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Nitrogenase Activity of Soybean Root Nodules Inhibited After Heat Stress

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Abstract: High temperature in general was found to be the major factor affecting symbiotic nitrogen fixation. High temperature of 35EC when applied to the roots only didn't effect nitrogen fixation. However, small variances were recorded at 40EC in all cultivars. Fixation was severely inhibited at 45EC in all cultivars. The cultivar sable was found to be the most tolerant to high temperatures and it also recovered from heat stress better than the other cultivars. Although, cultivar bragg was not as heat tolerant as others but this recover better than other cultivars studied. Which were very similar in their sensitivities to heat stress.

Key words: Soybean, nitrogenase activity, nodule, high temperature

Introduction

There are many different physiological and environmental factors affecting the rate of the nitrogen fixation in legume root nodules, such as temperature, waterlogging, water stress, salinity, combined nitrogen levels, pH, nutrients etc. Many of the above factors, including temperature, affect various aspects of nitrogen fixation and assimilation, as well as factors such as respiratory activity, gaseous diffusion and the solubility of dissolved gasses which ultimately affect plant growth. Most of the nitrogen in plants and indirectly in animals, comes from the soil in the form of inorganic ions absorbed by plant roots. Some nitrogen, however, is also fixed from the air, which is converted to ammonia and other nitrogenous substances by either free living or symbiotic nitrogen-fixing bacteria.

The adverse effects of root temperatures on nitrogen fixation have been shown for numerous legumes Gibson, (1977); Sprent and Minchin, (1984). The biological nitrogen fixation is often affected more by temperature than the general growth and photosynthesis of the plant Granhall, (1981). Steward (1966) has also suggested that nitrogen fixation is often especially inhibited by temperature extremes which have less effect on plant growth.

Some techniques used to study legume nitrogen fixation are destructive to the whole plant. For example, the Kjeldhal and ^{15}N-tracer methods usually involve the complete harvesting of the plant for its analyses. Acetylene Reduction Activity (ARA) has been widely used to estimate comparative rates of nitrogen fixation in soybean at different temperatures. However, the acetylene reduction method is not destructive. It has been widely used to assay nitrogen fixation in intact plants and also in excised root systems.

Materials and Methods

The experiment was conducted during 1995 at the School of Biological Sciences, University of Wales, Bangor, U.K. Seeds of three cultivars, Bragg (Sindh Agriculture Research Institute, Oil Seed Section, Tandojam), Davis (Pak. Agri. Res. Council (PARC), Islamabad), and Sable (Pak. Agri. Res. Institute, Peshawar, Pakistan) were grown in (10 x 5 cm) pots filled with vermiculite pre-washed with distilled water. The pots were incubated in a growth room (Vindon), which was run with a 27/20EC " 0.5EC day/night temperature regime and with a 16h photoperiod. The light intensity was 135-150 imols m^{-2} s^{-1} and the humidity was 65-67%.

The seeds were soaked for 30 min in an *B.rhizobium japonicum* RCR3407 inoculum (supplied by Institute of Grassland and Environmental Research (IGER), Aberystwyth, U.K.). Two seeds were sown in each pot. Seven days after sowing, the seedlings were thinned to one per pot and inoculated again with 3 cm^3 of *B.rhizobium japonicum* inoculum.

The plants were used for the heat-stress treatments five weeks after inoculation. Heat stresses of 2, 3 or 4h were applied at 35, 40 or 45EC in the water bath. After the heat stress treatments nitrogen fixation was measured and the plants were then allowed to recover for 72h at 27EC. Then again, after measuring its nitrogen fixation rate, the rooting system was removed from the pot and washed carefully. All root nodules were separated from the roots and counted. Their fresh weight was then determined and recorded. Next, the nodules were transferred into small paper bags and kept in the drying oven at 70EC for 48h before measuring their dry weight.

Heat stress treatments: Heat stress was applied in a shaking water bath (Grant). For these treatments, the pots were covered by a plastic lid which had two holes, one for the plant shoot and the other for injecting and withdrawing gas samples. The pots were sealed with electrician water-proofing compound (Centaure MFG) and transferred to the water bath for the heat stress treatment. The start of the heat stress was measured 30 min after transfer to the water bath, because it took this period for the required temperature to be reached inside the pots. Temperatures inside the pot were monitored by inserting a thermocouple probe into the vermiculite. After heat treatment, the pots were removed from the water bath and nitrogen fixation was measured.

Acetylene reduction assay: To determine nitrogen fixation rates, the acetylene reduction method was used Keerio and Wilson, (1996), Hardy, *et al.*, (1968); Habte, (1983) and Masterson and Murphy, (1980). The acetylene reduction method is cheap, easy and non-destructive to use. In this method, acetylene is reduced to ethylene by the nitrogenase complex in the root nodules and the ethylene product is measured. It involved injecting 30 cm^3 of pure acetylene gas into the pot contained in a closed container fitted with two Suba Seals to give a concentration of approximately 10% acetylene inside the pot. (The pot size was 10 cm high by 5 cm diameter, and its inside capacity was 350

cm^3). Before injecting the gas, a syringe needle was put into Suba Seal to allow the extra pressure to escape when 30 cm^3 of acetylene was added. When all the acetylene had been injected, both the injection needle and the vent needle were withdrawn leaving a sealed pot. At 10, 20 and 30 min after acetylene injection, 1 cm^3 samples were taken from the pot using an airtight syringe and analyzed for their ethylene content by gas chromatography (GC). The GC machine (Sveriges, Sweden) was fitted with a Dura-pack column and run with 50 cm^3/min of fresh air as the carrier gas supplied by a small air pump. The oven temperature was 45EC. The apparatus was calibrated with 4 X injections of 0.5 cm^3 of a 100 ppm ethylene standard before unknown samples were analyzed. The ethylene peaks were recorded by a chart recorder connected to the GC. After every 20 injections, the silicon septum of the GC was changed.

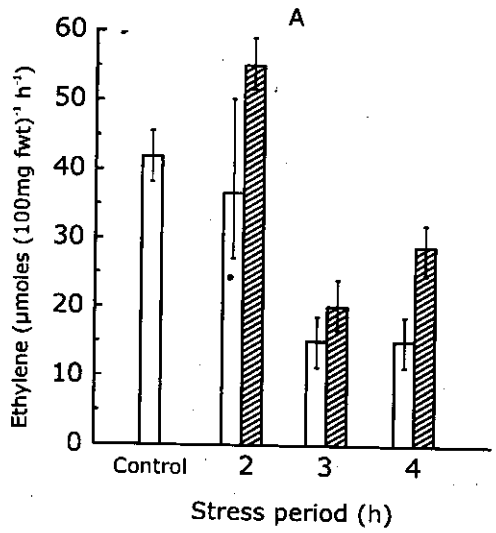
Statistical analysis: Statistical analysis by analysis of variance (ANOVA) was done using the personal computer (Mitac) with the statistical package Genstat.

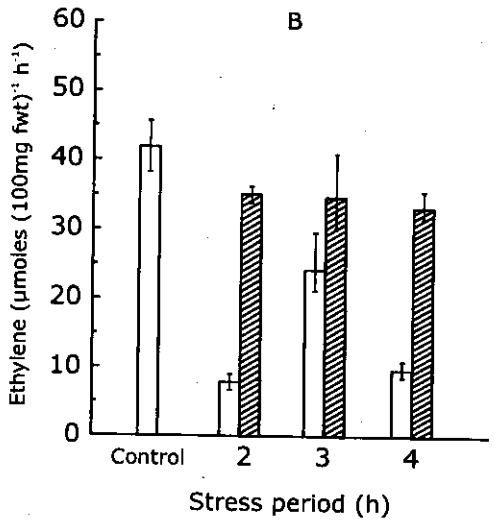
Results and Discussion

Acetylene reduction activity in the cultivar Sable is presented in (Fig. 1). A 2h heat stress at 35EC had little effect on activity. After 3 and 4h at 35EC, there was approximately 50% decrease in activity. At 40EC, the 2 and 4h stresses decreased nitrogen fixation more than the 3h stress and was strongly affected by heat stress at 45EC. After 72h recovery, the values in plants stressed for 2h at 35EC were higher than the controls and also better recovery occurred in other stressed plants. Plants which were stressed for either 2, 3 and 4h at 40EC showed the same degree of recovery of nitrogen fixation. Very slow recovery of nitrogen fixation occurred in plants heat stressed at 45EC.

Fig. 2 contains the data for cultivar Bragg. The rate of ethylene production in control plants, 17.1 $\mu moles^{-1}h^{-1}$, was recorded. When subjected to heat stress at 35EC, a 50-65% decrease in activity occurred after 2, 3 or 4h and in each case a very small recovery was found after 72h recovery at 27EC. At 40EC, approximately 70, 80 and 90% reductions occurred after 2, 3 or 4h of heat stress respectively. This recovered to a level higher than the control value in the 2h-stressed plants, however, and it fully recovered in the 3h-stressed plants. Activity did not reach the control value in the 4h-stressed plants after recovery. Following heat stress at 45EC, approximately 90-95% reductions in the ethylene production rate were found after 2, 3 or 4h and 80, 70 and 50% of the control value was regained in the 2, 3 and 4h stress plants respectively after 72h of recovery.

The control fixation rate of Davis (Fig.3) was only second to that of Sable at 24.7 $\mu moles^{-1}\ h^{-1}$ ethylene produced. Overall, the cultivar Davis showed a similar pattern of nitrogen fixation to Sable, when exposed to stress at 35EC. Ethylene production was not inhibited by 2 or 4h stresses, but declined after a 3h stress. At 40EC, similar decreases in activity were found in all treatments. At 45EC, activity was more affected by 2h stress than by 3 or 4h stresses. After recovery, the values were higher in plants stressed for 2 or 4h at 35EC compared with controls, and fixation recovered fully in 4h-stressed plants. Also, a better recovery was found in plants stressed at 40EC than in the 35EC stressed plants, but activity did not reach control values during recovery. Although nitrogen fixation recovered in plants stressed for 2h at 45EC, no





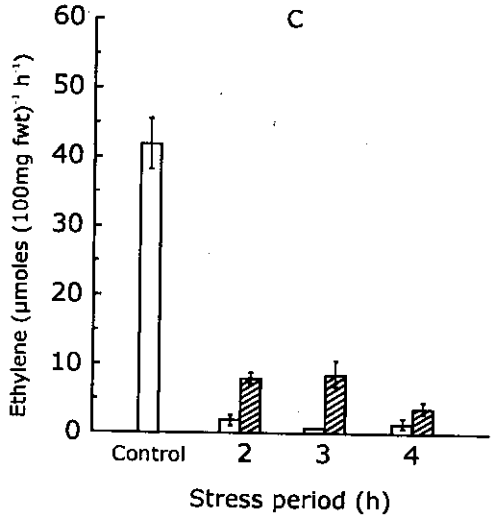
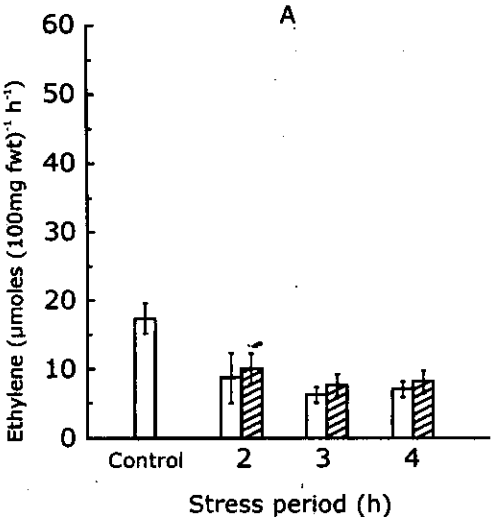
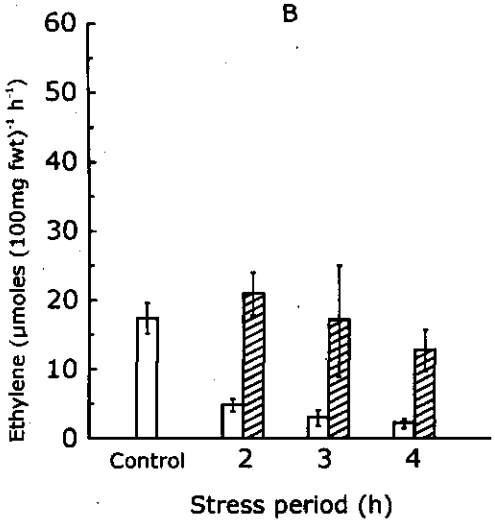


Fig. 1: Effect of root heat stress on nitrogen fixation in cv. Sable. A, 35; B, 40; C, 45°C; at the end of heat stress period; following recovery for 72 hr





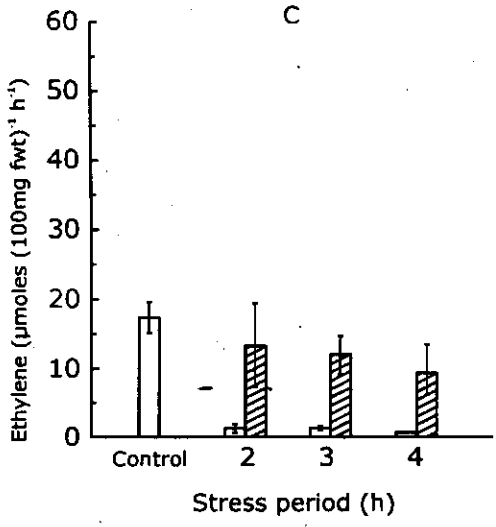
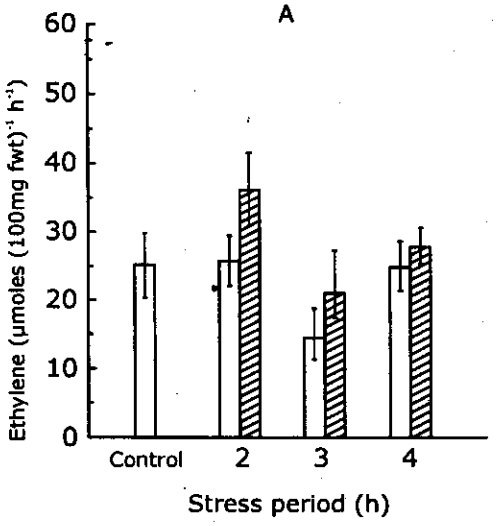
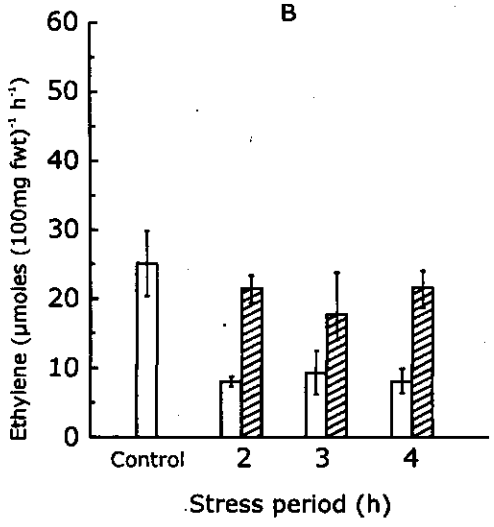


Fig. 2: Effect of root heat stresson nitrogen fixation in cv. Bragg. A, 35; B, 40; C, 45°C; at the end of heat stress period; following recovery for 72 hr





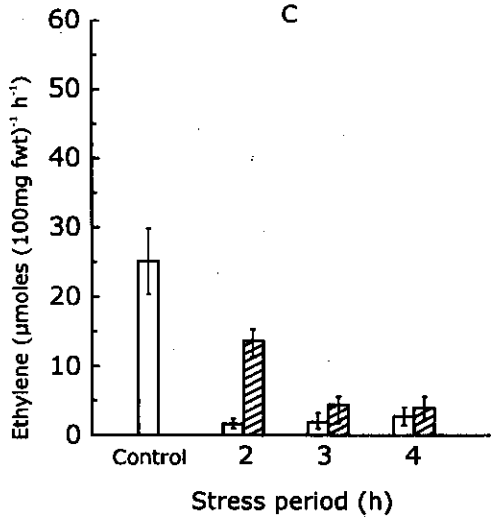


Fig. 3: Effect of root heat stresson nitrogen fixation in cv. Davis. A, 35; B, 40; C, 45°C; at the end of heat stress period; following recovery for 72 hr

recovery was observed in the plants stressed for 3 or 4h at this temperature.

Statistical analysis showed that, ethylene production was strongly dependent upon the cultivar, temperature and stress period. There were also significant interactions between the cultivar, temperature and stress period. It is understood that there are several possible causes of nitrogen fixation inhibition at high temperatures.

It is known that nitrogen fixation in legume nodules is affected by a wide range of adverse environmental and physiological conditions such as temperature, salinity, drought, chemicals etc. Our results are in general agreement with many reports from other researchers. Thus, some *Rhizobium* strains produce a large mass of nodules with low specific activity, while others can fix the same amount of nitrogen with a smaller nodule mass Duque *et al.*, (1982) and Rosendahl, (1984). Pulver *et al.* (1982) also found that genotypic variation exists in soybean with respect to its ability to established an effective symbiosis with local populations of *Rhizobium spp.*

These results roughly agree with those of Dart and Day (1971) and Dart *et al.* (1975), who reported that acetylene reduction by nitrogen-fixing organisms occurred over a wide temperature range with maximum activity between 24 and 33EC, rapidly declining at higher temperatures. Sinclair and Weisz (1985) observed that acetylene reduction rates in soybean increased with soil temperatures up to 30EC. Rates then declined slightly up to 34EC, but above this temperature they were greatly reduced.

In the present study, the responses which were observed in the different soybean cultivars were presumably due to the combined effects of high temperature on many different processes involved in the plant-rhizobium symbiotic association. These include the survival of the bacterial cell in the root nodules, nodule metabolism, changes in the physiology of the host plant and the heat sensitivity of the nitrogenase enzyme. Also, the role of leghaemoglobin in delivering this oxygen to the respiratory sites without inhibiting the oxygen-sensitive nitrogenase enzyme system Brun, (1978) may be affected by high temperatures. It has been suggested that in clover Hartwig *et al.*, (1987) and soybean (Vessey *et al.*, 1988) the nitrogenase is limited by oxygen supply (and thereby ATP availability) rather than by reductant (reduced ferredoxin) availability. Heat stress may affect the permeability of the nodules to O_2 by interfering with the normal mechanisms which regulate diffusion of oxygen into the nodules. These aspects are at present poorly understood Kuzma and Layzell, (1994).

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