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The Rate of Nitrogen Fixation in Soybean Root Nodules After Heat Stress and Recovery Period

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ABSTRACT

The results of the heat stress applied to the roots showed that nitrogen fixation (ethylene production) was inhibited by heat stress of 40°C and fixation activity generally declined with increasing periods of heat stress from 2 to 6 h. In both the 4 and 6 h stresses at 40°C, the fixation rate had declined by 70%, although after 2 h of stress the rate had only declined by 15%. In spite of these large decreases in the fixation rate after 4 and 6 h of heat stresses, the plants were able to recover much of their fixation capacity between 24 and 144 h after the end of the heat stress. The maximum recovery occurred 72 h after cessation of heat stress. Fixation capacity recovered better in the 4 h-stressed plants than in 6 h-stressed plants.

Key Words: Soybean; Nitrogen fixation; Nodule; Heat stress; Recovery

INTRODUCTION

There are many different physiological and environmental factors affecting the rate of the nitrogen fixation in legume root nodules, such as temperature, water logging, water stress, salinity, combined nitrogen levels, pH, nutrients etc. Research on these factors in different legumes has been performed in relation to temperature by Kuo and Boersma (1971), Pankhurst and Sprent (1976) and Sinclair and Weisz (1985). Many of these factors, including temperature, affect many 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.

Some techniques used to study legume nitrogen fixation are destructive to the whole plant. 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 (Lawn & Brun, 1974). In soybean study, soil temperatures between 30 and 33°C caused little change in the fixation rate, but temperatures above 34°C had a negative effect (Sinclair & Weisz, 1985). Munevar and Wollum (1981) also found high rates of fixation at 28°C in soybean and very low rates at 38°C. Similarly, Waughman (1977) found that fixation rates of detached soybean nodules increased to a maximum at 30°C and then decreased rapidly at 35°C. For soybean, soil temperatures between 30 to 35°C have been reported to interfere with the development and function of root nodules compared with lower temperatures (Munevar & Wollum, 1981, 1982).

MATERIALS AND METHODS

Preparation of the *B. rhizobium japonicum* inoculum. The liquid culture medium contained 10 g mannitol, 1 cm^3 of 10% K_2HPO_4, 4 cm^3 of 10% KH_2PO_4, 2 cm^3 of 10% $MgSO_4$, 1 cm^3 of 10% NaCl, and 0.4 g of yeast extract in 1 L of distilled water, pH 7.0, contained in 2 x 500 cm^3 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 1 g of stock sample to each bottle) and kept in an incubator for 10 days at 25°C.

Plant material. Seeds were soaked for 30 min in *B. rhizobium japonicum* inoculum and germinated in (10 x 5 cm) pots (2 seeds per pot) filled with vermiculite that had been pre-washed with distilled water. The pots and their contents were incubated in the growth room. The growth room was set at 24°C ± 0.5°C with a 16 h photoperiod. The light intensity in the room was 82-85 μmols m^{-2} s^{-1}. Five days after sowing, the seedlings were thinned to one per pot. Plants were irrigated with half-strength (nitrogen free) Long Ashton nutrient solution (Hewitt, 1966). One-week-old plants were inoculated with 3 cm^3 of *B. rhizobium japonicum* inoculum.

Effect of heat stress on nitrogen fixation. First experiment was conducted to investigate the effects on nitrogen fixation of heat stress applied to the roots. Seeds of cultivar Sable were germinated and inoculated seven days after sowing as described above. The plants were used when they were five weeks past-inoculation. Heat stress was applied to the roots for 2, 4 or 6 h (2 h daily) at 40°C in a water bath. 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. To determine the nitrogen fixation rate, the acetylene reduction method was used (Masterson & Murphy, 1980; Keerio & Wilson, 1998; Keerio, 2001). Nitrogen fixation was measured at 10, 20 and 30 min after injecting the acetylene. The experiment was carried out with five replicates for each treatment.

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 70°C for 48 h before measuring their dry weight.

Effect of heat stress and subsequent recovery. In the 2nd experiment the seeds of the cultivar Sable were grown and inoculated as described above. The plants were used for the experiment five weeks after the inoculation. Using the same method of heat stress as described above, heat stresses of 4 and 6 h duration at 40°C were applied to the roots and nitrogen fixation was measured immediately after the stress and after 24, 48, 72 and 144 h of recovery at the normal (24°C) growing temperature. In each case, nitrogen fixation was measured at 10, 20 and 30 min after the injection of acetylene into the pots. At the end of the experiment, nodules were counted and their fresh and dry weights were recorded as mentioned above. The experiment was conducted with five replicates for each treatment.

Statistical Analysis. Means, standard deviations and standard errors were determined using a pocket scientific calculator and they were checked by personal computer. Statistical analysis by analysis of variance (ANOVA) was done using the computer with the Minitab statistical package (version 10.2). In all figures, vertical bars show the standard errors of the mean values.

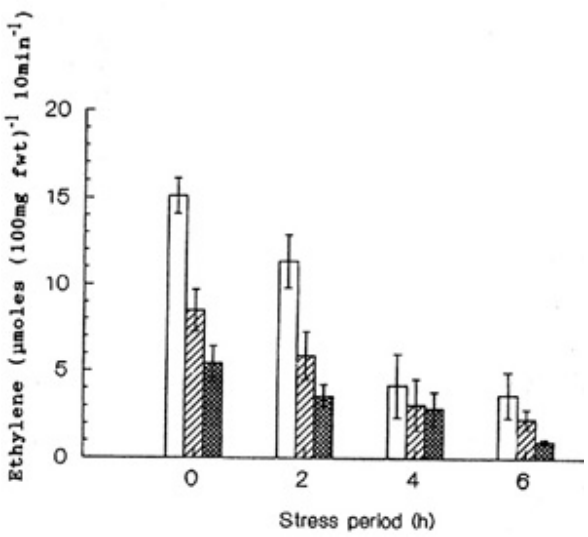
RESULTS AND DISCUSSION

Effect of heat stress on nitrogen fixation. The data in Fig. 1 show that 15.1 µmoles (100 mg fwt)$^{-1}$ h^{-1} of ethylene were produced 10 min after acetylene addition to the control plants. The rate then decreased with increasing incubation periods of 20 and 30 min after the acetylene addition. Approximately 25% reduction in the rate of ethylene production occurred as a result of a single 2 h heat stress treatment. A further 50% reduction was observed after 4h heat stress. Finally, another small decrease was observed after 6 h of heat stress. Similar patterns of declining activity were observed at 10, 20 and 30 min after acetylene injection in all cases. The data also show that both heat stress and the injection of acetylene had adverse effects on ethylene production.

The data were analysed statistically using the ANOVA test, which showed that, the ethylene production rate was significantly ($P<0.05$) dependent upon the length of the stress period and the timing of the acetylene production assay. There were no significant interaction between the stress period and assay timing.

Effect of heat stress and subsequent recovery. Heat stresses of 4 or 6 h duration were applied at 40°C and recovery was checked after 24, 48, 72 and 144 h. The data in Fig. 2a show that ethylene production measured 10 min after acetylene injection decreased by 90% after 4 h heat stress compared with the control. After 24 h of recovery, however, the rate had increased again to approximately 40% of the control value. The ethylene production rate continued to increase with increasing recovery period up to 72 h and decreased onward. Similar results were recorded for assays carried out at 20 and 30 min after acetylene addition. After 6 h of heat stress (Fig. 2b), there was an approximately 80% decrease in activity, followed by a pattern of recovery similar to that seen in the 4 h heat-shocked plants. The rate of ethylene production similarly declined from 10 to 30 min following acetylene injection at all points in the experiment. Analysis by ANOVA showed that both the heat stress treatments and the recovery treatments had significant

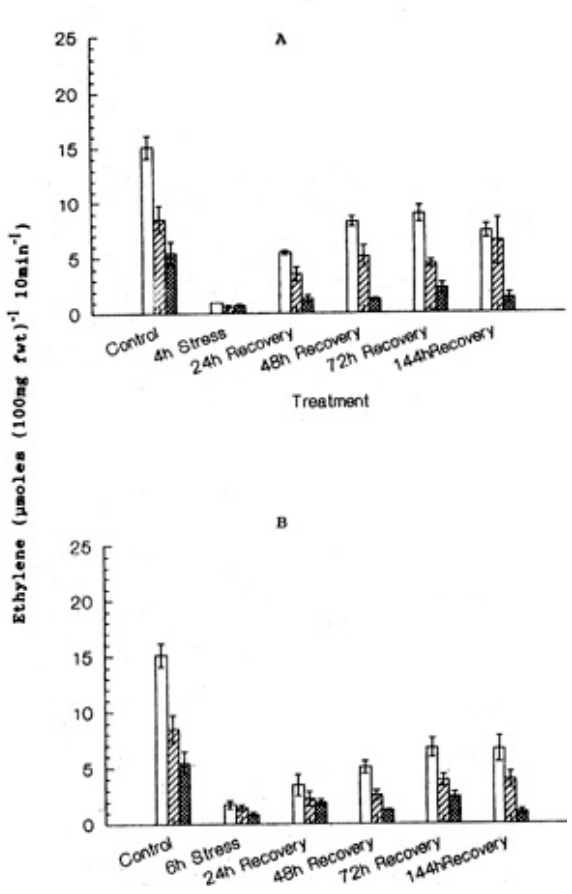
Fig. 1. Effect of root heat stress on nitrogen fixation in soybean cv. Sable, 10, 20 and 30 minutes after stress



($P<0.05$) effects on the ethylene production.

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. These results roughly agree with those of Keerio (2001) and Keerio and Wilson (1998) who found that nitrogen fixation was seriously inhibited at high temperatures. Minchin *et al.* (1983) found that when the nodulated roots of some legumes were exposed to acetylene, the rate of ethylene production decreased by 40 to 60% during the first 30 min of the assay. In contrast, Mederski and Streeter (1977) suggested that in soybean a 60% decline in nitrogenase activity only occurred at the end of 6 days

Fig. 2. Effect of root heat stress on nitrogen fixation in soybean cv. Sable, A: 4 h stress, B: 6 h stress; 10, 20 and 30 minutes after stress



continuous exposure to an acetylene-air mixture. Dart and Day (1971) and Dart *et al.* (1975) reported that acetylene reduction by nitrogen-fixing organisms occurred over a wide temperature range with maximum activity between 24 and 33°C, rapidly declining at higher temperatures. Sinclair and Weisz (1985) observed that acetylene reduction rates in soybean increased with soil temperatures up to 30°C. Rates then declined slightly up to 34°C, but above this temperature they were greatly reduced. Gibson (1969) reported a linear decline in the rate of nitrogen fixation with long daily exposures to 30°C. His results indicated that the effect was transient, however, and directed towards some temperature-sensitive step or steps in the nitrogen fixation reaction.

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 and these aspects are at present poorly understood (Kuzma & Layzell, 1994). 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. Hartwig *et al.* (1987) working on clover and Vessey *et al.* (1988) working on soybean, have suggested that nitrogenase is limited by oxygen supply (and thereby ATP availability) rather than by reductant (reduced ferredoxin) availability.

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