

Growth Response of *Bradyrhizobium japonicum* RCR3407 to Heat Hardening Treatments

Mohammad Ibrahim Keerio, Mohammad Ashraf Mirjat,
Shanila Yasmeen Chang and Irfana Parveen Bhatti
Department of Plant Physiology and Biochemistry, Faculty of Crop Production,
Sindh Agricultural University, Tandojam, Pakistan

Abstract: Heat-hardening treatments indicates that *B. rhizobium japonicum* can grow better at high temperatures. During first 12h of incubation (after hardening at 40°C) the rhizobia sometimes grew less well than the controls, but growth then increased more quickly up to 36h of incubation compared to controls. A small decrease in cell population was measured after 48h, however, similar to that in the control. At 45°C, the heat-hardened rhizobia grew similar to the controls during the first 12h, then a sharp increase was found after 36h. Growth was then much better in the hardened cultures than controls. Same time at 40°C, the pH declined sharply after 12h and remained stable on further incubation. However, the pH of the culture which was incubated at 45°C did not decline during the first 24h of incubation and only a very slow decline was found after that. Similar results were found in the cultures which had been hardened at 45°C. In cultures which were incubated at 40°C, the pH decreased slightly after 12h of incubation and became stable on further incubation. At 45°C, decrease in the pH was not found up to 24h and then a small decline take place with increasing incubation periods.

Key words: *B. rhizobium japonicum*, heat-hardening, temperature, optical density, pH

Introduction

The bacteria capable of infecting the roots of legumes and stimulating nodule formation belong to several strains of the species *Rhizobium*. Each strain is only able to infect the roots of a specific group of legumes or only one species. *Rhizobium* belongs to the family *Rhizobiaceae* (Jordan and Allen, 1974) which consists of rod-shaped cells without endospores. They are gram negative, aerobic and motile, having either one polar or sub-polar flagellum or 2 to 6 peritrichous flagellae (Buchanan and Gibbons, 1974). Many studies have reported that the growth and survival of *Rhizobium* and *Bradyrhizobium* in soils are adversely affected by high soil temperatures (Munevar and Wollum, 1981a, 1982; Osa-Afiana and Alexander, 1982; Kluson *et al.*, 1986 and Kennedy and Wollum, 1988). The effects of high temperatures on the survival of rhizobia in soil has been reported by many researchers. The evaluation of the temperature responses of rhizobia in pure culture may, therefore, be useful in the search for *R. japonicum* strains better suited to environments in which high soil temperatures are present. Munevar and Wollum (1981a) found only one *Bradyrhizobium* strain able to survive in liquid culture at 49°C, but it was not effective in nitrogen fixation. Hafeez *et al.* (1991) reported that two *Bradyrhizobium* strains Vm1 and Vr16 were able to survive and grow at 48°C on agar plates. The experiment was conducted to observe the growth of *Bradyrhizobium japonicum* and pH changes in the culture media after applying heat-hardening treatment.

Materials and Methods

The bacterium *Bradyrhizobium japonicum* RCR3407 was supplied by Dr. F.R. Minchin, Institute of Grassland and Environmental Research (IGER), Aberystwyth, U.K. All glassware was washed and autoclaved before use. The liquid culture medium contained 10 g mannitol, 1 cm³ of 10% K₂HPO₄, 4 cm³ of 10% KH₂PO₄, 2 cm³ of 10% MgSO₄, 1 cm³ of 10% NaCl, and 0.4 g of yeast extract in 1L of distilled water, pH 7.0, contained in 2 X 500 cm³ bottles. The bottles and their contents were autoclaved for 20 min. at 15 psi. After cooling, the medium was inoculated with *B. rhizobium*

japonicum RCR3407 (Approximately 1g of stock sample to each bottle) and kept in an incubator for 10 days at 25°C. Culture medium was prepared and autoclaved as described above. Then 10 cm³ volumes of the medium were transferred to McCartney bottles and again autoclaved. The pH was checked and readjusted to 7.0 if necessary. After cooling, the bottles were inoculated with cultures of *B. rhizobium japonicum*, which had been grown at 40°C or 45°C, were used to inoculate new media (0.1 cm³ to 10 cm³ of fresh medium). These media were then incubated at 40°C or 45°C in a water bath. At 12, 24, 36 and 48h after inoculation, the growth (as optical density) was determined at 600 nm using a spectrophotometer also the pH was measured. Means, standard deviation and standard error values were calculated. Further statistical analysis by analysis of variance (ANOVA) was done with Minitab for Windows (version. 10.2).

Results and Discussion

Growth of *B. rhizobium japonicum* in un-inoculated (control) culture media: The un-inoculated (control) tubes showed no change in optical absorbance at any temperature or at any time of incubation. Also, no changes in pH occurred in these un-inoculated control media.

Growth of *B. rhizobium japonicum* after Heat-hardening treatment: Heat-hardening treatments at 40 or 45°C were given to *B. rhizobium japonicum* and growth was then observed after 12, 24, 36 and 48h of incubation at either 40 or 45°C. In the first treatment, the bacteria were heat hardened for 7 days at 40°C, re-inoculated into new culture media and kept at either 40 or 45°C for 48h. The growth of pre-hardened cultures was initially (up to 24h) greater (Fig. 1) at 40°C than it was at 45°C. At 36h and 48h, however, the two cultures had similar optical densities. More importantly, the growth of the pre-hardened cultures after 24h at both 40°C and 45°C was greater than their corresponding control. This suggest that heat hardening had taken place. In the second treatment, the *B. rhizobium japonicum* was heat hardened for 7 days at 45°C, then re-inoculated into new culture media and grown at either 40 or 45°C. The results

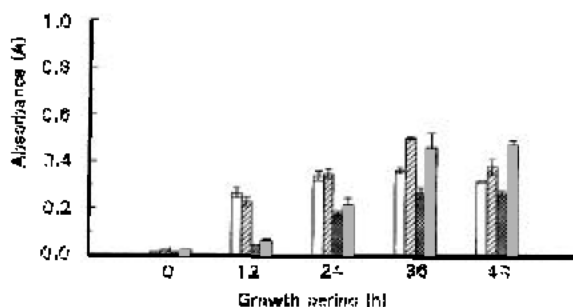


Fig. 1: Effect of heat-hardening on growth of *B. rhizobium japonicum*.
 □ Control grown at 40°C; ▨ pre-hardened at 40°C then grown at 40°C; ▩ control grown at 45°C; ▪ pre-hardened at 45°C then grown at 45°C.

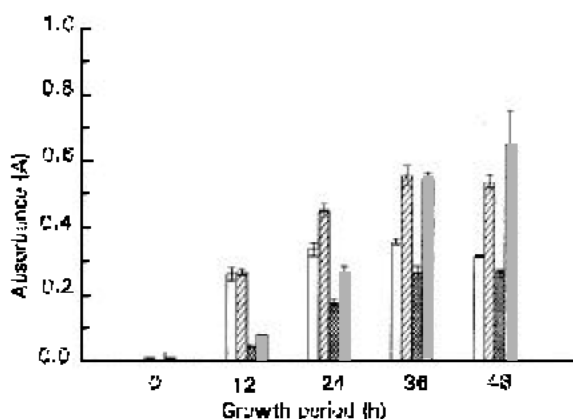


Fig. 2: Effect of heat-hardening on growth of *B. rhizobium japonicum*.
 □ Control grown at 40°C; ▨ pre-hardened at 45°C then grown at 42°C; ▩ control grown at 45°C; ▪ pre-hardened at 45°C then grown at 45°C.

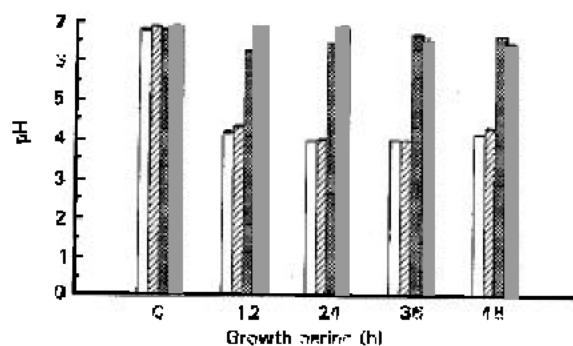


Fig. 3: Change in pH of *B. rhizobium japonicum* cultures following heat-hardening.
 □ Control grown at 40°C; ▨ pre-hardened at 15°C then grown at 40°C; ▩ control grown at 45°C; ▪ pre-hardened at 15°C then grown at 45°C.

from this experiment were very similar to those from the first experiment and they provide more clear evidence for heat hardening (Fig. 2). The pre-hardened cultures again grown faster at 40°C than at 45°C, at least up to 24h. After 24h, their optical densities were similar. The growth of heat-hardened cultures after 24h was clearly greater than the

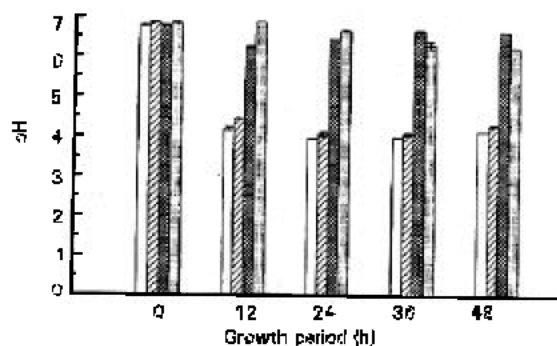


Fig. 4: Change in pH of *B. rhizobium japonicum* cultures following heat-hardening.
 □ Control grown at 40°C; ▨ pre-hardened at 45°C then grown at 40°C; ▩ control grown at 45°C; ▪ pre-hardened at 45°C then grown at 45°C.

corresponding controls. Statistically the bacterial growth was strongly dependent ($P < 0.05$) upon temperature and time. There was no interaction ($P > 0.05$) between temperature and time.

The growth of *B. rhizobium japonicum* RCR3407 was evaluated at 25, 35, 40 and 45°C after 12, 24, 36 and 48h of incubation. The optimal growth temperature for this strain of *B. rhizobium japonicum* is 25 to 35°C, Keerio *et al.* (1999) and Chang *et al.* (2000). The optimum temperature for the growth of *B. rhizobium* was found between 27.7 and 35.2°C by Munevar and Wollum (1981b). La Favre and Eaglesham (1988) found that strains of cowpea rhizobia tolerated up to 43°C, and Karanja and Wood (1988) found strains of *R. leguminosarium* biovar *phaseoli* capable of multiplying at 47°C. Munevar and Wollum (1981a) have suggested three characteristic temperature ranges for rhizobia. An optimum temperature range from 27.4 to 35.2°C, a maximum permissible growth temperature of 29.8 to 38°C and, finally, a maximum survival temperature from 33.7 to 48.7°C (depending on strain).

Changes in pH of the growth media of the heat-hardened *B. rhizobium japonicum*: Before and during the incubations, the pH was recorded in all the growth tubes. For the first treatment (heat-hardening at 40°C). The pH of the 40°C culture media declined to 4.0 with increasing time of incubation up to 36h and it then slightly increased again to 4.3 at 48h (Fig. 3). There was no change in pH after 12h in the 45°C culture and only a very small reduction in pH was observed at 48h of incubation. A similar pattern of pH change was observed in the second treatment using the 45°C-hardened bacteria (Fig. 4). A striking feature of the results was the absence of any difference between the pH of these heat-hardened cultures and the pH of their corresponding controls. This was in contrast to the differences observed between the optical densities of the cultures.

The pH in cultures grown at 40°C was strongly dependent ($P < 0.05$) upon the incubation period, while the pH of the 45°C cultures were not influenced ($P > 0.05$) by the incubation period. These significances were true for both the control and heat-hardened cultures. The pH was also significantly ($P < 0.05$) affected by temperature. There were no interactions ($P > 0.05$) between the temperature and time.

Results similar to the earlier experiments (Keerio *et al.*, 1999 and Chang *et al.*, 2000) were observed with respect to the pH measurements in cultures. The data showed that the pH declined with increasing bacterial growth at optimal

temperatures. Clearly, rhizobial cultures change pH during growth. If peptone were the principal organic nutrient of *Rhizobium*, it is known that the pH rises due to ammonia formed from the deamination of amino acids (Meynell and Meynell, 1965). In contrast, if fermentable sugars are present (as in these experiments), the pH falls due to acid production. At pH 6.8, some glucose is also converted to other sugars, including gentiobiose (Khan and Walker, 1958). The pH declined with increasing growth at 25, 35 and 40°C but not at 45°C. There was no close quantitative correlation between the bacterial growth and the pH change however. This may be explained by differences in metabolism at low and high temperatures. Generally, fast growing rhizobia can be expected to utilize a wider range of carbon compounds than slow growing rhizobia (Stowers, 1985). Most *Rhizobium* strains can produce strongly ureolytic enzymes. Many slow-growing strains, however, fail to reduce nitrate or reduce it at extremely slow rates (Graham and Parker, 1964). In contrast, Neal and Walker (1935) have suggested rapid nitrate utilization by slow-growing root nodule bacteria. Oxygen can play an important role in the growth of *Rhizobium*. Growth at low oxygen tension appears to be essential for the development of nitrogenase activity in free-living rhizobia. The exclusion of oxygen inhibits both growth and enzymatic activity, whereas high levels of oxygen stimulate growth but the bacteria are devoid of nitrogenase activity (Evans and Keister, 1976). Postgate (1971) has reported that, with the aerobic nitrogen fixing bacteria, nitrogen fixation is most effective at hypobaric oxygen pressures and this also seems to be the case of symbiotic rhizobia.

A particularly interesting observation was the pH changes in heat-hardened *B. rhizobium* cultures grown at 40°C and 45°C was similar to the pH changes in non-hardened cultures although the bacterial growth rates were different in two cases. This indicates that the metabolism of the hardened bacteria is different from that of untreated bacteria. In general, the results from the heat-hardening experiments indicate that *B. rhizobium japonicum* grew better at high temperatures after heat-hardening treatments. These findings confirmed earlier observation that there is not a strict quantitative correlation between bacterial growth and the pH change. Heat-hardening treatments were shown to be effective in permitting better growth at high temperatures. This experiment indicate that, in the natural environment of the soil, heat acclimation of the *B. rhizobium japonicum* may take place which would allow nitrogen fixation in soybean at high temperatures.

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