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Inhibition of Ferricyanide Reduction in Chloroplasts Prepared from Water-stressed Cotton Leaves[1]

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ABSTRACT

Water stress was applied to cotton (*Gossypium hirsutum* L.) chloroplasts and leaves by the following treatments: (i) prepared chloroplasts were placed in assay media of different osmotic potentials; (ii) cut shoots were allowed to transpire under illumination; (iii) whole potted plants were held for 5 days without irrigation; and (iv) cell sap was expressed from individual leaves via the xylem in a pressure bomb. These treatments caused a decrease in ferricyanide reduction when isolated chloroplasts were assayed potentiometrically for the Hill reaction. The above treatments caused (i) 1.7%, (ii) 1.4%, (iii) 1.8%, and (iv) 1.9% decrease of initial activity respectively per bar of water potential decline. This decrease in activity, which was nearly linear with the degree of stress, occurred from —3 to —30 bars leaf water potential in treatments 1, 3, and 4, and from —15 to —30 bars in treatment 2. These results indicate that water stress in the leaf can directly influence the photochemical activity of the chloroplasts.

Additional index words: **Water potential, Hill reaction.**

THE effects of water stress on photosynthesis in higher plants have been studied extensively. There is ample evidence of increased stomatal resistance limiting photosynthesis in water-stressed plants (10). Evidence, however, is also accumulating that shows a more direct inhibition of the photochemical process in the chloroplasts of stressed plants. Prepared chloroplasts have shown decreased ferricyanide reduction and photophosphorylation when stressed in osmotic media before and during the assay (6). When plants were stressed prior to the preparation of chloroplasts, photophosphorylation was diminished in spinach (*Spinacia* sp.) and fodder beets (*Beta* sp.) (6), swiss chard (*Beta vulgaris cicla*), (5), and cotton (*Gossypium hirsutum* L.) (9). Under the same treatments ferricyanide reduction was partially inhibited in swiss chard (5) and cotton (3, 9). Oxygen evolution decreased in leaves of pea (*Pisum sativum* L.) and sunflower (*Helianthus annus*) under moderate water stress (2). This study includes various methods of obtaining water stress in cotton leaves and chloroplasts, and compares the corresponding inhibition of ferricyanide reduction in the chloroplasts with levels of stress.

MATERIALS AND METHODS

Cotton plants (*Gossypium hirsutum* L.) of the glandless variety 'M-8' (4) were grown in pots containing sand-vermiculite-peat (1:1:1) mixture and were watered with a dilute nutrient solution. All sympodial branch growth was removed in the bud stage. Leaves and shoots were preconditioned to a maximum moisture equilibrium by enclosing potted plants from which they were obtained in polyethylene bags in the dark for 12 hours. The resulting water potentials of about —3 bars were found uniform among the leaves utilized for the experiments. Leaf discs were sampled from 23±3-day-old leaves at about the eighth node from the apex.

The leaf water potentials were either estimated from the pressure bomb measurements (7) or were determined graphically from the relationship of relative water content measurements to the pressure bomb measurements using the same plant variety and culture as in the experiments. Corrections for the osmotic potential of the xylem sap were not included, although cryoscopic measurements of the sap indicated that the average value was about —0.4 bars. The different methods of obtaining decreased water potentials in the chloroplasts, leaves, shoots and plants are described under RESULTS for each treatment.

The chloroplasts were prepared in 10% polyethylene glycol 6,000 in 0.1*M* potassium phosphate buffer adjusted to a pH of 7.6. The osmotic potential was measured at about —8 bars. The homogenate of 2-g leaf samples was squeezed through nylon parachute cloth and centrifuged at 8,000 × g for 3 min. Ferricyanide reduction by illuminated chloroplasts was assayed potentiometrically in a 0.5 *M* sucrose buffer. The uncoupling effect of the ammonium ion at 0.01 *M* was utilized in all assays to maximize activity. Although fewer chloroplasts were prepared from flaccid leaf discs than from turgid discs, the variation introduced from this source was avoided by basing the assays on the chlorophyll content. A detailed description and discussion of the above method was given in a previous paper (3).

RESULTS

Osmotic Stress in Assay Medium

A single volume of chloroplasts was prepared from preconditioned leaves and was stored in the isolation medium at 1 C. The ferricyanide reduction activity was assayed in a set of sucrose concentrations of decreasing osmotic potentials from —2 to —40 bars. A second set of assays followed immediately with increasing osmotic potentials, and the combined results were averaged to compensate for the loss of activity during storage. Figure 1 shows the regression for three replications. Increasing sucrose concentrations in the assay medium progressively inhibited the ferricyanide reduction and between the osmotic potentials of —2 and —40 bars (1.08 *M*) the activity had decreased 37%. The data of Santarius and Ernst (6) indicated that the Hill reaction activity decreased about 24% when spinach chloroplasts were placed in 1 *M* sucrose before and during the assay.

Stressing of Illuminated Shoots

From preconditioned plants shoots were excised in the dark, leaf discs were sampled, and the chloroplasts were prepared and assayed for ferricyanide reduction (Fig. 2). The shoots were then moved to a small controlled environmental chamber furnished with fluorescent and tungsten lamps giving 21,000 lux. They were held at 26 C and 50% relative humidity for 20 min, at which time humidity was increased to nearly saturation and held there for the balance of the experimental period of approximately 3 hours. Periodically, additional samples were taken and assayed from the leaves that were sampled initially. The water potentials of adjacent leaves were measured in the pressure bomb, and the results of four replications

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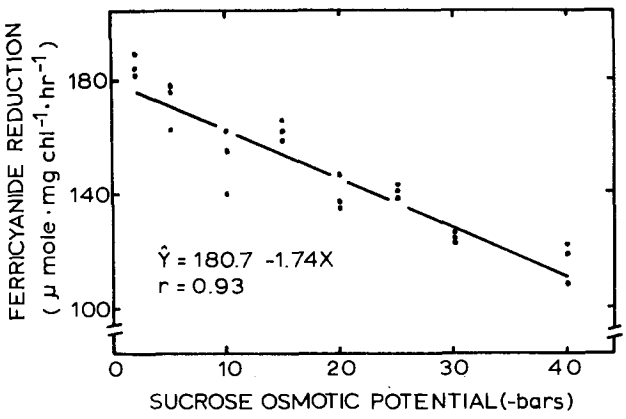

Fig. 1. The effect of different osmotic potentials in the assay medium on ferricyanide reduction in cotton chloroplasts.

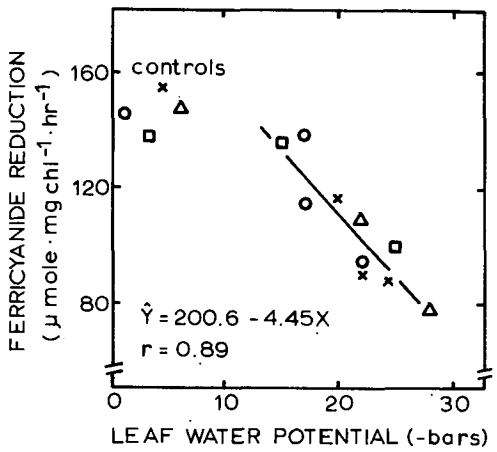

Fig. 2. The effect of different leaf water potentials on ferricyanide reduction in cotton chloroplasts. The leaves were partially dehydrated on cut shoots while illuminated. The controls represent the chloroplast activity before treatments. The different symbols represent four shoots.

are shown in Fig. 2. As the leaf water potentials decreased from —3 (controls) to —15 bars, only a small amount (4%) of the initial activity was lost. Boyer and Bowen (2) found a similar relation between leaf water potential and O_2 evolution in peas at —4 to —12 bars and in sunflower at —4 to —8 bars. In cotton leaves, however, at water potentials below —15 bars inhibition of ferricyanide reduction increased progressively, and at —27 bars about 46% of the initial activity was lost. The latter results compare favorably with the other treatments showing a general inhibition as water stress developed.

In the leaves having water potentials between —3 and —15 bars the resistance of the chloroplasts to activity decline might be explained by the enhancement effect of light on the chloroplast activity after the plants had been held in the dark for 12 hours. To show this, leaves were treated as above except that a high humidity was maintained so that leaf water potentials remained above —5 bars. The resulting chloroplast activity increased 31% over the activity obtained when shoots were excised after preconditioning. Apparently in Fig. 2, the amount of inhibition expected in the chloroplasts as the water potential decreased to —15 bars was balanced by the light enhancement resulting in very little change in activity. Below —15 bars the water potential was a dominant factor responsible for the loss of chloroplast activity.

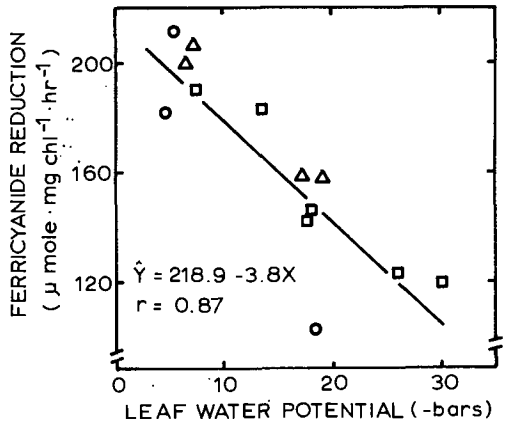

Fig. 3. The effect of prolonged water stress in potted plants on the ability of the chloroplasts to reduce ferricyanide. The different symbols represent three sets of plants.

The light effect may have been related to a soluble sugar increase that offered the chloroplasts some protection from desiccation (6). Preliminary assays for soluble sugars in leaves of cotton plants held in the dark for 12 hours showed that the level was already low. Only a small increase was obtained from leaves that were stressed in the dark; however, in the leaves stressed in the light, the sugar content doubled.

Stressing of Potted Plants

Whole potted plants were selected from greenhouse stock for uniformity and were preconditioned in the dark overnight in polyethylene bags. Two leaf discs were sampled from each of five turgid leaves of six plants in the dark, and the samples were divided evenly between two assays of 30 discs each, five of which furnished the relative water content data. After the plants were sampled they were returned to the greenhouse until midafternoon, when they were placed again in the dark. This treatment was repeated for 5 days, during which time irrigation was withheld from the plants to develop a water stress. The water potential was estimated from a predetermined relationship of pressure bomb measurements and relative water content. Figure 3 shows the results of three experiments. A progressive inhibition of the ferricyanide reduction in the chloroplasts is apparent under increasing water stress. A 40% decrease in activity occurred in 4 to 5 days as the water potential decreased from —4 to —26 bars. The slope of this regression is like that of Fig. 2, although the desiccation took several days. The rate of decrease for both the leaf water potential and chloroplast activity increased rapidly on the 4th and 5th days of treatment. The chloroplast activity of Fig. 3 is generally higher than that of Fig. 2, showing the effect of increased sunlight for greenhouse cotton during May (3). The chloroplasts in Fig. 2 were assayed in February.

In one of these experiments the plants were watered at the end of the stress period. After 24 hours the plants recovered 43% of the initial leaf water potential drop and 22% of the lost chloroplast activity. In another experiment where the re-watered plants were

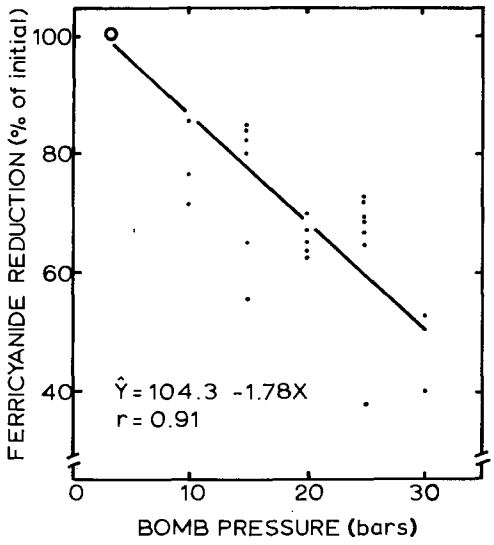

Fig. 4. The effect of lowered leaf water potentials on the ability of cotton chloroplasts to reduce ferricyanide. Each point represents the percentage loss of chloroplast activity after the leaf water was expressed with a pressure bomb (7). The initial activities, which varied between 100 and 250 μ moles ferricyanide reduced · mg chlorophyll^{-1} · hr^{-1}, were taken as 100% (circle).

held in saturated moisture conditions overnight, the chloroplasts recovered 31% of the lost activity, and the leaves, nearly 100% of the water potentials. Swiss chard (*Beta vulgaris cicla*) chloroplasts have been reported to recover about 36% of lost activity after the restoration of leaf turgor (5). In similar conditions for sunflower (*Helianthus* sp.), Boyer (1) suggested that the incomplete recovery of photosynthetic activity in severely droughted leaves was caused by stomatal resistance. In the present experiment the photochemical activity was directly affected by drought. The inhibition was partially reversible in 24 hours, even though wilting in the greenhouse had reduced leaf water potential to —26 bars for at least 16 hours.

Pressure Bomb Treatments

Scholander et al. (7) showed that leaf water potential can be lowered by expressing essentially pure water from a leaf or shoot by means of the pressure bomb. As the leaf loses water, the turgor decreases to 0 and there follows a decrease in the osmotic potential. This method was applied here to stress cotton leaves. Individual preconditioned leaves were placed in the bomb and stressed at selected pressures from 10 to 30 bars for 2 to 3 hours. Assays of chloroplast activity were made from the same leaf before and after the treatment. Initial samples of leaf discs were taken around the edge of the leaf where pressure bomb readings were not significantly affected as long as the larger veins were not cut. Subsequent samples were taken nearer the center of the leaf. Tests showed that there was no significant difference in chloroplast activity in turgid leaves between the edge and the center of the leaf. Sap expression had nearly ceased at the end of each treatment. The pressure bomb readings of the same leaf after once releasing the treatment pressure were within ± 1 bar of the treatment pressure. The direct effect of pressure and its sudden release on chloroplast activity was not determined.

In Fig. 4 the results from 23 leaves show a definite decline in chloroplast activity when the leaves were stressed in the above manner. The data show that a 50% reduction would be expected after stressing the leaves with 30 bars pressure. The results from lower pressure treatments were intermediate but somewhat scattered, possibly because of a diurnal effect on the activity-decline being measured. It was noticed that of the leaves given the 15-bar treatment there was a 23% decline in chloroplast activity for measurements made around 1130, while at 1345 and 1600 hours there was 7 and 3% decline, respectively. If this is an effect of time, other factors being constant, the chloroplasts apparently develop a greater resistance to internal changes by water stress as the day proceeds.

DISCUSSION

The above results show a general decline in the ability of cotton chloroplasts to reduce ferricyanide when they are at a water potential below maximum both *in vitro* and *in vivo*. Methods of chloroplast preparation and the assay for activity were the same for all the experiments to reduce extraneous variables. Water potential was assumed to be the primary factor influencing chloroplast activity. During their preparation, the chloroplasts were subjected to a water potential decrease from about —4 bars in the leaf to about —8 bars osmotic potential in the isolation medium (3). Upon placing the chloroplasts in the assay medium of a higher osmotic potential than the isolation medium their activity was apparently higher than if assayed at the same osmotic potential (Fig. 1). Their activity was also lowered when placed in assay media of more negative osmotic potentials. This change in activity occurred within 4 to 5 min after the chloroplasts were mixed with the assay medium. The influence of the isolation medium on activity was apparently reversible and transient in that the activity was readily increased when the chloroplasts were subjected to osmotic potentials above the —8 bar level.

A more permanent effect of leaf water stress on chloroplast activity is shown in the experiments where leaves, shoots, and plants were stressed before the preparation of chloroplasts. Here the inhibition that developed in the stressed leaves persisted in the chloroplasts during their preparation and assay. In the plants that were watered after the stress period, the leaf water potential increased to maximum (ca.—4 bars) in 24 hours, yet the chloroplasts recovered only 31% of their original activity, indicating a more permanent damage to the chloroplasts induced by dehydration.

Santarius and Ernst (6) have sugested that excessive loss of water leads to reversible uncoupling of photophosphorylation from electron transport. Previous work has shown that in cotton the ammonium ion will enhance ferricyanide reduction about 170% over control (3). Additional data (Fry, unpublished) from 11 experiments show an average 180 and 187% increase in ferricyanide reduction due to the ammonium for turgid and wilted leaves, respectively. This suggests that if uncoupling was caused by wilting in the leaves, it was probably reversed during the chloro-

plast preparation (24 min), and was not a part of the more permanent inhibition discussed above.

A comparison of the activities lost in each of the dehydration methods was made by expressing the rate of percent activity-decline between —4 and —30 bars leaf water potential. Chloroplasts assayed in the varied osmotica showed a decline of 1.7% per bar decrease in osmotic potential. This is twice the rate of spinach chloroplasts in sucrose as estimated from the data of Santarius and Ernst (6). Similar amounts of inhibition were obtained in the whole plant and pressure bomb treatments in which activity declined 1.8% and 1.9% per bar of leaf water potential, respectively. In the cut shoots a mean decline rate between the water potentials of the controls (—3 bars) and —27 bars was about 2.1% per bar although the actual rate between —15 and —27 bars was 3.5%. The increased rate may have resulted from the combined effects of water stress and a degeneration of the light enhancement.

These percentages might be compared to those derived from the work of Vieira-da-Silva and Veltkamp (9), who stressed plants in nutrient solutions with a maximum osmotic potential of about 11.1 bars as obtained with polyethylene glycol 6,000. Although no leaf water potential was reported, a value of 17.7 bars was estimated from Slatyer's data for the soil-plant relationships of Pima S-1 cotton (8). This conversion when applied to the data of Vieira-da-Silva and Veltkamp gives a 1.8% decline per bar for *G. hirsutum* var. 'HAR 444.2,' which is comparable to the present results. A greater decline of 4% was estimated for *G. thurberi*. The data of Boyer and Bowen (2) show a 2.5 and 7.0% decline of oxygen evolution per bar leaf water potential for pea below —12 bars and sunflower below —8 bars leaf water potential, respectively. Apparently there are specific and genetic differences in the loss of chloroplast activity as influenced by water stress. Such variations if present in cultivars of cotton may provide the breeder with a quick test for drought resistance.

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