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# Effects of high temperature on nodulation and nitrogen fixation by *Phaseolus vulgaris* L.

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**Abstract**

Screening of *Rhizobium leguminosarum* bv. *phaseoli* strains showed some that were able to nodulate common beans (*Phaseolus vulgaris* L.) at high temperatures (35 and 38°C/8 h/day). The nodulation ability was not related to the capability to grow or produce melanin-like pigment in culture media at high temperatures. However, nodules formed at high temperatures were ineffective and plants did not accumulate N in shoots. Two thermal shocks of 40°C/8 h/day at flowering time drastically decreased nitrogenase activity and nodule relative efficiency of plants otherwise grown at 28°C. Recovery of nitrogenase activity began only after seven days, when new nodules formed; total incorporation of N in tops did not recover for 2 weeks. Non-inoculated beans receiving mineral N were not affected by the thermal shock, and when growing continuously at 35 or 38°C had total N accumulated in shoots reduced by only 18%.

## Introduction

Symbiotic $N_2$ fixation by legumes is strongly affected by temperature conditions and nodulated legumes grow optimally within narrower temperature ranges than those receiving mineral N (Lie, 1981).

High temperature is a major factor limiting $N_2$ fixation in tropical, and even some subtropical regions. It can depress root hair formation, reducing the sites for nodulation (Frings, 1976; Jones and Tisdale, 1921) and affect adherence of bacteria to hairs (Frings, 1976). The infection process seems to be particularly sensitive (Barrios et al., 1963; Frings, 1976; Lie, 1981). High temperature also affects nodule development (Dart and Mercer, 1965; Jones and Tisdale, 1921; Piha and Munns, 1987) and functioning (Hernandez-Armenta et al., 1989; Meyer and Anderson, 1959; Munévar and Wollum, 1981a; Piha and Munns, 1987) and may accelerate nodule senescence (Pankhurst and Gibson, 1973; Sutton, 1983).

Legume species vary in their temperature tolerance, and common bean (*Phaseolus vulgaris* L.) is thought to be more sensitive to high temperature than cowpea and soybean (Piha and Munns, 1987). Although the upper limits for $N_2$ fixation with bean were considered to be between 30°C (Graham and Halliday, 1977; Pankhurst and Sprent, 1976) and 33°C (Barrios et al., 1963), declines in $N_2$ fixation have been observed even above 28°C (Piha and Munns, 1987). Soil temperatures in tropics often exceed 40°C (Dennet, 1984).

The development of a methodology to allow the selection of temperature-tolerant *Rhizobium* strains under laboratory conditions could contribute greatly to the success of a nitrogen fixing symbiosis in high temperature conditions. Based

on the reports showing that the information for nodulation and for a melanin-like pigment (Beynon et al., 1980) were harbored in the same plasmid of *R. leguminosarum* bv. *phaseoli* (Borthakur et al., 1987; Lamb et al., 1982), Oliveira et al. (1983) isolated eighteen strains that were able to produce dark pigment in culture medium under fluorescent light. In an attempt to use pigment production as an indicator in their selection program, Oliveira et al. (1983) submitted these strains to high temperature, which caused loss of pigment production or capacity to nodulate, but not simultaneously, suggesting that either the genes for those characters are not on the same plasmid, or that they are on the same plasmid but are subject to different temperature control.

In the experiments reported in this paper, we describe the attempts to detect $N_2$ fixation in beans with some thermal-tolerant strains (both melanin producers and unpigmented) identified by Oliveira et al. (1983). We also examine the effects of a thermal shock on the function of nodules containing a relatively temperature-tolerant strain.

## Materials and methods

Two experiments were performed under greenhouse conditions at EMBRAPA-CNPBS, km 47, Rio de Janeiro, Brazil.

In the first, seven strains of *R. leguminosarum* bv. *phaseoli* that were able to grow in plates exposed continuously to high temperature (38°C) were tested for nodulation with common bean (*Phaseolus vulgaris* L. cv. Negro Argel) at 28, 35 and 38°C/8 h/day. Seed N content was of 7.5 mg N/seed. Four of the strains produced melanin-like pigment when grown in TY medium (Beringer, 1974) under the conditions established by Beynon et al. (1980).

For plant inoculation, strains were grown in YM medium (Vincent, 1970) for five days at 28°C. Bean seeds were surface sterilized (Vincent, 1970) and soaked for 1 h in 1-mL of inoculum (about $10^8$ cells $mL^{-1}$) for each fifteen seeds. Five seeds were planted per pot, and covered with a layer of sterile sand. Pots were made of PVC tubes filled with two thick plastic bags, and received 2.5 kg of sterile sand and vermiculite (1/2, v/v). Four days after emergence (DAE) plants were thinned to two per pot. At the time of sowing, pots were put into water tanks so that the water level was just above that of the substrate inside the pot. Thermostats controlling the water temperature at 28, 35 or 38°C were turned on from 7:00 a.m. to 5:00 p.m., taking about 2 h to reach the desired maxima. A pump circulated the water in the tank during the hot period. After the heating was turned off, the temperature of the pots quickly dropped to 23°C. Temperatures around the shoots averaged 29/23°C, day/night.

Pots were watered through a capillary 'candle filter' system in each pot to maintain the pots at the moisture of about 75% of maximum water holding capacity (Boddey et al., 1987).

Nutrient solution without mineral N was provided weekly and contained: 0.725 m*M* $NaH_2PO_4$; 0.725 m*M* $KH_2PO_4$; 3.1 m*M* $MgSO_4$ $7H_2O$; 2.9 m*M* $CaSO_4$ $2H_2O$; 1.5 m*M* $K_2SO_4$; 1 $\mu M$ $MnSO_4$ $4H_2O$; 0.1 $\mu$M $CuSO_4$ $5H_2O$; 0.1 $\mu M$ $ZnSO_4$ $7H_2O$; 5.0 $\mu M$ $H_3BO_3$; 10.0 $\mu M$ NaCl; 0.5 $\mu M$ $NaMoO_4$ $2H_2O$; 0.02 $\mu M$ $CoSO_4$ $6H_2O$ and 5.0 $\mu M$ Fe-EDTA; pH 6.0 to 6.2. The treatment without inoculation received a total of 8 mg of N as $KNO_3$/plant/day from 5 to 26 DAE.

The experiment was laid out in a split-plot design, each temperature replicated in two tanks with two pots in each tank. Plants were harvested at 28 DAE (early flowering) and the following parameters were evaluated: shoot dry weight and total N (Boddey et al., 1987), nodule number and dry weight.

In the second experiment, of similar experimental design, cv. Negro Argel was inoculated with strain SEMIA 487 (IPAGRO, RS, Brazil), or received mineral N (10 mg N as $KNO_3$/plant/day, starting at 5 DAE). Inoculation and planting were as described for the first experiment. Plants were grown in the water tanks at 28°C during the day and 23°C at night. At 30 DAE (early flowering), a thermal shock of 40°C for 8 h was applied to half of the pots, and the shock was repeated on day 31. Plants were harvested at 30, 31, 32, 33, 36, 38 and 47 DAE.

Acetylene reduction activity was evaluated on excised roots using a continuous flow system with

12% of acetylene in air at a rate of 100 mL/min, as described before (Boddey et al., 1987). Previously (Hungria and Neves, 1987a), a comparative experiment was performed to evaluate whether the nitrogenase was affected by the presence of acetylene (Minchin et al., 1983). Plants were transfered to a continuous flow system, detopped and flushed with an air stream containing 12% of $C_2H_2$. Samples were taken after 1, 3, 4, 5, 6, 7, 10, 15 and 30 minutes. Percent decreases in nitrogenase activity for thirty symbiotic combinations (five bean cultivars and six *Rhizobium* strains) were just 4.5% after 30 minutes. In the experiments reported in this paper, we detopped the plants, immediately incubated with acetylene and samples were taken after 4 minutes with acetylene. Although we have not investigated the effect of detopping, it is unlikely that any significant effect would take place in this short interval.

Hydrogen evolution by the nodules was measured on excised root systems that were incubated in air in 280-mL jars under normal atmosphere for 10 minutes, and hydrogen was analyzed by gas chromatography as described previously (Boddey et al. 1987). Relative efficiency of nitrogenase was determined according to Schubert and Evans (1976).

Nitrate reductase activity of leaves, stems and roots was evaluated according to Hungria and Neves (1987b). Nodules were examined for texture (hard/soft) and internal color (pink/brown) and classified as senesced (soft and/or brown) or non-senesced (hard and pink). Number and dry weight of nodules were also evaluated. Plant dry weight and total N (shoots + roots) were analyzed according to Boddey et al. (1987).

## Results

All seven *R. leguminosarum* bv. *phaseoli* strains were able to nodulate beans when root systems were exposed at 35 and 38°C/8 h/day. This ability was not related to their growth or melanin-like pigment production in plates exposed continuously to 38°C (Table 1). However, nitrogenase activity was not detected and plants had a very low N content. On the other hand, plants receiving mineral N were not greatly affected, and total N accumulated in shoots decreased by only 18% at 35 or 38°C (Table 1).

A thermal shock of 40°C/8 h/day at 30 DAE, of plants previously grown at 28°C, resulted in a 70% loss of nitrogenase activity 24 h after, and with a second thermal shock at 31 DAE, there was a further loss in activity of 15% (Fig. 1). The proportion of electrons diverted to hydrogen

*Table 1.* Nodulation and N accumulation in shoots of common bean, cv. Negro Argel, inoculated with seven *Rhizobium* strains or receiving mineral N (8 mg N/plant/day from 5 to 26 DAE) and grown at 28, 35 or 38°C/8 h/day and 23°C at night. Plants were harvest at early flowering (28 DAE) and data represent the average of four replicates

| *Rhizobium* strains | Production of dark pigment in TY medium at 28°C | Growth in YM medium | | Nodulation | | | | | | Shoot N (mg N/plant) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Number | | | Dry weight | | | | | |
| | | 35 | 38 | 28 | 35 | 38 | 28 | 35 | 38 | 28 | 35 | 38 |
| SEMIA 487[a] | + | + | + | 95 | 63 | 39 | 50 | 40 | 23 | 52 | 18 | 18 |
| SEMIA 4021[a] | + | + | + | 79 | 77 | 13 | 50 | 23 | 10 | 50 | 20 | 24 |
| F 413[b] | + | + | + | 84 | 70 | 32 | 40 | 43 | 33 | 54 | 24 | 20 |
| F 413 Mn3[c] | + | + | – | 82 | 61 | 13 | 40 | 27 | 17 | 40 | 18 | 20 |
| C05[d] | – | – | – | 59 | 33 | 13 | 47 | 27 | 20 | 46 | 26 | 20 |
| CIAT 57[c] | – | – | – | 115 | 79 | 15 | 73 | 37 | 17 | 54 | 18 | 18 |
| CIAT 255[c] | – | – | – | 96 | 87 | 13 | 84 | 30 | 13 | 58 | 18 | 20 |
| Mineral N | | | | 0 | 0 | 0 | 0 | 0 | 0 | 92 | 76 | 74 |
| Analyses of variance | | | | | | | | | | | | |
| Temperature ($p < 0.05$) | | | | | 17 | | | 12 | | | 9 | |
| Inoculation ($p < 0.05$) | | | | | 20 | | | 16 | | | 8 | |

[a]IPAGRO, RS, Brazil; [b] Nitragin Co., USA; [c]F 413 isolated from soils with high levels of manganese; [d]CENA, SP, Brazil; [c]CIAT, Cali, Colombia.

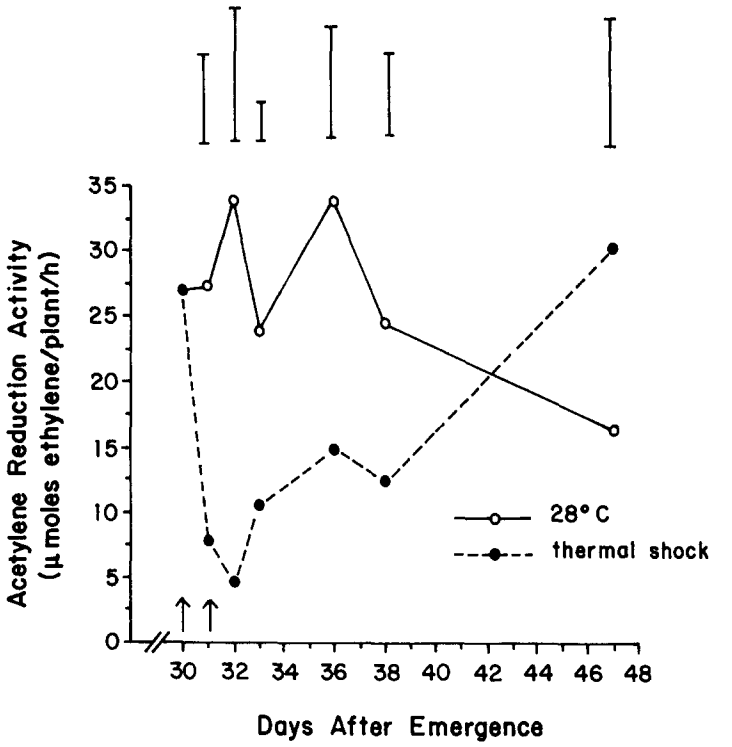


*Fig. 1.* Effects of two thermal shocks (40°C/8 h/day) at 30 and 31 DAE on the nitrogenase activity of bean, cv. Negro Argel, inoculated with Rhizobium strain SEMIA 487 and grown at room temperature (28°C). Means of four replicates and vertical bars denote LSD for each harvest ($p = 0.05$).

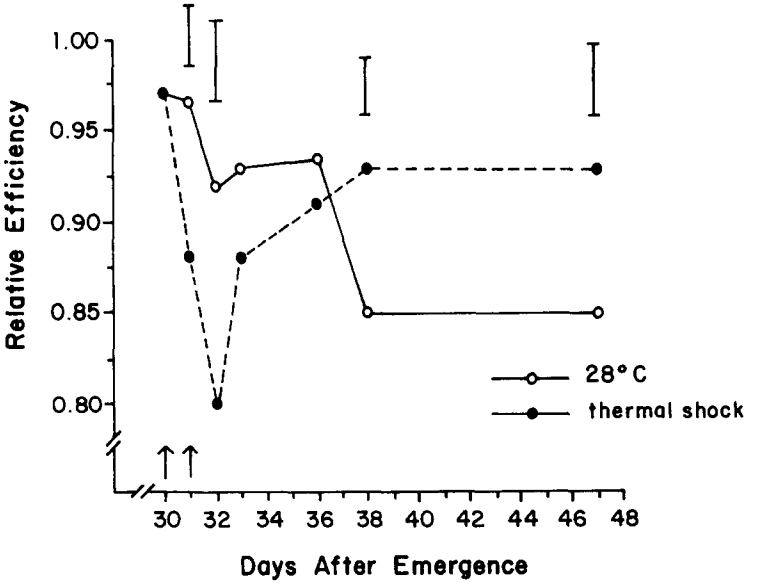


*Fig. 2.* Effects of two thermal shocks (40°C/8 h/day) at 30 and 31 DAE on the relative efficiency of nitrogenase of bean cv. Negro Argel, inoculated with SEMIA 487 and grown at room temperature (28°C). Means of four replicates, and vertical bars denote LSD for each harvest ($p = 0.05$).

evolution was also changed, and the relative efficiency decreased with the increase in the temperature (Fig. 2). By 33 DAE, both nitrogenase (Fig. 1) and relative efficiency (Fig. 2) had begun to recover, but 7 days after the second thermal shock, the nitrogenase activity was still less than half of the control level. However, total nitrate reductase (nitrate reductase of leaves + stems + roots) was not affected by the thermal shocks, declining only at 36 DAE for both treatments (Fig. 3).

Five days after the thermal shock, the number of senesced nodules increased by 33%, but 7 days after the plants had been submitted to 40°C new nodules could be seen, increasing nodule number per plant and decreasing significantly the percentage of senesced nodules. So, at 47 DAE, only 17 days after the first thermal shock, the number of nodules in stressed plants was 54% higher than that of the control (Fig. 4). New nodules led to recovery of nitrogenase activity and relative efficiency, with values significantly higher in stressed plants at 47 DAE (Figs. 1 and 2).

There was no effect of high temperature shock on N accumulation by the plants that received mineral N (Fig. 5). Also at the final harvest there was no difference between plants given mineral N and those inoculated with strain SEMIA 487 and growing at optimal temperatures.

## Discussion

Other workers have also shown that the ability of rhizobia and bradyrhizobia to grow in culture medium at high temperatures (up to 47°C) does not necessarily relate to the infectivity and $N_2$ fixation under temperature-stress conditions (Karanja and Wood, 1988; La Favre and Eaglesham, 1986). Also the attempts to use a genetic marker, such as the production of melanin-like pigment, to identify temperature-tolerant rhizobia have not been successful (Oliveira et al., 1983). The role of melanin-like pigment production by *R. leguminosarum* strains is still unclear. We found no relation between the production of this pigment in plates and the nodulation ability of *R. leguminosarum* bv.

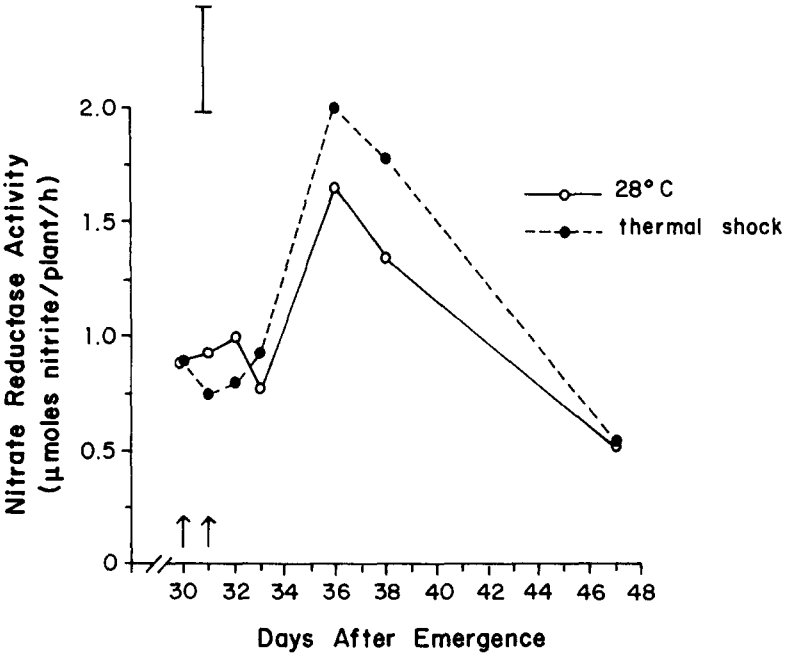


*Fig. 3.* Effects of two thermal shocks (40°C/8 h/day) at 30 and 31 DAE on nitrate reductase activity in leaves of beans, cv. Negro Argel, receiving 10 mg N/plant/day and grown at room temperature (28°C). Means of 4 replicates and vertical bars denote LSD for the effect of harvesting time ($p = 0.05$).

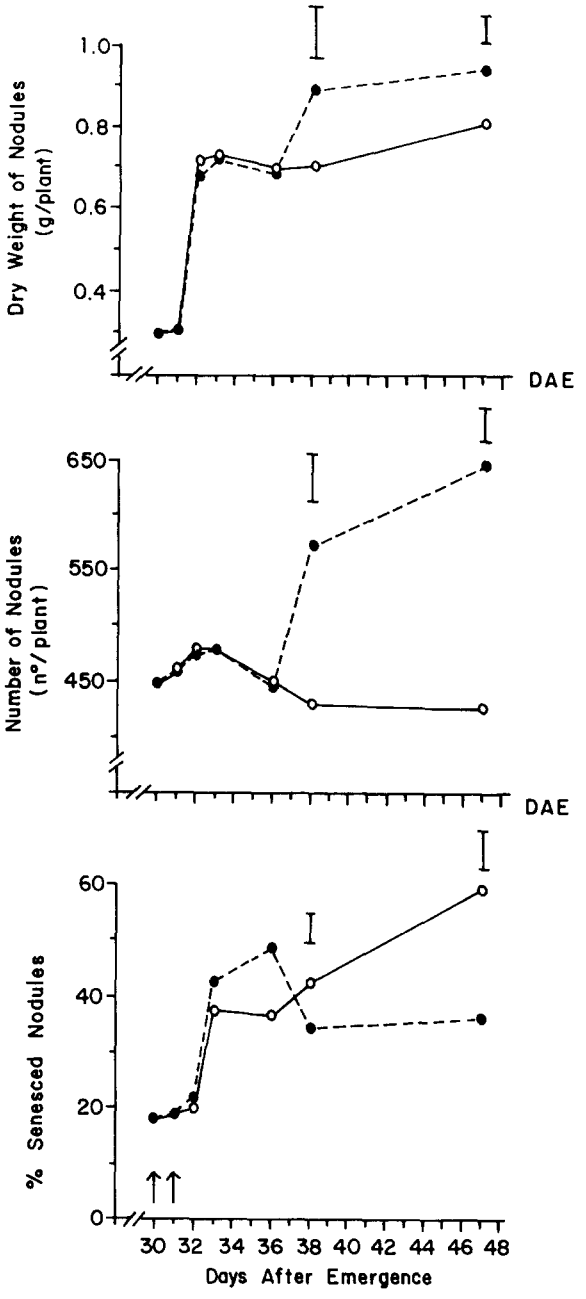


*Fig. 4.* Effects of two thermal shocks (40°C/8 h/day) at 30 and 31 DAE on nodulation of beans cv. Negro Argel inoculated with SEMIA 487 and grown at 28°C. Mean of 4 replicates and vertical bars denote LSD for each harvest ($p = 0.05$).

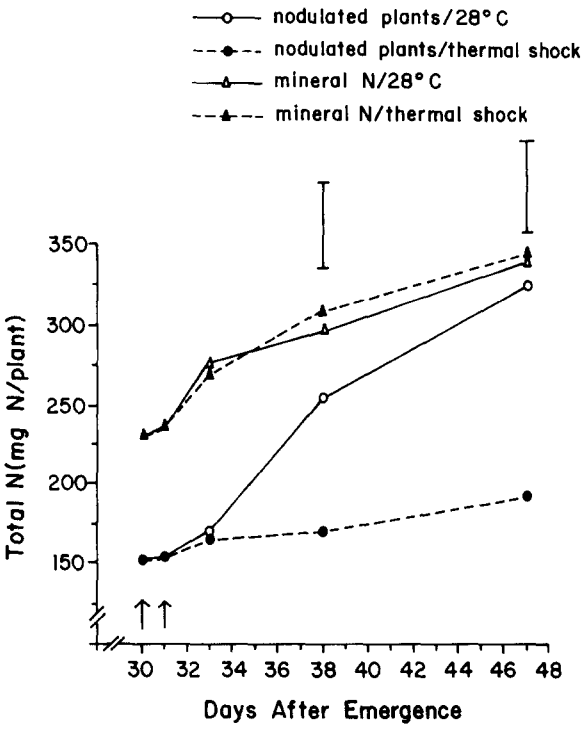


*Fig. 5.* Nitrogen accumulation by bean cv. Negro Argel, inoculated with strain SEMIA 487 or receiving 10 mg N/pl/day. Plants were grown at room temperature (28°C) and received or not thermal shocks of 40°C/8 h/day at 30 and 31 DAE. Means of 4 replicates and vertical bars denote LSD for each harvest ($p = 0.05$)

*phaseoli* strains. Also in *R. leguminosarum* bv. *viceae*, Hynes et al. (1988) showed that the loss of dark-pigment production had no effect on the symbiotic performance or competitive ability of this strain.

The effect of a thermal shock is unlikely to reflect simply the loss of Sym plasmid, since Soberón-Cháves et al. (1986) showed that strains of *R. leguminosarum* bv. *phaseoli* lost symbiotic properties at a frequency of 62% after growth at 37°C for five days, but maintained the same electrophoretic pattern and the same plasmid molecular weight. In that work, the loss of Fix phenotype resulted from genomic rearrangements that could only be shown by hybridizing techniques (Soberón-Cháves et al., 1986).

Although in the first experiment seven Rhizobium strains were capable of nodulating beans when root day temperatures were 35 and 38°C, data on the acetylene reduction activity, total N accumulated by plants (Table 1) and number of infected cells (data not shown) showed that these nodules were not functional.

Similar results were also obtained for beans by Gitonga et al. (1989). There was also no relation between the origin of the strains (from tropical or temperate regions) and the resistance to high temperatures, as also observed by Munévar and Wollum (1981a).

Differences among strains in tolerance to high temperature were observed for *Bradyrhizobium japonicum* (Dart et al., 1976; La Favre and Eaglesham, 1986; Munévar and Wollum, 1981a,b) and *R. leguminosarum* bv. *phaseoli* strains (Karanja and Wood, 1988; Piha and Munns, 1987), but as in our experiments, there was almost no $N_2$ fixation with temperatures above 33°C. Since plants receiving mineral N in experiment 1 had total N decreased by only 18%, the limitation appears to be in the symbiosis rather than in the growth potential of the bean plant.

In the second experiment, plants grown under optimal temperature conditions and exposed to a thermal shock at flowering time decreased nitrogenase activity by 70% after 24 h (Fig. 1). Similar results were obtained by Hernandez-Armenta et al. (1989) confirming that the symbiosis of *Phaseolus vulgaris-R. leguminosarum* bv. *phaseoli* is extremely sensitive to high temperatures. The proportion of electrons allocated to the proton reduction was increased after 38°C (Fig. 2), as it was also observed before for nodulated cowpea (Rainbird et al., 1983). In our experiment, plants started to recover after one week, due to the development and/or formation of new nodules (Fig. 4).

The data presented here demonstrate that high temperature (35°C or above) can affect the symbiosis of *P. vulgaris-R. leguminosarum* bv. *phaseoli* and none of the strains tested was able to fix $N_2$ under these stressed conditions. However, in tropical and even some subtropical regions, soil temperatures between 40–60°C are often observed, and prolonged periods at 38°C in the surface 4 cm of soil were reported in North Carolina, USA (Munévar and Wollum, 1981a). Since we have shown that the plant growth per se was not greatly affected by heat (35 or 38°C/8 h/day during growth or a thermal shock of 40°C/8 h/day at flowering), there would still seem to be potential for improvement of bean yields from inoculation if the appropriate strain could be found. To this end we have investigated other sources of Rhizobium for $N_2$ fixation with beans at high temperatures, and we describe this selection work in the following paper (Hungria et al., 1993).

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