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# EFFECTS OF HIGH TEMPERATURE ON SURVIVAL, SYMBIOTIC PERFORMANCE AND GENOMIC MODIFICATIONS OF BEAN NODULATING *RHIZOBIUM* STRAINS

Patrícia P. Pinto[1], Ruy Raposeiras[1], Andrea M. Macedo[2], Lucy Seldin[3], Edilson Paiva[4], Nadja M.H.Sá[1]*

[1]Departamento de Botânica and [2]Departamento de Bioquímica, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil. 3Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil. 4EMBRAPA, Centro Nacional de Pesquisa de Milho e Sorgo, CNPMS, Sete Lagoas, MG, Brasil.

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## ABSTRACT

High temperatures can affect the survival, establishment and symbiotic properties of *Rhizobium* strains. Bean nodulating *Rhizobium* strains are considered particularly sensitive because on this strains genetic recombinations and/or deletions occur frequently, thus compromising the use of these bacteria as inoculants. In this study *R. tropici* and *R. leguminosarum* bv. *phaseoli* strains isolated from Cerrado soils were exposed to thermal stress and the strains' growth, survival and symbiotic relationships as well as alterations in their genotypic and phenotypic characteristics were analyzed. After successive thermal shocks at 45ºC for four hours, survival capacity appeared to be strain-specific, independent of thermo-tolerance and was more apparent in *R. tropici* strains. Certain *R. leguminosarum* bv. *phaseoli* strains had significant alterations in plant dry weight and DNA patterns obtained by AP-PCR method. *R. tropici* strains (with the exception of FJ2.21) were more stable than *R. leguminosarum* bv. *phaseoli* strains because no significant phenotypic alterations were observed following thermal treatments and they maintained their original genotypic pattern after inoculation in plants.

**Key words**: bean nodulating *Rhizobium* strains, high temperatures

## INTRODUCTION

High soil temperature in tropical regions is one of the major constrains for biological nitrogen fixation in legume crops. Temperatures in these regions average above 40ºC (4) may affect symbiotic relationships, nitrogen content and plant production (2,7). These effects are particularly accentuated in *Phaseolus vulgaris* L. (15) which is a very important staple crop in the tropics. Rhizobia strains of *P. vulgaris* L. from different tropical soils vary in heat tolerance (6,11,19) and high temperatures may affect this bacteria's survival, establishment (8) and symbiotic properties (22). Bean nodulating *Rhizobium* strains are considered to be particularly sensitive because of their genetic characteristics. Genes responsible for nodulation and $N_2$ fixation in these *Rhizobium* strains are located on a single replicon, the symbiotic plasmid (Psym). The genome is complex, containing many reiterated DNA sequences that may provide sites for recombination and genomic rearrangements (5,9). With temperature increases, plasmid deletions (25) and genomic rearrangements (22) may occur, resulting in alterations or in loss of symbiotic properties. Consequently, this genetic instability is compromising these *Rhizobium* strains' use in commercial inoculum production. Total or partial plasmid deletions, under high temperature conditions, have been occurring more frequently in sensitive strains (30). However, individual reaction to a given temperature varies within the strains. Using cured plasmid derivatives of *Rhizobium leguminosarum* bv. *trifolii*, Baldani *et al.* (1) showed that some strains were cured at 28ºC, others at 39ºC and some were not cured even at 44ºC. It was also observed that the more heat tolerant *R. leguminosarum* strains retained their Psym even after longer incubation periods at 37ºC and others maintained $N_2$ fixation even at temperatures above 38ºC (7).

In order to select stable bean nodulating *Rhizobium* strains for tropical conditions, the objetive of this study was to evaluate the effects of heat on growth, survival, symbiotic performance and genomic modifications in effective *R. tropici* and *R. leguminosarum* bv. *phaseoli* strains isolated from Cerrado soils.

## MATERIALS AND METHODS

This study initially analyzed 43 strains of *Rhizobium* isolated from bean plants cultivated in Cerrado soils previously characterized phenotypically as *R. leguminosarum* bv. *phaseoli* (24 strains) and *R. tropici* (16 strains) (16).

**Determination of maximum growth temperature in bean nodulating *Rhizobium* strains isolated from bean plants cultivated in Cerrado soils** - Isolated colonies of each strain were grown up to a final log phase ($10^8$ cells/ml) in yeast mannitol medium (YM) at 29ºC (26). To determine the maximum growth temperature for each strain, transfers from the initial growth were made. Each inoculum corresponding to 1% of the total volume of the medium was incubated on shaker (Lab-line Orbit Environ - Shaker Model 3527) at a optimum temperature of 29ºC, and then at 35, 36, 37, 38, 39, 40, 41 and 42ºC in accordance with the method described in Munevar and Wollum (12). Three replicates of each strain were used. The maximum growth temperature was determined as the temperature at which the strain and the control (grown at 29ºC) had equivalent growth during the same incubation period (12). Growth was monitored by optical density (OD 600nm).

**Survival capacity and *Rhizobium* inoculation tests in bean plants after temperature stress** - *R. leguminosarum* bv. *phaseoli* and *R. tropici* strains with different levels of heat tolerance were inoculated in Cerrado soil and were incubated at 45ºC in a shaker for 4 hours (0.7 ml of inoculum with $10^8$-$10^9$ cells/g of soil). A second treatment consisted of repeating the above procedure 4 times at 48-hour intervals. To evaluate the survival capacity of the strains following high temperature exposure, viable cells were counted before and after each stress temperature using the pour plate dilution technique in YM agar medium. After each thermal shock, other replicates and their controls, with the same number of cells, were placed in Leonard jars (26) containing asseptically cultivated beans (cv. aporé). The experiments were carried out using four replicates of a completely randomized block design. All plants received N-free solution (7) for 30 days after emergence. The plants' dry weight was measured after drying at 65ºC for 48 hours and its nitrogen content determined by microkjeldahl method (23).

**Genomic pattern evaluation of *Rhizobium* spp. strains before and after successive exposures to high temperatures and plant inoculation, using Arbitrarily Primed Polymerase Chain Reaction (AP-PCR)** - *R. tropici* and *R. leguminosarum* bv. *phaseoli* strains with different levels of heat tolerance isolated from Cerrado soils were grown in YM

medium up to the log phase ($10^8$ cells/ml). The total DNA of *Rhizobium* was isolated using the method described by Sá *et al*. (19) which allowed high quality DNA isolation. Amplification was performed in a thermocycler (ERICOMP) in accordance with the technique reported by Steindel *et al*. (21). After two amplification cycles with denaturation at 95ºC for 5 min., annealing at 30ºC for 2 min., and extension at 72ºC for 30 sec., thirty-three amplification cycles were performed with annealing at 40ºC for two min. Final extension was carried out at 72ºC for 5 min. Each reaction mixture contained: 7.3 µl of distilled $H_2O$, 1.0 µl of PCR buffer 10X, 0.5 µl o dNTP (2.5 mM), 0.2 µl of Taq DNA polymerase (Taq Cembiot), 1.0 µl of one decamer primer (Operon Technologies, Inc., Alameda, CA, USA), and 1.0 ng of DNA. The amplification products were eletrophoretically separated on 5% acrylamide gel. The DNA bands were silver stained (20) and photographed.

## RESULTS AND DISCUSSION

Bean nodulating *Rhizobium* strains isolated from Cerrado soils varied in their capacity to tolerate heat when incubated at temperatures between 35ºC to 39ºC (Table 1). This same type of high temperature tolerance have also been reported by several researchers (12,13,14). *R. tropici* strains were more tolerant than *R. leguminosarum* bv. *phaseoli* strains. Among the *R. tropici* strains, 71.4% growth was observed at temperatures 37ºC compared to only 63% growth in *R. leguminosarum* bv. *phaseoli* strains at the same temperatures. These results are consistent with those obtained by Martinez-Romero *et al*. (10) who reported that besides being more heat tolerant, the analyzed *R. tropici* strains were also more stable because they retained Psym for longer periods of incubation at 37ºC.

**Table 1.** Maximum growth temperature of 24 *R. tropici* strains and 19 *R. leguminosarum* bv. *phaseoli* strains isolated from beans cultivated in Cerrado soils

| Species | Maximum temperature (ºC) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 |
| | % *Rhizobium* strains | | | | | | | |
| *R. tropici* | - | 28.6 | 23.8 | 23.8 | 23.8 | - | - | - |
| *R. leguminosarum* bv. *phaseoli* | 9.0 | 28.0 | 9.0 | 27.0 | 27.0 | - | - | - |

Survival capacity, determined by the number of viable cells after exposure to stress temperatures (45ºC for 4 hours), was specific to each strain and to each species independent of their thermo-tolerance (Table 2). These results are more evident when each strains' percent of variation in the number of cells before and after temperature stress is considered (Table 2). For example, BR 322 and SLP 1.3 strains (T. max 39ºC) had the same number of cells before and after exposure to one thermal shock therefore the percent of variation was 0, while FJ 2.2 (T. max 36ºC) had a drastic reduction in cell numbers, corresponding a 41.9% variation (Table 2). More sensitive strains like SLA 2.2 and FJ 2.21 (T. max 36ºC) also had different decreases in the number of viable cells after thermal stress, corresponding to 18.6% and 44.5% average variation, respectively. Mpepereki *et al*. (13) also reported that maximum permissive temperatures and maximum survival temperatures were not significantly correlated in indigenous Rhizobia isolated from tropical soils.

**Table 2.** Survival capacity of *R. tropici* and *R. leguminosarum* bv. *phaseoli* with different thermal tolerance and symbiotic performance in beans after one thermal shock at 45°C for 4 hours.

| *Rhizobium* strain | Max. Growth T °C | Viable cells number/ml ($log_{10}$) | | | Plant dry weight (g/plant) | | Plant total N (mg/plant) | |
|---|---|---|---|---|---|---|---|---|
| *R. tropici* | | A | B | C | D | E | D | E |
| BR 322 | 39 | 8.90 | 8.90 | 0.0 | 0.527 | 0.498 | 220 | 198 |
| FJ 2.2 | 39 | 8.60 | 5.00 | 41.9 | 0.450 | 0.446 | 229 | 270 |
| SLBR 3.12 | 39 | 9.04 | 5.60 | 38.0 | 0.418 | + | + | + |
| SLP 4.9 | 38 | 9.18 | 5.48 | 40.3 | 0.520 | + | + | + |
| FJ 2.21 | 36 | 8.60 | 4.78 | 44.5 | 0.739* | 0.399* | 286* | 143* |
| SLA 2.2 | 36 | 8.60 | 7.00 | 18.6 | 0.375 | 0.422 | 239 | 270 |
| SLA 3.2 | 36 | 9.48 | 7.00 | 26.1 | 0.522 | 0.511 | 270 | 254 |
| *R. leguminosarum* bv. *phaseoli* | | | | | | | | |
| SLP 2.10 | 39 | 9.30 | 8.30 | 10.8 | 0.382 | 0.387 | 249 | 278 |
| BR 322 | 39 | 8.90 | 8.90 | 0.0 | 0.527 | 0.498 | 220 | 198 |
| FJ 2.2 | 39 | 8.60 | 5.00 | 41.9 | 0.450 | 0.446 | 229 | 270 |
| SLBR 3.12 | 39 | 9.04 | 5.60 | 38.0 | 0.418 | + | + | + |
| SLP 4.9 | 38 | 9.18 | 5.48 | 40.3 | 0.520 | + | + | + |
| FJ 2.21 | 36 | 8.60 | 4.78 | 44.5 | 0.739* | 0.399* | 286* | 143* |
| SLA 2.2 | 36 | 8.60 | 7.00 | 18.6 | 0.375 | 0.422 | 239 | 270 |
| SLA 3.2 | 36 | 9.48 | 7.00 | 26.1 | 0.522 | 0.511 | 270 | 254 |
| *R. leguminosarum* bv. *phaseoli* | | | | | | | | |
| SLP 2.10 | 39 | 9.30 | 8.30 | 10.8 | 0.382 | 0.387 | 249 | 278 |
| SLP 5.8 | 39 | 8.90 | 8.78 | 1.4 | 0.394 | 0.393 | 273 | 263 |
| SLA 1.5 | 39 | 8.30 | 5.30 | 36.1 | 0.393* | 0.283* | 314* | 201* |
| SLP 1.3 | 39 | 8.30 | 8.30 | 0.0 | 0.485 | 0.465 | 218 | 204 |
| SLP24.1 | 38 | 8.95 | 7.00 | 21.8 | 0.449 | 0.504 | 253 | 265 |
| BR 10.026 | 38 | 8.60 | 6.48 | 24.7 | 0.330 | 0.304 | 265 | 253 |
| BR 10.028 | 36 | 8.85 | 6.30 | 28.8 | 0.375 | 0.373 | 292 | 245 |
| SLP 4.4 | 36 | 8.95 | 6.78 | 24.3 | 0.361 | 0.356 | 293 | 288 |

A: Number of cells before thermal shock
B: Number of cells after thermal shock
C: Average % of variation between A and B
D: Control strain
E: Strain submitted to thermal shock

* Significant differences (Duncan 5% probability) values given represent the mean data of 4 repetitions.
+ SLBR 3.12 and SLP 4.9 strains lost their ability to nodulate after thermal shock, therefore plant dry weight and total N were not analyzed.

Differences in the effect of high temperatures on strains' symbiotic properties are showed in Table 2. Two strains, SLBR 3.12 and SLP 4.9, lost their ability to nodulate, and two strains, *R. Tropici* - FJ 2.21 and *R. leguminosarum* bv. *phaseoli* - SLA 1.5, had decreased nitrogen fixation levels as measured by plant dry weight and total N after only one thermal shock (Table 2). The latter two strains presented high thermo-tolerance (T. max 39ºC) but low survival capacity compared to all other analyzed strains.

Certain strains with contrasting characteristics in relation to temperature were submitted to 4 successive thermal shocks at 48-hour intervals. Their survival capacity was evaluated after each shock and then inoculated in bean plants. Again, the results showed that survival capacity was specific to each strain and species independent of their thermo-tolerance (Table 3). While cell numbers in FJ 2.21 were drastically reduced, corresponding to 77.7% average variation, BR 10,026 and SLP 2.10 were practically not affected (7.0% and 7.5% respectively), and BR 322 had a slight decrease (29.2% variation).

**Table 3.** Survival capacity of *R. tropici* and *R. leguminosarum* bv. *phaseoli* with different thermal tolerance and symbiotic performance in beans after four thermal shocks at 45°C for 4 hours.

| *Rhizobium* strain | Max. growth T°C | Viable cells number/ml weight (g/plant) | | | | | | Plant dry ($log_{10}$) | | Number of nodule/plant | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *R. tropici* | | A | B | C | D | E | F | G | H | G | H |
| SLA 2.2 | 36 | 8.60 | 7.00 | 6.30 | 7.70 | 5.95 | 30.8 | 0.40 | 0.47 | 53 | 53 |
| SLA3.2 | 36 | 9.48 | 7.00 | 6.30 | 5.70 | 5.60 | 40.9 | 0.44 | 0.43 | 86 | 51 |
| FJ 2.21 | 36 | 7.60 | 5.00 | 3.60 | 1.70 | 1.70 | 77.7 | 0.60 | 0.50 | 77 | 46 |
| BR 322 | 39 | 8.90 | 8.90 | 8.30 | 7.70 | 6.30 | 29.2 | 0.40 | 0.43 | 85 | 50 |
| R. leguminosarum bv. *phaseoli* | | | | | | | | | | | |
| SLP 1.3 | 39 | 8.30 | 8.30 | 6.90 | 7.60 | 6.48 | 21.9 | 0.63 | 0.52 | 29 | 63 |
| SLP 2.10 | 39 | 9.30 | 8.30 | 8.30 | 8.30 | 8.60 | 7.5 | 0.77* | 0.48* | 92* | 38* |
| SLA 1.5 | 39 | 8.30 | 5.00 | 6.48 | 5.90 | 5.30 | 36.1 | 0.71* | 0.44* | 100* | 60* |
| BR 10.026 | 38 | 8.60 | 8.48 | 8.48 | 8.84 | 8.00 | 7.0 | 0.55 | 0.49 | 69 | 45 |

A: Number of cells before thermal shock
B: Number of cells after 1st thermal shock
C: Number of cells after 2nd thermal shocks
D: Number of cells after 3rd thermal shocks
E: Number of cells after 4th thermal shocks
F: Average % of variation between A and E
G: Control strain
H: Strain submitted to four thermal shocks

* Significant differences (Duncan, 5% of probability), values given represent the mean data of four repetitions.

Plant dry weight and number of nodules are shown in Table 3. Among the strains analyzed, the SLP 2.10 and SLA 1.5 strains of *R. leguminosarum* bv. *phaseoli* showed the largest significant differences in dry weight after heat exposure (Duncan 5% of probability). The remaining strains, did not showed statistically significant differences in dry weight and total N accumulation (Table 2). In relation to nodule numbers, significant differences were observed only in *R. leguminosarum* bv. *phaseoli* SLP 2.10 and SLA 1.5 strains. Taken together, these results suggest that the *R. tropici* strains were more stable.

No relationships were evident between thermo-tolerance, survival capacity and $N_2$ fixation after thermal stress within each species tested under axenic condition. Under such conditions, the number of viable cells apparently did not affect the dry weight and total N accumulation, contrary to what is expected in soils where competition with other strains and microorganisms naturally occurs.

High temperatures also affected the Rhizobia genome (3,27), especially in fast growing rhizobia like bean nodulating strains (22). The effects of high temperatures on genetic modifications were investigated using AP-PCR. According to Welsh and McClelland (28) and Williams *et al*. (29), this method is particularly useful in identifying strains within the same species and in detecting modifications in DNA nucleotides. In addition, AP-PCR provides an efficient assay for genetic variation studies in microorganisms. In this study, polymorphisms in the amplification of DNA products using the primer S34 (5' GGT TCG ATT GGG GGT TGG TGT AAT ATA 3') (Fig. 1), confirmed at the genetic level, alterations that were observed at the phenotypic level in *R. leguminosarum* bv. *phaseoli* SLP 2.10 (lanes 12 and 13) and SLA 1.5 (lanes 15 and 16). Other strains of this species (SLP 1.3 and BR 10,026) did not have significant differences in dry weight production, (Table 3) but they presented changes in their genomic patterns (Fig. 1). In this case, these alterations probably did not affect genes related to symbiosis or $N_2$ fixation. The BR 322, SLA 2.2 and SLA 3.2 strains of *R. tropici* did not have phenotypic alterations and they maintained similar PCR banding patterns after high temperature exposure and plant inoculation (Fig. 1). Similar results were obtained with other AP-PCR tests using 6 different primers and the reproducibility of the results was verified in independent experiments.

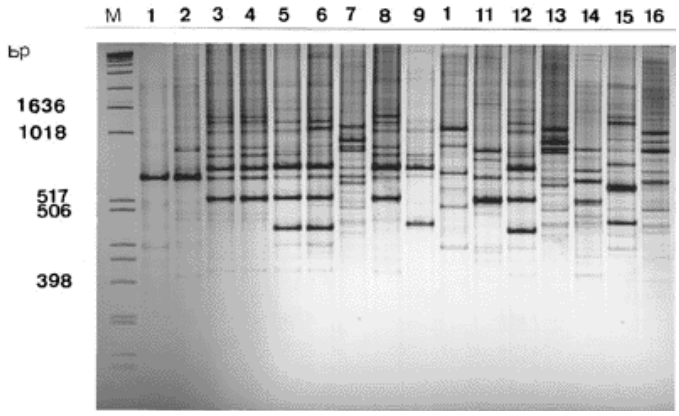


**Figure 1.** Amplification of genomic DNA from *R. tropici* (1-8) and *R. leguminosarum* bv. *phaseoli* (9-16) strains before (TB) and after thermal shock (TA) with random primer S34. M-DNA marker (1Kb ladder; Bethesda Research Laboratories). 1-BR322 (TB); 2-BR322 (TA); 3-SLA2.2 (TB); 4-SLA2.2 (TA); 5-SLA3.2 (TB); 6-SLA3.2 (TA); 7-FJ2.21 (TB); 8-FJ2.21 (TA); 9-BR10.026 (TB); 10-BR10.026 (TA); 11-SLP2.10 (TB); 12-SLP2.10 (TA); 13-SLP1.3 (TB); 14-SLP1.3 (TA); 15-SLA1.5 (TB); 16-SLA1.5 (TA).

High genetic variability due to reiterations in the genome of these bacteria caused deletions of certain genome elements at the frequency of $10^2$-$10^3$ (5). Flores *et al.* (5) reported that after cultivating *R. leguminosarum* bv. *phaseoli* CFN 285 strain for one year in a laboratory free of stress factors, nearly 35% of the cells presented differences compared to original cells.

More detailed studies to explore these genetic variations, especially related to high temperature, are currently underway in this laboratory. Results of analysis of some colonies isolated from BR 322 and SLA 2.2 strains of *R. tropici* and from SLA 1.5 and SLP 2.10 strains o *R. leguminosarum* bv. *phaseoli* show variable reactions in nodulation capacity and nitrogen fixation within and between colonies of the same strain following 4 thermal shocks. Some colonies of strains of *R. leguminosarum* bv. *phaseoli* lost their nodulation capacity. Analysis of DNA amplification products from these colonies showed on the genetic level, the variations observed in the phenotypic characteristics and the profiles of the colonies from *R. leguminosarum* bv. *phaseoli* strains were more heterogeneous compared to those of *R. tropici* strains (17).

The results described here indicate significant genetic stability in *R. tropici* strains compared to *R. leguminosarum* bv. *phaseoli* strains. Moreover, the strategies used in this study to evaluate survival capacity, $N_2$ fixation performance and genetic stability after thermal stress could be useful in selecting efficient, stable *Rhizobium* strains to be used as inoculum for bean plant cultivation in tropical soil conditions.

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## RESUMO

### Sobrevivência, fixação de nitrogênio e modificações genéticas em estirpes de *Rhizobium* sp. efetivas na nodulação do feijoeiro, expostas à altas temperaturas.

Altas temperaturas podem afetar a sobrevivência, estabelecimento e as propriedades simbióticas em estirpes de *Rhizobium*. As estirpes capazes de nodular o feijoeiro têm sido consideradas particularmente sensíveis, porque nessas estirpes é comum a ocorrência de recombinações e/ou deleções genômicas comprometendo, muitas vezes, a sua utilização como inoculantes. Neste trabalho, procurou-se avaliar a capacidade de crescimento e

sobrevivência em temperaturas elevadas de estirpes de *Rhizobium* efetivas na fixação de nitrogênio no feijoeiro isoladas dos cerrados, bem como avaliar suas características fenotípicas e genotípicas após choque térmico. A capacidade de sobrevivência à temperaturas elevadas, avaliada após choques térmicos sucessivos (45ºC por 4 horas) mostrou ser uma característica própria de cada estirpe, independente de sua termotolerância, que aparentemente foi mais acentuada nas estirpes de *R. tropici*. Algumas estirpes de *R. leguminosarum* bv. *phaseoli* mostraram alterações significativas (Duncan 5% de probabilidade) nas suas características fenotípicas (produção de matéria seca) após choques térmicos e nos seus padrões genômicos evidenciados pela técnica de AP-PCR. As estirpes de *R. tropici* foram aparentemente mais estáveis não sendo detectadas alterações fenotípicas significativas e com exceção da estirpe FJ2.21, após choque térmico e inoculação na planta hospedeira, mantiveram o padrão genômico original.

**Palavras-chave**: Estirpe de *Rhizobium* associadas ao feijoeiro, temperatura elevada.

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* Corresponding author. Mailing address: Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas, Departamento de Botânica, Pampulha. CEP: 31270-901 - Belo Horizonte, MG, Brasil. FAX: (+5531) 499-2673. E-mail: nadja@mono.icb.ufmg.br

***SBM***

**Av. Prof. Lineu Prestes, 1374**
**Cidade Universitária**
**05508-900 São Paulo SP - Brazil**
**Tel. / Fax: (55 11) 813.9647**

e-Mail

bjm@sbmicrobiologia.org.br