Rev. Microbiol., São Paulo, **24**(1): 38-48, 1993

SELECTION AND CHARACTERIZATION OF *RHIZOBIUM* SPP. STRAINS STABLE AND CAPABLE IN FIXING NITROGEN IN BEAN (*PHASEOLUS VULGARIS* L.)

Nadja M. Horta de Sá[1]
Maria Rita M. M. L. Scotti[1]
Edilson Paiva[2]
Avílio A. Franco[3]
Johanna Döbereiner[3]

SUMMARY

The variability in N_2 fixation effectiveness was determined by acetylene reduction tests of nodules formed by strains of *Rhizobium* spp before and after exposure to high temperatures. Nodules formed by strains more tolerant to high temperatures (*R. leguminosarum* bv. *phaseoli* and *R. tropici*) did not suffer any alterations in nitrogenase activity, plant dry weight and total plant nitrogen fixed. The protein pattern (eletrophoresis SDS-PAGE) differenciated strains among and within both species. The DNA hybridization using a nif probe marked through nick translation (biotin 14 dATP), when total DNA was digested with *Eco* RI, differenciated species of *R. leguminosarum* bv. *phaseoli* from *R. tropici* Polymorphism among the strains of *R. leguminosarum* bv. *phaseoli* after digestion with *Bam* HI and among *R. leguminosarum* bv. *phaseoli* and *R. tropici* after digestion with *Hind* III were also observed. Exposure to high temperature did not affect the protein or genomic patterns or nitrogenase activity. This may indicate that strains from both species (*R. leguminosarum* bv. *phaseoli* and *R. tropici*) that are tolerant to high temperature are also more genetic stable.

Key words: *Phaseolus vulgaris* L., *Rhizobium* termo tolerant, nitrogenase activity, protein and genomic patterns.

INTRODUCTION

Bacteria of the genus *Rhizobium* interact with legumes eliciting a simbiotic process, where nitrogen fixed by the bacterium is assimilated by the plant. However, it has been frequently observed that the strains of *Rhizobium leguminosarum* biovar *phaseoli* used in bean inoculation, present nodulating and/or simbiotic variability. On the other hand, the strains of *Rhizobium* that nodulate bean have been described as belonging to an heterogeneous group according to different criteria of characterization like protein patterns (9), antibiotic resistance (1), serological groups (18), multilocus

1. Departamento de Botânica - Instituto de Ciências Biológicas da UFMG - Cidade Universitária - 31270-901 - Belo Horizonte - MG - Brasil
2. EMBRAPA - CNPMS - 35700-000 - Sete Lagoas - MG - Brasil
3. EMBRAPA - CNPBS - 23851-970 - Seropédica - RJ - Brasil

enzyme eletrophoresis (17), DNA - DNA hibridization patterns (7) and plasmid profiles (12).

The genetic information which controls the symbiotic activity of *Rhizobium* strains nodulating beans is located on plasmids (12). These symbiotic plasmids (Psym) hold from one copy to several copies of the gene coding for nitrogenase (nif genes). The *Rhizobium* strains containing multiple copies of nif genes on plasmids have a narrow nodulation host range and were previously distinguished as type I, while those containing single nif genes copies on the plasmids, nodulate bean and *Leucaena* spp. and were distinguished as type II (10). Recently Martinez et al. (13), on the basis of the results of genetic analysis and phenotypic characteristics, considered the type II strains as a new species - *Rhizobium tropici*.

The reiterated regions of the nif genes seem to be normally necessary for the whole expression of the symbiotic effectiveness (19) and they can represent sites of recombinations producing genomic rearrangements which constitute the molecular basis of the variability and loss of the symbiotic properties in these strains of *Rhizobium* (4, 21, 24). Flores et al. (4) have analysed direct descendents of a single cell of *R. leguminosarum* bv. *phaseoli* after sucessive cultivations under normal laboratory conditions and in some cases, when they used recombining plasmids pMF101 and pMF122 as probes, they have detected alterations in their genome in relation to the patterns of the original cells.

The genetic instability of *R. leguminosarum* bv. *phaseoli* presents a serious problem for inoculum production. This factor is agravated in tropical regions where stress conditions, such as high temperatures, can favor the instability through rearrangements (4, 21) or curing of plasmids (11, 25). On the other hand, Hungria & Franco (6) have observed high levels of nitrogen fixation of beans grown at temperature above 38°C when inoculated with rhizobia isolated from *Leucaena*.

Aiming at selecting strains of *Rhizobium* efficient and stable in nitrogen fixation in beans, experiments were developed in order to compare variability of nodulation and N_2 fixation, as well as protein and genomic patterns before and after growing several generations of each strain at the highest temperature they were able to grow.

MATERIALS AND METHODS

Experiment I - Nineteen strains of *Rhizobium* spp. from "Centro Nacional de Pesquisas em Biologia do Solo" (CNPBS) and the "Centro de Pesquisas Agropecuárias dos Cerrados" (CPAC) collections, were tested for their N_2 fixation capacity in *Phaseolus vulgaris* L. cv. Negro Argel in Leonard jars (23). Inoculation (2 seeds/jars) was performed with 2 ml of cells grown in yeast mannitol medium (YM) to the final logarithmic phase. Three repetitions were used, and a treatment were *Leucaena* was used as a host plant was also included. After 28 days of growth, the plants were harvested and 10 nodules/plant of uniform size (fresh weight between 5.0 and 7.0 mg), chosen at random, were detached from the roots and placed in flasks hermetically closed, where 10% of the atmosphere was substituted by acetylene. After incubation for 10 minutes the ethylene produced was measured using a gas chromatograph (Variant - 2440, flame ionization detector, column poropak N, carrier N_2).

Experiment II - Determination of the maximum temperature for growth - selection "*in vitro*" of clones growing at high temperatures.

Isolated colonies of each strain were grown up to the final log phase (10^8 cells/ml) in YM at 29°C. For determining the maximum temperature of growth for each strain, transfers from the initial growth were made, corresponding each inoculum to 1% of the total volume of the medium and incubated on a shaker at 29°C (optimum temperature) and at 35, 36, 37, 38, 39, 40, 41 and 42°C. The strains were grown again twice at the maximum temperature they would grow up to the final log phase and stored thereafter at 4°C.

Experiment III - Six strains, the most tolerant to high temperature, after exposure to their maximum growth temperature, as well as the parent strains, were tested and compared for their variability of N_2 fixation after analysis by acetylene reduction (ARA) in the same condition of Experiment I. Subsequently isolations from 10 nodules of each plant were performed according to Vincent (23). The dry weight of the plants was determined after drying at 65°C for 48 hours and its nitrogen content determined by microkjeldahl method (22).

Experiment IV - Protein pattern determinations. SDS-PAGE - Sodium dodecyl sulfate polyacrylamide gel eletrophoresis was conducted according to Laemmli (8) with modifications as described below. Wild type strains (especially the most tolerant to high temperature) of the *R. leguminosarum* bv. *phaseoli* and *R. tropici*, both before and after growth at high temperature, and their isolates from nodules of different levels of

ARA were grown in YM up to the beginning of the stationary phase, corresponding approximately to 10^9 cells/ml. The cells were centrifugated (8000 x g for 10 min at 4°C), washed and suspended in buffer PBS pH 7.4. Then they were disrupted through sonication (sonifier Branson, output-3, duty cycle 75%) for 4 min at intervals of 2 min. Desnaturized samples which were obtained by 5 min heating, in buffer Tris 62.5 mM, 2.3% SDS, pH 6.8 added of mercaptoetanol 5% were used. The amount of protein added to each well was 18 mg. The protein concentration was determined according to the procedure described by Bradford (2). The concentration of the acrylamid gradient used, varied from 7.5 to 17.5%. The running buffer used was the Tris-glycine with SDS (3.03 g Tris HCl; 14.41 g glycine and 1.0 g SDS per litre). The samples were submited to an initial current of 12 mA followed by application of 24 mA after penetration on the stacking-gel. At the end of the eletrophoresis the gel was dyed with comassie blue 0.2%.

Experiment V - Genomic pattern determinations. Wild type strains, especially the most tolerant to high temperature, after exposure to their maximum growth temperature, and their isolates from nodules of different levels of ARA, were grown in YM up to the log phase (10^8 cells/ml). The total DNA of the strains was isolated, using Hahn & Hennecke's method (5) with the following modification. After precipitation of the DNA, by addition of 1/10 vol 5M NaCl and 2 volumes ethanol (-20°C) it was directly transferred to 76% ethanol, 0.2 M NaOAC and then resuspended and allowed to stay overnight in TE buffer (10 mM Tris, 1 mM EDTA pH 8.0).

After spectrophotometric padronization, the total DNA was digested by the restriction enzymes *Eco* RI, *Hind* III and *Bam* HI and submited to eletrophoresis in agarose gel 0.6% 30 volts/16mA for 18 hours. Then, the DNA was transferred to membranes of nitrocellulose, according to Sambrook et al. (20). The probe used was a plasmid recombinant; pcQ15 which carries a 4.7 Kilobase *Eco* RI insert with nitrogenase structural genes (nif genes). The probe was marked through nick translation in the presence of 14 dATP-biotin. The labelling was made according to the description of the DNA Detection System Instruction Manual - BRL, being the biotinilated probe separated in sephadex G-50 columns. The hybridization procedure of the DNA was done according to Medeiros et al. (14) at the temperature of 42°C for 18 h and the final detection of the probe connection to the DNA target was done according to the Blue Gene Kit of BRL - non radioactive nucleic acid detection system.

RESULTS AND DISCUSSION

Determination of the nitrogen fixation variability of Rhizobium *strains capable of nodulating bean.* - Among the nineteen strains tested, all nodulated bean, six did not nodulate *Leucaena* (*R. leguminosarum* bv. *phaseoli*) and thirteen nodulated both bean and *Leucaena* (*R. tropici*) (Tab. 1). The acetylene reduction activity evidenced a great variability among individual nodules of the same strain (data not show), following a normal distribution, similarly to what was found with soybean nodules by Peres et al. (16). Differences were also observed in the mean activities of nodules of each strain (Tab. 1). Three strains of each species were able to grow at temperatures equal or superior to 38°C (tab. 1). These strains more tolerant to heat, after growing twice at the maximum temperature (38 or 39°C), were grown at 29°C and were inoculated into beans for comparison of their N_2 fixation activities with their parent strains. High temperatures, among other stress conditions, can increase the frequency of alterations verified in the characteristics of effectiveness of N_2 fixation in *Rhizobium* capable of nodulating bean (21). However in the present experiment acetylene reduction activity of the strains more tolerant to heat from both species of *Rhizobium* were not altered. The same was also verified for plant dry weight and total plant nitrogen (tab. 2). The distribution of the individual nodule activity of these strains (Fig. 1) was quite similar, except as CPAC H_{14} (*R. leguminosarum* bv. *phaseoli*) and CENA CO_5 II (*R. tropici*) which presented less homogeneous distributions when compared to the parental strains. However, their mean activities did not differ significantly either (table 2).

Protein patterns - The comparison of the protein patterns showed differences both among the strains of *Rhizobium* of the same species and between the species. The strains of *R. tropici* presented more homogeneous profiles while in *R. leguminosarum* bv. *phaseoli* differences were evident in a great number of polypeptides (Fig. 2). Strains of both species, when submitted to high temperatures, did not present alterations in their protein patterns in relation to the parent strain (Fig. 3). Isolates of nodules of different levels of

TABLE 1 - Nodulation of bean (*Phaseolus vulgaris* L.) and *Leucaena leucocephala* and acetylene reduction of individual nodules inoculated with 6 strains of *R. leguminosarum* bv. *phaseoli* and 13 strains of *R. tropici* as well as their maximum growth temperature.

Strains	Bean Nodulation	*Leucaena* Nodulation	Maximum growth temperature (°C)	Nitrogenase activity (a) (nmoles C_2H_4/h/nodule)
R. leguminosarum bv. *phaseoli*				
Br 365 (CNPAF 146)	+	–	37	104.13
Semia 476 (CPAC H_{19})	+	–	38	91.01
CPAC H_{30} (IPAGRO 1102)	+	–	37	62.02
CPAC H_{35} (IPAGRO 1378)	+	–	39	48.42
CPAC H_{23} (V_{23} RGS)	+	–	37	37.80
CPAC H_{14}	+	–	39	29.75
R. tropici				
CIAT 899 (Br 322)	+	+	38	132.38
UFP 491 (CPAC H_{21})	+	+	37	125.38
CPAC H_{20}	+	+	38	105.02
Na 82 (Br 10.013)	+	+	37	80.55
Br 817 (NGR8)	+	+	36	51.56
Br 818 (TAL1145)	+	+	35	45.06
CENA CO_5 II (Br 266, Semia 492)	+	+	39	36.86
Car 22 (Br 10.014)	+	+	37	30.01
CPAC H_{36}	+	+	37	27.83
USA 1070 (CPAC H_{38})	+	+	36	22.48
CPAC H_{26} (IPAGRO 1020)	+	+	36	22.30
Br 814 (DF10)	+	+	36	14.75
CFN 299	+	+	<35	7.04

(a) Means of 60 nodules/strain.

TABLE 2 - Mean activity of acetylene reduction in 60 bean nodules produced by strains of *R. tropici* and *R. leguminosarum* before and after growth at high temperatures, as well as plant dry weight and total plant N.

Strains	Growth temperature (°C)	Acetylene reduction (nmoles C_2H_4/h/nodule)	Plant dry weight (g/plant)	Total Plant N (mg/plant)
CPAC H_{20} (a)	29	58.75	0.61	142
CPAC H_{20} (b)	38	50.02	0.58	110
CENA CO_5 II (a)	29	38.14	0.74	180
CENA CO_5 II (b)	39	39.30	0.70	161
CIAT 899 (a)	29	107.30	0.82	171
CIAT 899 (b)	38	105.73	0.85	180
CPAC H_{14} (b)	29	36.6	0.52	61
CPAC H_{14} (b)	39	32.7	0.43	52
Semia 476 (a)	29	40.10	0.66	159
Semia 476 (b)	38	35.32	0.53	120
CPAC H_{35} (a)	29	32.40	0.44	122
CPAC H_{35} (b)	39	35.15	0.34	91

(a) Parent strain and (b) strains grown twice in the temperature indicated. No significant differences (Ducan, 5% of probability) in the activities of acetylene reduction, dry weight and total N were observed.

acetylene reduction activity of a strain, did not show any variation in protein patterns either (data not shown).

Genomic patterns - The results of hybridization of the total DNA of the parent strains of *Rhizobium*, after digestion with *Eco* RI, showed differences among the DNA patterns between strains of *R. leguminosarum* bv. *phaseoli* and *R. tropici*, but not among strains of the same species. Confirming the information of Martinez et al. (12, 13) that the strains of *R. leguminosarum* bv. *phaseoli* present multicopies of the nif genes while those of *R. tropici* present only one copy (Fig. 4). However, when the total DNA of these strains were digested with *Bam* HI (Fig. 5), the DNA of the strains of *R. leguminosarum* bv. *phaseoli* present-

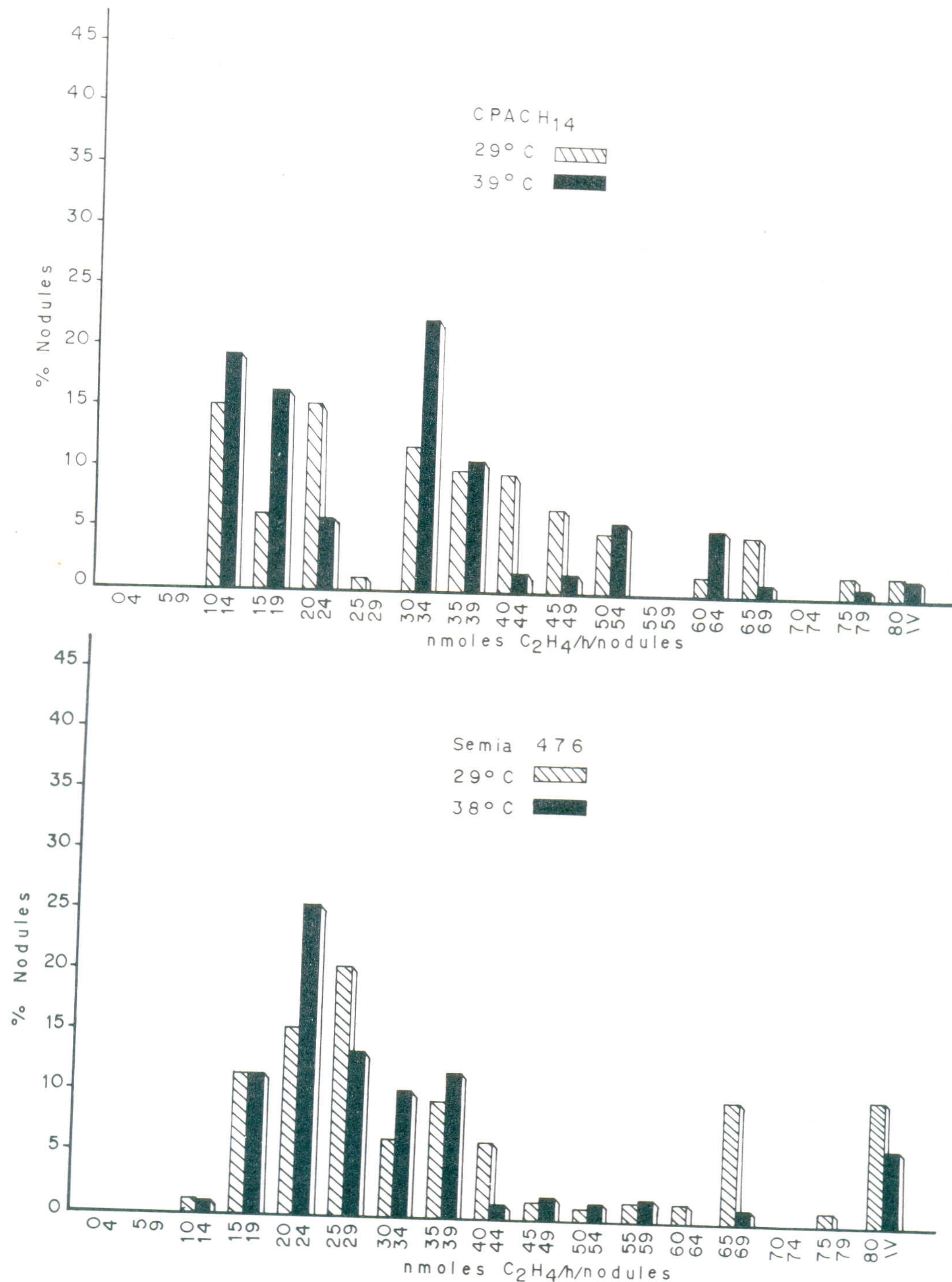
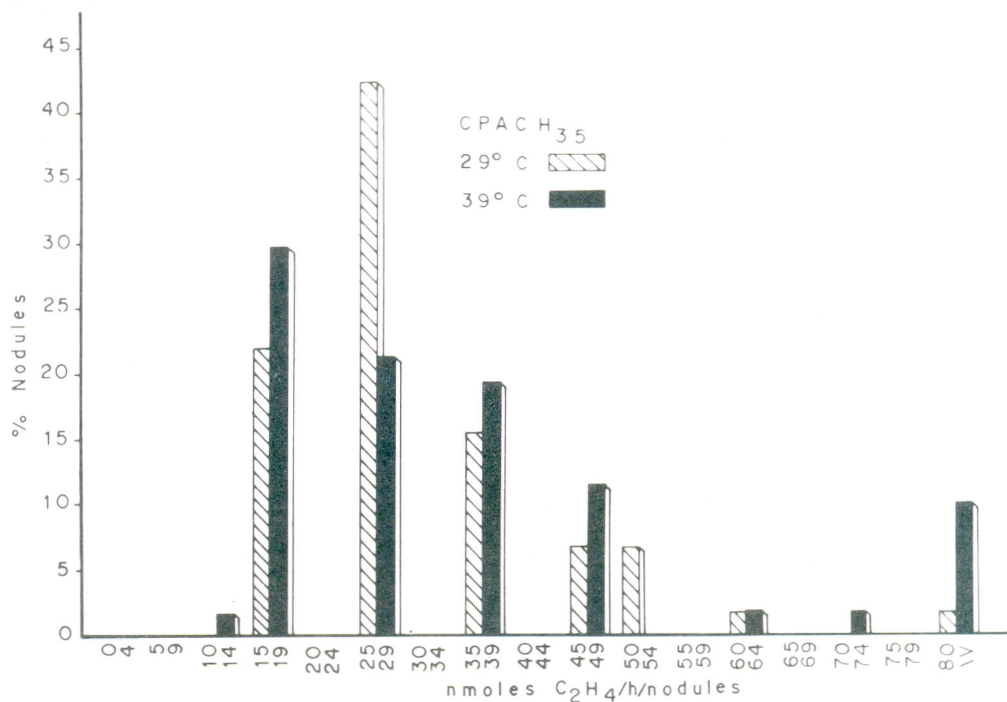
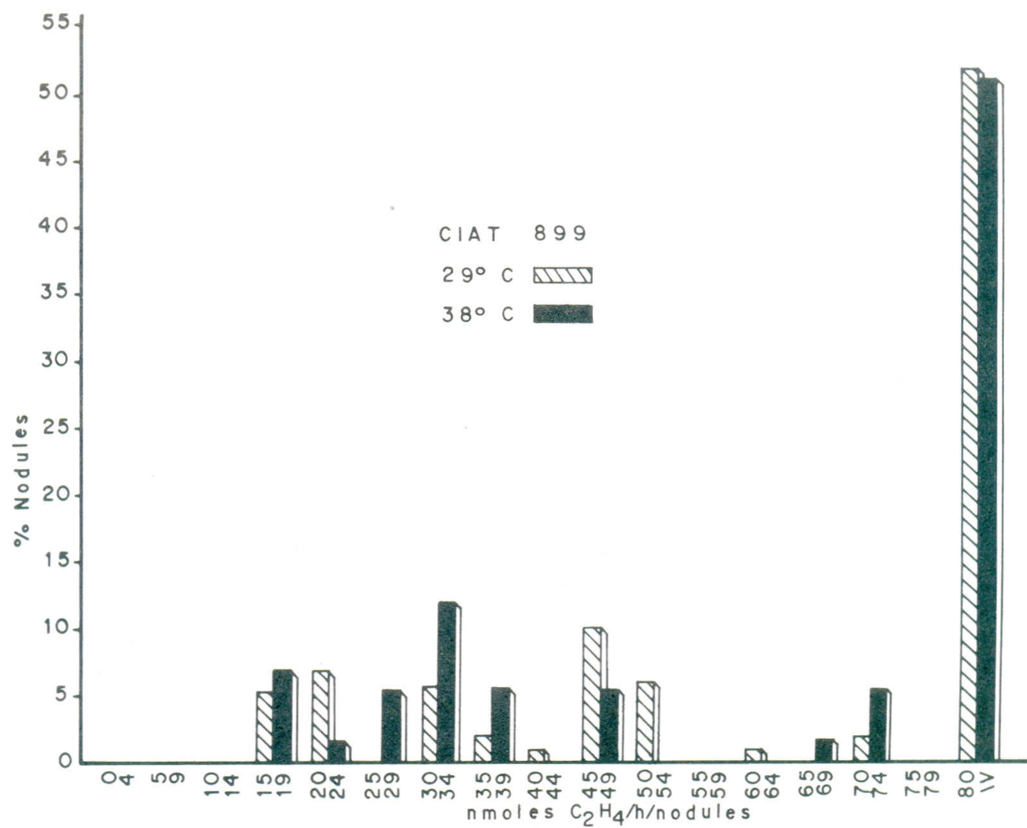


FIGURE 1 - Activity of acetylene reduction in 60 individual nodules of bean after inoculation with *R. leguminosarum* bv. *phaseoli* strains: Semia 476 parent (29°C), Semia 476 exposed to high temperature (38°C); CPAC H_{14} parent (29°C), CPAC H_{14} exposed to high temperature (39°C); CPAC H_{35} parent (29°C), CPAC H_{35} exposed to high temperature (39°C) and *R. tropici* strains: CIAT 899 parent (29°C), CIAT 899 exposed to high temperature (38°C); CENA CO_5 II parent (29°C), CENA CO_5 exposed to high temperature (39°C); CPAC H_2O parent (29°C), CPAC H_2O exposed to high temperature (38°C).

Continuação da **FIGURA 1**





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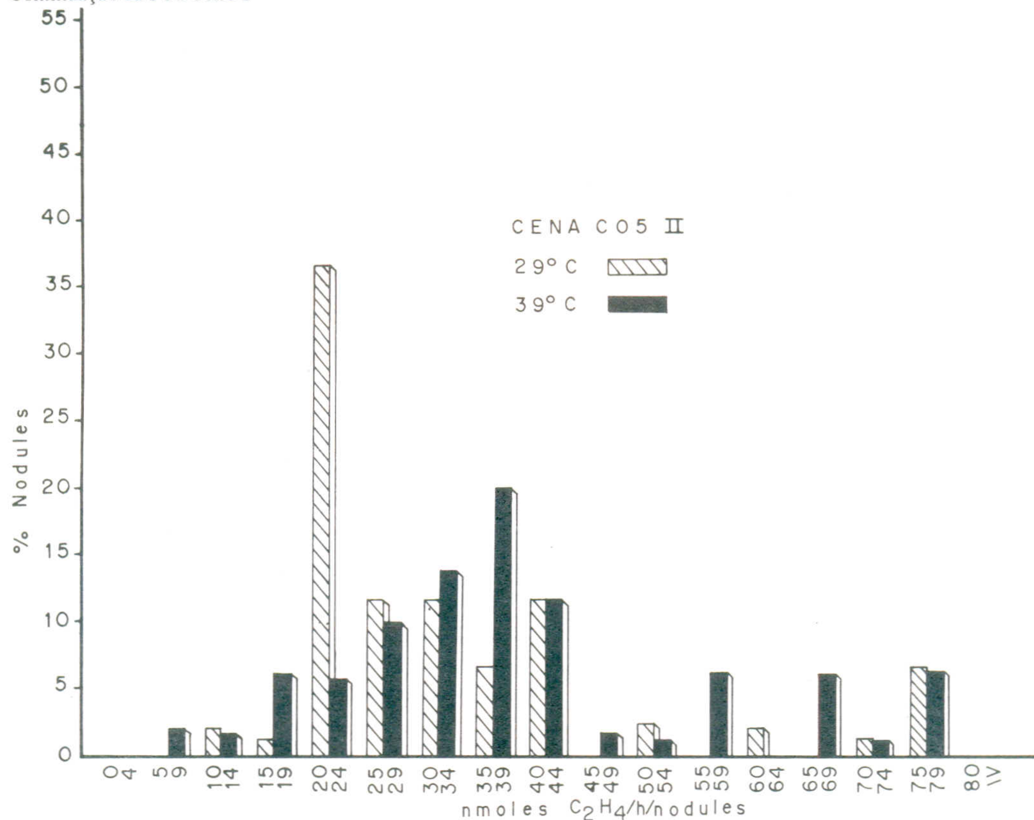
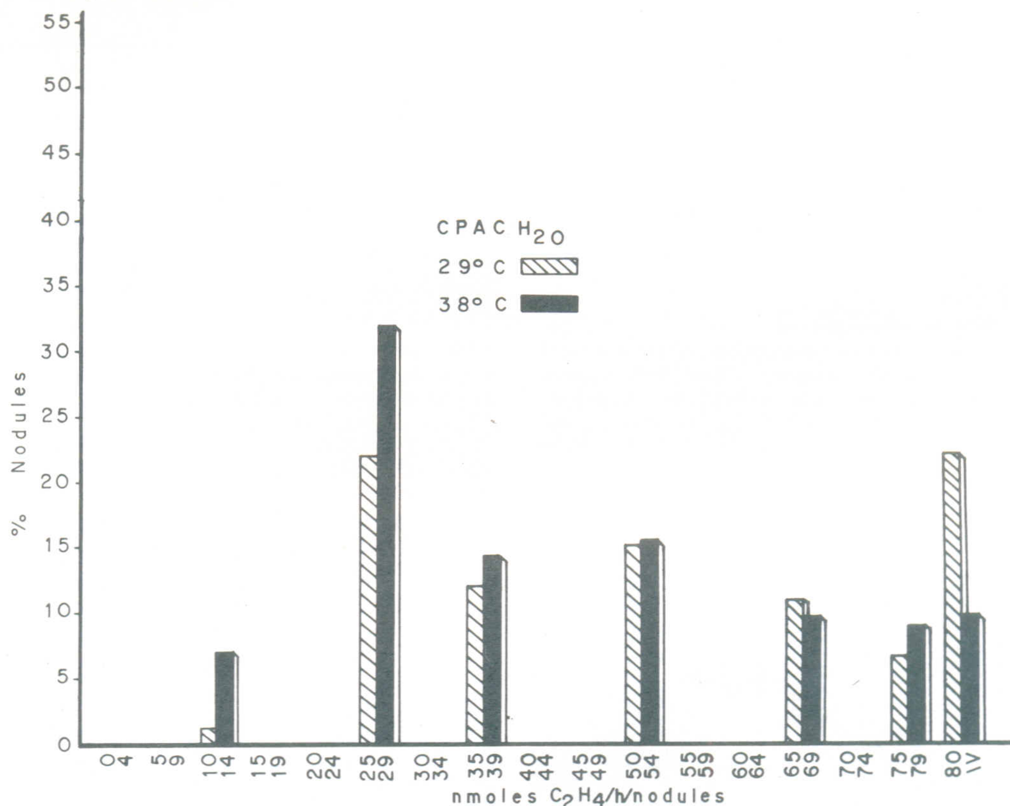


ed polymorphism and conservation of the central band in all of them. Strain Semia 476 presented a different pattern from the others; strains CPAC H_{14} and CPAC H_{35} presented identical patterns between them, however different from the others, similarly to what happened to strains Br 365 and CPAC H_{23}. Strains of *R. tropici*: CIAT 899, CENA CO_5 II and CPAC H_20 presented homologous patterns among them, with the presence of two hybridization bands in the same position. When the DNA was digested with *Hind* III, the polymorphism was much more evident. The strains presented specific cleavage patterns of the DNA indicating genetic variations at the level of their nucleotide sequencies (fig. 6). Among the strains of *R. leguminosarum* bv. *phaseoli*, strain Semia 476, similarly to what happened after digestion with *Bam* HI, also presented a different pattern from the others, strains Br 365 and CPAC H_{14} presented the same patterns between them, however different from the others, similarly to what happened to strains CPAC H_{35} and CPAC H_{23}. However, the strains of *R. tropici* after digestion with *Hind* III presented polymorphism not shown when digested with *Eco* RI and *Bam* HI.

Strains CIAT 899 and CENA CO_5 II presented the same pattern in their total DNA, but different from the others, as to what happened to strains CPAC H_{20} and Na 82. With *Hind* III, strains Br 814, Br 817 and Br 818 present different patterns among them, as well as in relation to the other strains tested.

In an attempt to detect modifications in the molecular characteristics of strains of *Rhizobium* after exposure to high temperature, the hybridization patterns of their total DNA with the nif probe were compared to their parent pairs, after digestion with *Eco* RI. The results, Fig. 7, show that there were no alterations in relation to the original patterns in the strains of both *Rhizobium* species, showing that the Sym plasmids of these strains were not modified or cured by *in vitro* growth at 38°C. This constrasts with results obtained by others (3, 11) who used strains that were not selected for tolerance to high temperature. The results of hybridization test with isolates of different levels

Continuação da FIGURA 1



of nitrogenase activities obtained from *R. leg.* bv. *phaseoli* (Strain Semia 476) and *R. tropici* (strain CIAT 899) did not show polymorphism in their total DNA with nif probe, after digestion with *Eco* RI (Fig. 8).

The differences evidenced both in the protein patterns (Fig. 2) and in the total DNA hybridization patterns (Fig. 4, 5, and 6) between species (*R. leg.* bv. *phaseoli* and tropici) and in the parent strains, confirm the data of the literature about heterogeneity of the strains of *Rhizobium* nodulating bean. However, our results suggest a diversification which is more evident in the strains of *R. leg.* bv. *phaseoli* than in those of *R. tropici*. The analysis of the more homogeneous protein patterns of *R. tropici*, rather than those of *R. leg.* bv. *phaseoli* (Fig. 2)

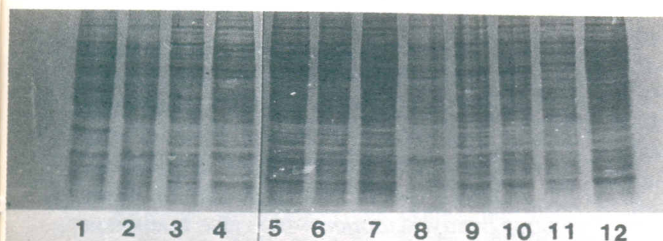


FIGURE 2 - Eletrophoresis SDS-PAGE. Total protein of patterns of *R. leguminosarum* bv. *phaseoli* (1) Semia 476, (2) CPAC H_{35}, (3) CPAC H_{14}, (4) Br 365 and of *R. tropici:* (5) ciat 899, (6) cena cos II, (7) CPAC H_2O, (8) Car 22, (9) Br 814, (10) Br 818, (11) Br 817, (12) Na 82.

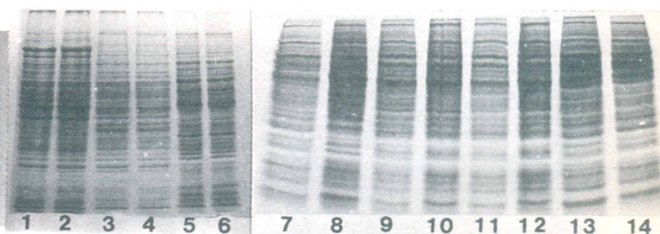


Figure 3 - Eletrophoresis SDS-PAGE. Total protein patterns of *Rhizobium* spp.: (1) CPAC H_{35} º, (2) CPAC H_{35}*, (3) CPAC H_{14} º, (4) CPAC H_{14}*, (5) Semia 476º, (6) Semia 476*, (7) CPAC H_{20}º, (8) CPAC H_{20}*, (9) CIAT 899º, (10) CIAT 899º, (10) CIAT 899*, (11) CENACO $_5$IIº, (12) CENACO $_5$II*, (13) Car_{20}º, (14) Car_{22}*, º - parent strain,
* Strain grown twice at maximum growth temperature.

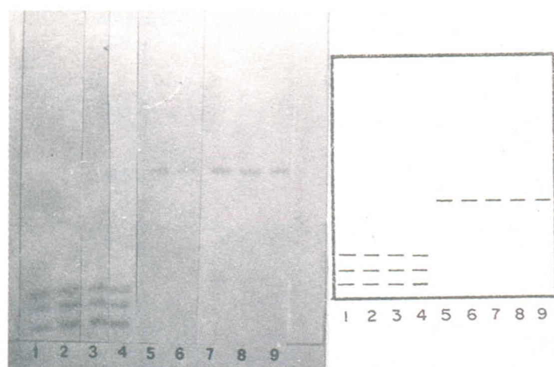


FIGURE 4 - Southern blot hibridization, nif probe marked with biotine through nick translation. Total DNA digested with *Eco* RI of the original strains of *R. leg.* bv. *phaseoli* (1) Semia 476, (2) CPAC H_{14}, (3) CPAC H_{35}, (4) CPAC H_{23} and of *R. tropici*, (5) CIAT 899, (6) CENA CO_5 II, (7) H_{20}, (8) Br 817, (9) Na 82.

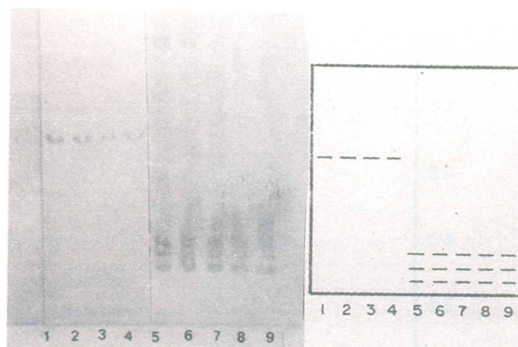


FIGURE 7 - Southern blot hibridization, nif probe marked with biotine through nick translation. Total DNA digested with *Eco* RI of the strains (1) Semia 476 º, (2) Semia 476*, (3) CPAC H_{35} º, (4) CPAC H_{35}*, (5) CPAC H_{14} º, (6) CPAC H_{14}*, (7) CIAT 899 º, (8) CIAT 899*, (9) CENA CO_5 II º, (10) CENA CO_5 II*, (11) CPAC H_{20}º, (12) CPAC H_{20}*.

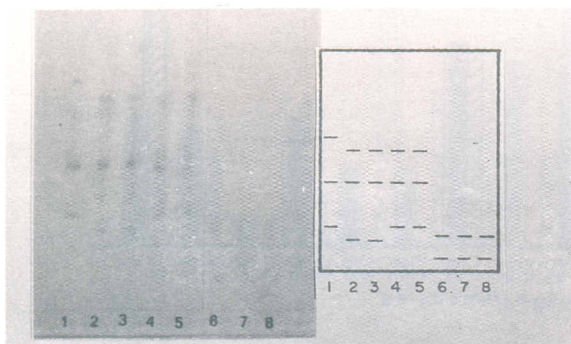


FIGURE 5 - Southern blot hibridization, nif probe market with biotine through nick translation. Total DNA with *Bam* HI of the original strains of *R. leg.* bv. *phaseoli* (1) Semia 476, (2) CPAC H_{14}, (3) CPAC H_{35}, (4) Br 365, (5) CPAC H_{23} and of *R. tropici*, (6) CIAT 899, (7) CENA CO_5 II, (8) CPAC H_{20}.

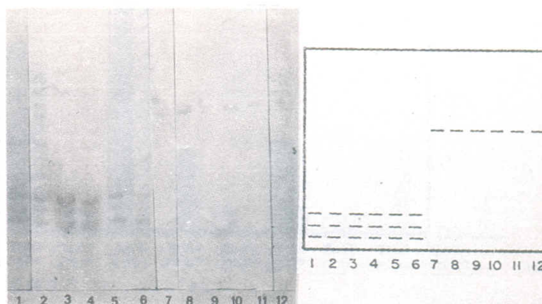


FIGURE 8 - Southern blot hibridization, nif probe marked with biotine through nick translation. Total DNA digested with *Eco* RI of isolates from nodules with different levels of effectiveness of CIAT 899 (1 low, 2 medium, 3 and 4 high effectiveness) and Semia 476 (5 and 6 low, 7 medium 8 and 9 high).

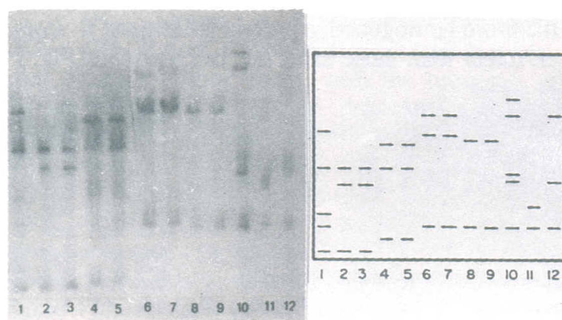


FIGURE 6 - Southern blot hibridization, nif probe marked with biotine through nick translation. Total DNA digested with *Hind* III of the original strains of *R. leguminosarum* bv. *phaseoli* (1) Semia 476, (2) Br 365, (3) CPAC H_{14}, (4) CPAC H_{35}, (5) CPAC H_{23} and of *R. tropici*, (6) CIAT 899, (7) CENA CO_5 II, (8) CPAC H_{20}, (9) Na 82, (10) Br 814, (11) Br 817, (12) Br 818.

and the tests of the DNA hybridization, where a greater number of hybridization bands was found, as well as a more acentuated polymorphism among the strains of *R. leg.* bv. *phaseoli* show a greater genetic variation in these strains. Recent results from Moreira et al. (15) indicate that fast growing rhizobia show greater variability in protein pattern than the strains of slow growth. *R. tropici* seems to be a link between the typical fast growing *R. leguminosarum* bv. *phaseoli*, specific for bean and the more promiscuous *Bradyrhizobium* which also nodulates *Leucaena*, a species which is nodulated by fast and slow growing rhizobia (13).

Flores et al. (4) and weaver et al. (24) showed that, in the majority of the cases, genetic studies of *R. leguminosarum* bv. *phaseoli* developed after cultivation through several generations

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and submitted to hybridization tests with various kinds of probes and comparison of plasmid profiles did not present genome differences in relation to their original clones. Only in some cases was it possible to show some kind of alterations. These observations show the difficult in detecting molecular alterations in strains exposed to high temperature or among isolates of the same strain showing different levels of nitrogenase activity. The alterations resulting from genetic rearrangements are probably confined to certain regions of the genome (4) and, having in mind this perspective, additional experiments would be necessary, as for example the use of other probes to detect them. It is also possible that the lack in detection of alterations in the strains submitted to high temperature actions is a result of the previous selection of the strains tolerant to high temperature and may represent an effective procedure to identify more genetic stable strains. All these studies could contribute to the understanding of the frequent alterations verified in the symbiotic characteristics of the strains of *Rhizobium* that nodulate beans. On the other hand, the selection and characterization of strains toleranting high temperatures and capable of maintaining their relevant characteristics in these conditions could represent a promising alternative for the bean inoculation in tropical soils.

RESUMO

Seleção e caracterização de estirpes de *Rhizobium* estáveis e capazes de fixar nitrogênio em feijão (*Phaseolus vulgaris* L.)

Determinou-se a variabilidade na capacidade de fixação de N_2, através de testes de redução de acetileno em nódulos formados por estirpes de *Rhizobium*, antes e após exposição das bactérias "*in vitro*" à temperaturas elevadas (38-39°C). Nódulos formados tanto por estirpes de *R. leguminosarum* bv. *phaseoli* como *R. tropici* mais tolerantes a altas temperaturas, quando inoculadas em feijão, não sofreram alterações nas características simbióticas tais como, atividade de nitrogenase, peso seco de planta e nitrogênio total fixado. O padrão de proteínas (eletroforese SDS-PAGE) diferenciou estirpes entre e dentro das espécies. A hibridização do DNA total usando "nif probe" marcada via "nick translation" (biotina 14 dATP), quando a digestão foi efetuada com *Eco* RI, diferenciou a espécie de *R. leguminosarum* bv. *phaseoli* de *R. tropici*. Foi observado polimorfismo entre as estirpes de *R. leguminosarum* bv. *phaseoli* após digestão com *Bam* HI e entre *R. leguminosarum* bv. *phaseoli* e *R. tropici* após a digestão com *Hind* III. Não foram detectadas alterações nos padrões protéicos ou genômicos e na atividade da nitrogenase da mesma estirpe antes e após crescimento a temperaturas elevadas, indicando que as estirpes de ambas as espécies (*R. leguminosarum* bv. *phaseoli* e *R. tropici*), tolerantes a altas temperaturas são também mais estáveis geneticamente.

Palavras-chave: *Phaseolus vulgaris* L., *Rhizobium* termo-tolerantes, a atividade da nitrogenase, padrões protéicos e genômicos.

ACKNOWLEDGEMENTS

The authors thank the laboratory technicians, Paulo Figueiredo, Renato Simões and Miguel Reis for their collaboration, as well as Dr. R. Palacios (Centro de Investigacion sobre Fijacion de Nitrogeno México) for the remittance of the probe with nif genes. We also thank FINEP-PADCT CNPq and EMBRAPA for their financial support.

"This work is part of the first author's Ph.D thesis, taking place at the UFRJ Microbiology Institute".

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Received for publication in 7/2/92.