter directly correlated to nitrogen fixation (8,15,16).

3. Determination of plasmid profile

Isolated colonies were multiplied in TY medium and the plasmid profile was determined through the methodology described by Eckhardt (4) and modified by Hynes and McGregor (7).

4. Determination of genomic patterns via AP-PCR

Bacterial total DNA was extracted according to the method of Sá *et al.* (17). DNA amplification of each colony was carried

out in accordance with Steindel *et al.* (21). Amplification reactions were carried out in a Perkin-Elmer 9600 thermocycler and consisted of 40 cycles of: denaturation (94°C for 15 sec.), annealing (35°C for 30 sec.) and extension at 72°C for 1 min. and a final extension at 72°C for 7 min. Each reaction was performed in 25.0ml using: 2.5 mM MgCl₂, 10.0mM TrisHCl, 0.05mM each dNTP, 1.0U Taq DNA polymerase, 0.4mM primer and 5.0ng DNA. The following primers from the Operon Kit (Operon Technologies, Inc.) were tested: OPAC 10 (5'GGCGAGTGTG3'), OPAC 04 (5'GTCGGTTTGTC3') and primer S34 (5'GGT TCG ATT GGG GGT TGG TGT AAT ATA3'). After the AP-PCR reaction the products were electrophoretically separated on 5% polyacrylamide gels. The gels were silver stained (17) and photographed. The DNA bands were scored as present or absent. Only bands with good resolution were considered. These data were used to determine the pairwise genetic distances among the isolated colonies in relation to the original strains according to the UPGMA method (Unweighted Pair-Group Average) using the statistics Program Copyright Statsoft Inc 1995-CNMN06/74.

RESULTS AND DISCUSSION

The dry matter of plants inoculated with the different isolated colonies of *R. tropici* (BR322 and SLA 2.2) and *R. leguminosarum* bv. *phaseoli* (SLP 1.3 and BR 10.026) strains before and after exposure to consecutive thermal shocks was evaluated. A decrease in dry matter of plants inoculated with colonies from original *R. leguminosarum* bv. *phaseoli* SLP 1.3 strain exposed to high temperature was observed (Table 1). The dry matter was significantly different in the plants inoculated with colonies 7 and 10. When compared among themselves the ten colonies isolated from the original SLP 1.3 culture, submitted to the thermal shock, displayed a great variability in dry matter yield of plants inoculated, unlike what was observed in the ten colonies isolated from this strain, not subjected to the thermal treatment (Tukey 5%). Exposure of isolated colonies from BR 10.026 strain to 45°C caused alteration in dry matter in plants inoculated with three out of the ten isolates (Tukey 5%) - Table 1. The dry matter was lower in plants inoculated with colonies 9 and 10 and higher in those inoculated with colony 4. Although there was variation in dry matter both in plants inoculated with colonies submitted or not to high temperature, such variation was more noticeable in plants in which the inoculating colonies were subjected to the thermal treatment. In addition the average dry matter of plants inoculated with isolated colonies from *R. leguminosarum* (SLP 1.3 and BR 10.026) submitted to thermal shock was also lower than that of the control plants inoculated with original strains.

Of the two *R. tropici* strains tested, the BR 322 strain seemed to be less affected by temperature. According to Table 1, the SLA 2.2 strain showed significant differences in dry weight of

Table 1. Dry matter of bean plants inoculated with isolated colonies of *R. leguminosarum* bv. *phaseoli* and *R. tropici* strains before and after exposure to high temperature.

<i>Rhizobium</i> strain	Exposure to high temperature	Isolated colonies number									
		1	2	3	4	5	6	7	8	9	10
Dry matter (mg/plant)											
<i>R. tropici</i>											
SLA2.2	A	326.7 ^{bcd}	*380.3 ^{abc}	337.5 ^{abcd}	327.5 ^{bcd}	500.8 ^a	312.0 ^{cd}	511.8 ^a	*171.8 ^d	221.7 ^{cd}	265.0 ^{cd}
	B	203.0 ^{ab}	*165.5 ^{ab}	356.7 ^a	213.7 ^{ab}	424.8 ^a	285.2 ^{ab}	328.8 ^a	*106.0 ^b	295.0 ^{ab}	176.7 ^{ab}
	C	287.0									
BR322	A	207.8 ^a	289.0 ^{ab}	366.2 ^a	231.7 ^a	357.8 ^a	309.8 ^a	301.7 ^a	296.7 ^a	362.7 ^a	330.5 ^a
	B	234.8 ^{bc}	300.3 ^{abc}	431.8 ^a	249.0 ^{bc}	414.8 ^a	326.7 ^{abc}	384.7 ^{ab}	243.0 ^{bc}	212.2 ^c	278.5 ^{abc}
	C	299.0									
<i>R. leguminosarum</i> bv. <i>phaseoli</i>											
SLP1.3	A	295.0 ^a	295.0 ^a	288.0 ^a	263.0 ^a	308.0 ^a	275.0 ^a	*283.0 ^a	273.0 ^a	265.0 ^a	*255.0 ^a
	B	253.0 ^{abc}	193.0 ^{bc}	275.0 ^{ab}	297.0 ^a	268.0 ^{ab}	270.0 ^{ab}	*188.0 ^{bc}	227.0 ^{abc}	303.0 ^a	*170.0 ^c
	C	302.0									
BR10026	A	228.0 ^{bc}	242.0 ^{bc}	280.0 ^{bc}	*243.0 ^{bc}	240.0 ^{bc}	267.0 ^{bc}	310.0 ^{ab}	212.0 ^c	*400.0 ^a	*288.0 ^{bc}
	B	260.0 ^{ab}	273.0 ^{ab}	208.0 ^{ab}	*298.0 ^{ab}	198.0 ^{ab}	337.0 ^a	250.0 ^{ab}	262.0 ^{ab}	*170.0 ^b	*210.0 ^b
	C	252.0									

- A – Inoculated plants with isolated colonies before exposure to high temperature; B - Inoculated plants with isolated colonies after exposure to high temperature; C - Control plants inoculated with original strains;

*- Significant differences (Tukey 5% probability) values between before and after high temperature exposure;

a, b, c, d - Mean values followed by the same letters not significantly different (Turkey 5% probability) within the colonies group before or after high temperature;

- The values given (plant dry matter) represent of mean data of 4 repetitions.

the plants inoculated with colonies 2 and 8 (Tukey 5%). Significant differences were also noticed in the productivity of plants inoculated within each group of colonies subjected or not to thermal stress. Nevertheless, average production in plants inoculated with the colonies subjected to thermal shock was lower than the average production of those not subjected to this treatment and similar to that of the control plants inoculated with original strain. Differences in dry matter yield were not observed when plants inoculated with colonies from BR 322 strain subjected to high temperature were compared to those inoculated with colonies not subjected to this treatment or to control plants inoculated with the original strain. On the other hand, the variability of the results obtained with plants inoculated with the group of colonies which underwent the thermal treatment was apparently greater and was reflected in the statistically significant differences in the values of dry matter of the plants.

The results presented above confirm the reports that high temperature may alter symbiotic characteristics of *Rhizobium* strains (2,10). It was also noticed that temperature enhanced variability in nitrogen fixation efficacy naturally found within *Rhizobium* strains (17,26) and that such effect varied according to the *Rhizobium* strains. The BR 10.026, SLP 1.3 (*R. leguminosarum* bv. *phaseoli*) and SLA 2.2 (*R. tropici*) were

apparently more affected by this factor than the BR 322 strain (*R. tropici*).

Plasmid profiles of colonies from *R. tropici* (BR 322 and SLA 2.2) strains were identical to the original strain regardless of the temperature treatment, as shown for BR 322 in Fig. 1(i). As for the profiles of colonies from the *R. leguminosarum* bv. *phaseoli* (BR 10.026 and SLP 1.3) strains, alterations were detected after the thermal shocks. As shown by colonies from the BR 10.026 original strain Fig. 1(ii), there was plasmid loss in two colonies without the thermal stress and in eight colonies after exposure to high temperature. Plasmid profiles of the original SLP 1.3 strain and those derived from it were identical when not submitted to the temperature treatment. However, after the heat shock one band was missing in the derived colonies when compared to the original SLP 1.3 strain (data not shown).

Although reports on the instability of the *Rhizobium* strains that nodulate bean plants are common, it has been difficult to detect alterations utilizing solely plasmid profile data. *Rhizobium* strains are able to maintain their plasmids after being exposed to temperatures about 44°C as evidenced in *R. leguminosarum* bv. *trifolii* (1). On the other hand, total or partial losses of cryptic plasmids (plasmids with unknown functions) do not always compromise N₂ biological fixation; and there are reports on

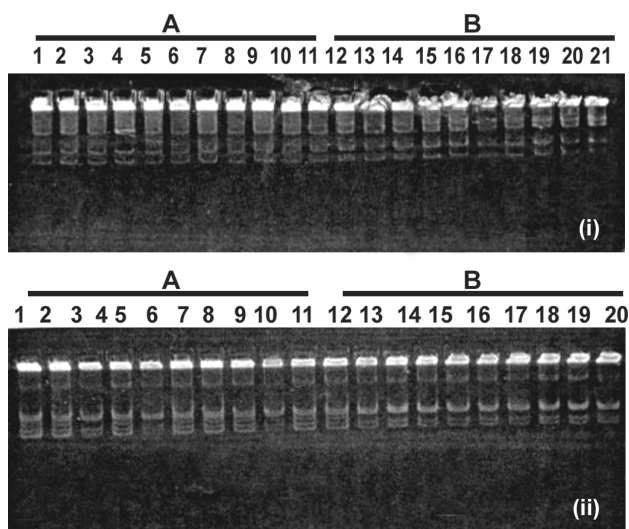


Figure 1. Plasmid profiles of isolated colonies before (A) and after (B) exposure to high temperature. (i) *Rhizobium tropici* BR322; 1= original strain, 2 to 11= isolated colonies (A) and 12 to 21= isolated colonies (B). (ii) *Rhizobium leguminosarum* BR10026; 1= original strain, 2 to 10= isolated colonies (A) and 11 to 20= isolated colonies (B).

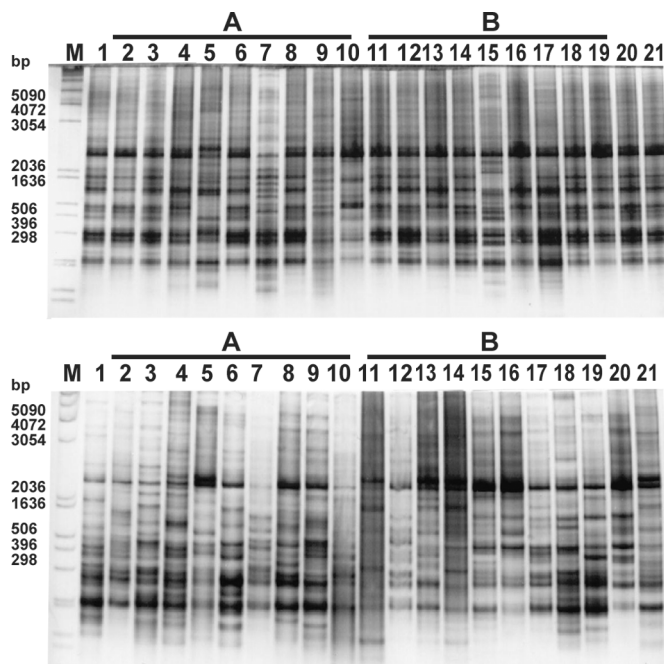


Figure 2. Amplifications of genomic DNA of isolated colonies before (A) and after (B) exposure to high temperature. M - DNA marker (1Kb ladder, BRL), (i) *Rhizobium tropici* SLA2.2; 1= original strain, 2-11= isolated colonies (A) and 12-21= isolated colonies (B) (ii) *Rhizobium leguminosarum* SLP1.3; 1= original strain, 2-11= isolated colonies (A) and 12-21= isolated colonies (B).

Table 2. Average variation in AP-PCR profiles of ten colonies isolated from *Rhizobium* sp strains, before (A) and after (B) high temperature exposure, with three different primers.

<i>Rhizobium</i> strains	primer	Percent of disagreement in relation to original strains	
		A	B
<i>R. tropici</i>			
BR322	OPC10	0.26	0.28
	OPC04	0.27	0.30
	S30	0.24	0.27
SLA2.2	X	0.26	0.28
	OPC10	0.28	0.30
	OPC04	0.30	0.32
	S30	0.28	0.28
	X	0.29	0.30
<i>R. leguminosarum</i> bv. <i>phaseoli</i>			
BR10026	OPC10	0.21	0.32
	OPC04	0.21	0.34
	S30	0.29	0.37
SLP1.3	X	0.24	0.34
	OPC10	0.34	0.42
	OPC04	0.32	0.41
	S30	0.33	0.38
	X	0.33	0.40

Rhizobium strains that had their N₂ fixation ability actually increased after elimination of some of these plasmids (19,23).

Therefore, genotypic alterations that would reflect in phenotypic changes in *Rhizobium* strains capable of nodulating bean seem to originate especially from genomic rearrangements within specific areas of the genome; otherwise they could be due to small deletions not detectable through traditional plasmid visualization techniques or the use of specific DNA probes (20,25). In this sense, the effects of high temperature on potential genetic changes in these organisms were analyzed using the AP-PCR method (Arbitrarily Primed Polymerase Chain Reaction), described by Welsh and McClelland (27). According to these authors and Williams *et al.* (28), this methodology allows not only identification of bacterial strains such as in other conventional methods of DNA analysis, but also allows the detection of modifications at the nucleotide level as evidenced by alterations in the number of amplified segments. Thus AP-PCR provides an useful tool in the study of genetic variation among organisms.

The analysis of the genomic patterns generated by AP-PCR showed alterations for all colonies after they had been exposed to high temperature. However, colonies derived from the *R. tropici* strains seemed to be less affected than the *R. leguminosarum* bv. *phaseoli* strains, as shown in Figs. 2(i) and 2(ii) with the SLA 2.2 (*R. tropici*) and SLP 1.3 (*R.*

leguminosarum bv. *phaseoli*) strains. The rates of genetic variation average (Table 2) obtained for colonies before and after exposure to high temperature in relation to the original strains were higher for *R. leguminosarum* bv. *phaseoli* strains, ranging from 0.24 to 0.34 (BR 10.026) and 0.33 to 0.40 (SLP 1.3). The *R. tropici* strains showed lower genetic variation rates: 0.26 to 0.28 (BR 322) and 0.29 to 0.30 (SLA 2.2). This genetic variation, enhanced by high temperature, affected on response of plant inoculation. The alterations of plant dry matter were especially observed in inoculated plants with the isolated colonies of *R. leguminosarum* bv. *phaseoli* strains submitted to high temperature. Furthermore, these results confirm the high genetic instability among the *Rhizobium* species capable of nodulating bean plants and the greater stability of the *R. tropici* strains shown by other authors and in our previous works (14). These results allow us to recommend especially the use of strains of *R. tropici* in the production of commercial inoculants for bean grown in tropical soil.

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RESUMO

Variabilidade de colônias isoladas de estirpes de *Rhizobium* efetivas na nodulação do feijoeiro, antes de após exposição temperatura elevada

A irregularidade de resposta do feijoeiro à inoculação com rizóbio tem sido atribuída, entre outros fatores, à baixa capacidade competitiva, baixa eficiência em fixar N₂ e instabilidade genética do simbiote. Esta instabilidade genética decorrente de frequências elevadas de rearranjos genômicos e/ou deleções plasmidiais pode ser acentuada após exposição a temperaturas elevadas, comprometendo a utilização dessas estirpes como inoculantes, especialmente nos solos tropicais. Nesse estudo, foi avaliada a variabilidade de colônias isoladas derivadas de estirpes efetivas de *R. leguminosarum* bv. *phaseoli* (SLP1.3 e BR10.026) e *R. tropici* (SLA2.2 e BR322), antes e após exposição à temperatura elevada (4 choques térmicos sucessivos a 45°C). Essa avaliação foi efetuada através da análise de características fenotípicas (medida pela produção de matéria seca das plantas inoculadas) e genotípicas (perfil plasmidial e padrões genômicos via RAPD). Os resultados obtidos indicaram que à temperatura elevada acentuou a variabilidade na performance natural entre as colônias isoladas das estirpes testadas, especialmente da espécie *R. leguminosarum* bv. *phaseoli*. Os perfis plasmidiais das colônias derivadas das estirpes de *R. tropici* antes e após exposição à temperatura

elevada apresentaram-se idênticos entre si e em relação a estirpe original. Os padrões genômicos determinados via RAPD mostraram mais alterações e variação genética nas colônias isoladas das estirpes de *R. leguminosarum* bv. *phaseoli*, indicando que as estirpes de *R. tropici* são mais estáveis e foram menos afetadas pela ação da temperatura.

Palavras-chave: *Rhizobium tropici*, *Rhizobium leguminosarum* bv. *phaseoli*, feijoeiro, solos de cerrado, temperatura elevada.

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