

# The Effects of Water Stress on the Development of the Photosynthetic Apparatus in Greening Leaves<sup>1</sup>

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## ABSTRACT

The effects of low and high relative humidity and of polyethylene glycol-induced root water stress on chlorophyll accumulation, on formation of the lamellar chlorophyll-protein complexes, and on the development of photosynthetic activity during chloroplast differentiation were examined. Low relative humidity or polyethylene glycol-induced root water stress (stress conditions) resulted in a 3 to 4 hour lag in chlorophyll accumulation, retarded the rate of chlorophyll *b* accumulation, and reduced the rate of formation of the light-harvesting chlorophyll *a/b* protein. All of these effects could be overcome by high relative humidity (nonstress) conditions. Concomitant measurement of leaf water potential showed that under stress conditions greening leaves were subjected to initial water deficits of -8 bars which decreased to -5 bars after 3 to 4 hours of illumination corresponding to the end of the lag phase. Leaves greening under nonstress conditions did not experience leaf water deficits greater than about -5 bars. It seems that the attainment of a minimum leaf water potential of -5 bars may be critical in the control of early chloroplast development. These results demonstrate that the lag phase is not indicative of a programmed event in chloroplast development, but rather is attributable to environmental conditions prevailing during leaf development and greening.

The macromolecular differentiation of chloroplasts during greening of etiolated tissue is responsive to specific environmental factors, most particularly to temperature and to water status. Temperature affects such specific aspects of chloroplast development as the rate of prolamellar body formation (22) and the rate of Chl accumulation (27, 33). Similarly, tissue water status exerts control over the chloroplastic developmental processes; leaves subjected to low level water stress display depressed Chl accumulation rates (8, 16, 37), reduced incorporation of <sup>3</sup>H-uracil into RNA (8), and impaired ultrastructural differentiation (7, 15). A number of studies on both

water-stressed leaf material and isolated chloroplasts have revealed that such photosynthetic activities as CO<sub>2</sub> exchange rates (9, 10), Hill activity (12, 18, 19), photophosphorylation (28, 34), and O<sub>2</sub> evolution (11) are dramatically affected by low level water stress. Thus, it is clear that the light-energy transducing system of the plant cell is quite sensitive to tissue water status.

Realizing the significance of the role of water stress in plant growth and development at both the cellular and whole plant level (20, 23), the present study was undertaken to examine some specific effects of low level water stress on the biochemical differentiation of the chloroplast. The effects of low and high relative humidity on the formation of the lamellar Chl protein complexes and on the development of photosynthetic function during differentiation of etioplasts to chloroplasts were studied. An attempt was also made to mimic the effects of atmospheric water stress on chloroplast development by inducing low level water stress in the root systems of hydroponically grown seedlings.

## MATERIALS AND METHODS

**Plant Material.** Jack bean (*Canavalia ensiformis* L. DC.) seeds were soaked in running water (30-35 C) for about 18 hr until fully imbibed. For some experiments seeds were planted in coarse vermiculite and germinated in total darkness at 28 to 29 C, and in 80 to 85% RH<sup>3</sup> in one treatment and 30 to 35% RH in the other. The experiments were conducted in controlled environment chambers of the Duke University unit of the Southeastern Phytotron. In other experiments, imbibed seeds were germinated on moist paper towels in total darkness under the same conditions as vermiculite-grown seedlings. When the radicles of the etiolated seedlings reached about 5 to 10 cm in length, the seedlings were transferred to aerated hydroponic culture in half-strength Hoagland's solution. These plants were grown either under high or low RH conditions in darkness. Aqueous solutions of PEG 4000 were pumped into the nutrient solutions over a 12-hr period one day before illumination of cultures to be subjected to water stress.

All seedlings were illuminated on the seventh day after the start of germination. In some experiments, seedlings were allowed to green under low (30-35%) RH conditions, whereas in others, high (80-85%) RH conditions were maintained. At specific times during the illumination period, primary leaves were harvested for Chl determinations, chloroplast lamellar analysis, photochemical activity determinations, and water status analysis.

**Chlorophyll Determinations.** At specific times during green-

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<sup>3</sup> Abbreviations: RH: relative humidity; PEG: polyethylene glycol; SDS: sodium dodecyl sulfate; CPI: the P700 chlorophyll *a* protein; CPII: the light-harvesting chlorophyll *a/b* protein.

ing, primary leaves (at least 5 leaf pairs) were harvested from control and experimental seedlings for determination of leaf Chl *a/b* ratios and rate of total Chl accumulation. The leaf pigments were extracted in 80% acetone (v/v); Chl determinations were made using the equations of Arnon (4).

**Preparation and Characterization of Lamellar Chlorophyll-Protein Complexes.** Chloroplast lamellae were prepared from greening tissue at specific times during illumination following the procedures of Alberte *et al.* (1). The washed chloroplast lamellar fragments were then solubilized with SDS or Triton X-100. The SDS-treated lamellae were fractionated into two Chl-protein complexes, CPI and CPII, derived from photosystems I and II by SDS polyacrylamide gel electrophoresis (1, 36) or by hydroxylapatite chromatography (24). The Triton-treated lamellae were used for purification of the CPI complex as described elsewhere (35). Distribution and quantity of Chl and protein between the Chl-protein complexes were determined from spectral analysis of the fractionated CPI and CPII components and from polyacrylamide gels scans (1, 24, 36).

**Measurement of Light-induced Absorbance Changes.** The reversible, light-induced bleaching of P700 was measured in Triton-solubilized lamellae in a Cary 14 with a sample chamber adapted for cross illumination or an Aminco DW-2 dual beam spectrophotometer following the procedures of Dietrich and Thornber (14, 35). The concentration of P700 was estimated from the light-oxidized *versus* reduced difference spectra using  $100 \text{ mm cm}^{-1}$  as the differential extinction coefficient for P700 at 697 nm, the wavelength of maximum bleaching.

**Measurement of Oxygen Evolution.** Leaf discs were prepared from greening primary leaves during the early stages of greening.  $\text{O}_2$  exchange was assayed in 0.5%  $\text{NaHCO}_3$ , and estimated polarographically on a Clark-type electrode (YSI-53) after the procedures of Cockburn *et al.* (13). Measurements were made at 24 or 28 C with saturating illumination.

**Determination of Leaf Water Potentials.** Leaf discs of 0.6-cm diameter were removed from greening tissues, and their total water potentials were measured with Peltier cooled thermocouple psychrometers. At least five samples were taken for each determination. In the hydroponic experiments, the nutrient solution osmotic potentials were determined in a similar manner by wetting filter paper discs with test solutions.

## RESULTS

**Chlorophyll Accumulation.** Chlorophyll accumulation during greening of etioplasts is sensitive to water stress. Figure 1 shows that this accumulation is linear in primary leaves of jack bean which were grown and greened under nonstress conditions; whereas, leaves grown and greened under stress conditions show a 3- to 4-hr lag in Chl accumulation. The same greening rate of seedlings subjected to humidity stress could be obtained in hydroponically grown plants greened at high RH when the root systems were subjected to low level ( $-1.4$  to  $-2.4$  bars) stress by the addition of PEG 4000 to the culture solution (Fig. 1). The greening rates of hydroponically grown seedlings not subjected to root water stress but greened under high or low RH showed greening rates indistinguishable from those plants grown in well watered vermiculite and greened under similar RH conditions (Fig. 1).

Water stress during early greening also resulted in alteration of the rate of change of the leaf Chl *a/b* ratios (Fig. 1). Seedlings greening under nonstress conditions (*i.e.*, high RH and either in hydroponic culture with no root water stress, or in well-watered vermiculite) showed leaf Chl *a/b* ratios similar to those described earlier for jack bean (1) (Fig. 1). Leaf chlorophyll *a/b* ratios of stressed plants were higher during

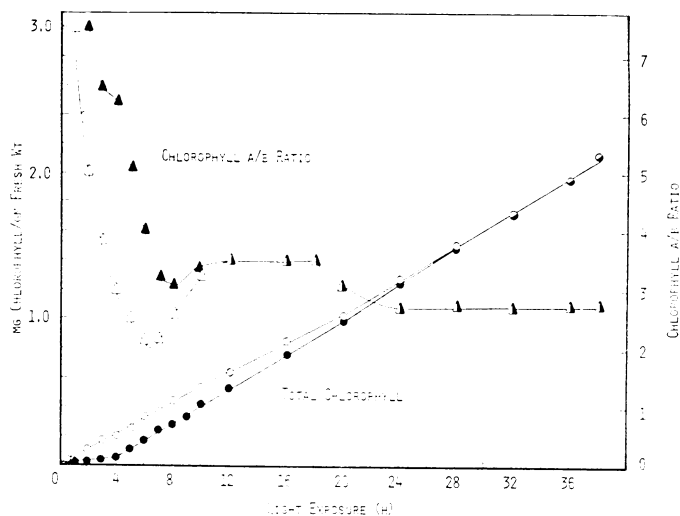


FIG. 1. Time course of total Chl accumulation in primary leaves of jack bean seedlings greened under high (80–85%) RH (—○—) either in well watered vermiculite or hydroponically grown; greened under low (30–35%) RH, or grown hydroponically and greened under high RH and subjected to PEG-induced root water stress (—●—). The leaf Chl *a/b* ratios are shown for the respective illumination periods and for the stressed (—▲—) and nonstressed (—△—) treatments.

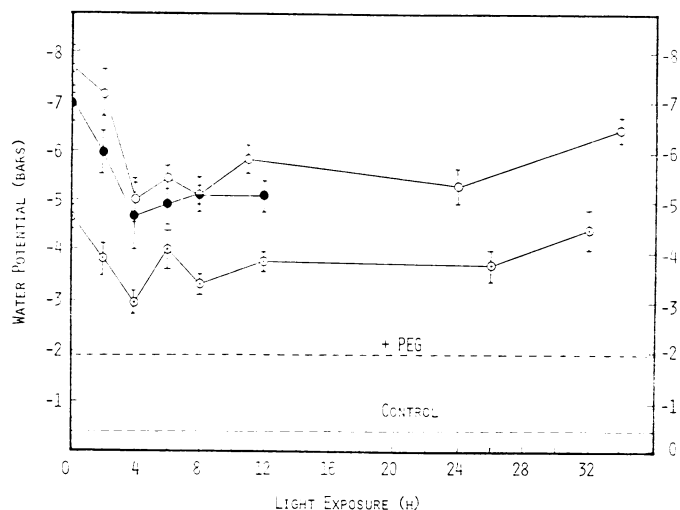


FIG. 2. Leaf water potential patterns of greening jack beans greened at low RH (○), high RH (○), and at high RH with PEG-induced root water stress (●). The water potentials for the control nutrient solution and the PEG-nutrient solution are also shown.

the lag phase (0–4 hr) of Chl accumulation. In these plants, there was a distinct delay in the decrease of the Chl *a/b* ratios, indicating a more reduced rate of Chl *b* production than of Chl *a* (Fig. 1). This lag is found concomitant with the lag phase of total Chl accumulation and has been observed previously (6, 29). After about 8 hr of illumination, leaf Chl *a/b* ratios of stress and nonstress tissues are similar (Fig. 1).

**Leaf Water Potentials.** A previous investigation (8) showed that jack bean seedlings greening in low RH possessed leaf water deficits in the range of  $-5$  to  $-8$  bars after 4 hr of greening; however, no data are available describing the changes in leaf water potentials during the course of chloroplast development. Figure 2 shows the leaf water potential patterns for seedlings greened under high and low RH, and subjected to PEG-induced root water stress. Leaf water potentials for

etiolated seedlings grown in well watered vermiculite or hydroponically in high RH environments, fluctuate between  $-2.8$  and  $-4.8$  bars during 32 hr of greening (Fig. 2). They initially increase from  $-4.8$  bars at 0 hr to  $-2.8$  at 4 hr, after which they decrease to about  $-4$  bars. Water potentials of leaves subjected to low RH atmospheric water stress whether grown in soil or hydroponically increase very sharply from about  $-7.5$  bars to about  $-4.8$  bars during the first 4 hr of greening and then decrease to a level of about  $-5.5$  bars (Fig. 2). Attainment of a minimal leaf water potential approaching  $-5.0$  bars after 4 hr of illumination corresponds to the end of the lag phase in Chl accumulation (Fig. 1).

The leaf water potential patterns obtained with seedlings greened in low RH could be mimicked in seedlings greened under high RH with low level ( $-1.4$  to  $-2.0$  bars) PEG-induced root water stress. The leaf water potentials increased in a manner similar to that for the low RH seedlings and stabilized between 4 and 14 hr of greening at about  $-5.0$  bars (Fig. 2). Leaf water deficits increased slightly again after 24 hr of greening in both stressed and nonstressed plants (Fig. 2).

**Oxygen Evolution.** Leaf discs were prepared from stressed and nonstressed primary leaves at various times during the first 6 hr of greening. The rate of dark respiration ( $O_2$  consumption) of stressed leaves was the same as that for nonstressed leaves. Similarly, the rates of net  $O_2$  evolution in the light were indistinguishable in stressed and nonstressed material.  $O_2$  evolution was detected in both groups after 2 hr of greening ( $20$ – $23 \mu\text{moles } O_2 \text{ hr}^{-1} \text{ mg Chl}^{-1}$ ). It is important to realize, however, that this method of detection cannot be used to discern whether or not  $O_2$  evolving capacity was retarded in stressed leaves, because the leaf discs most probably equilibrated rapidly with the osmotic potential of the bathing solution ( $0.5\%$   $\text{NaHCO}_3$ ). In this respect, another study (11) using a polarographic method for the estimation of  $O_2$ , found that  $O_2$  evolution was not inhibited in leaf material subjected to water deficits less than  $-8$  bars.

**Appearance of Light-induced Changes in P700.** The time course of appearance of light-induced bleaching of P700 in Triton-solubilized lamellae from both stressed and nonstressed leaves indicates that there is no detectable P700 present in the early stages of greening in agreement with earlier reports (1, 2). Photo-oxidation of P700 was detected after 6 hr of illumination in both cases. Further, the leaf Chl-P700 ratios of the stressed and nonstressed tissues were the same from first detection of P700 throughout the greening period and similar to those reported earlier for jack bean (2).

**Separation and Characterization of Lamellar Proteins.** The pigment and protein band patterns on SDS-polyacrylamide gels of lamellar extracts during the greening of seedlings under stress and nonstress conditions were identical and indistinguishable from those reported previously (1). In all cases a pigment-protein band corresponding to CPII, the Chl *a/b* protein, was detected after 2 hr of greening. However, in stressed leaves there was a 50% reduction in the amount of CPII observed on gels or obtained from hydroxylapatite chromatography between 2 and 4 hr of illumination (Table I). The reduction in amount of CPII corresponds to the lag in Chl accumulation and specifically with the lag in Chl *b* accumulation (Fig. 1). Further, it was noted that the early formed CPII isolated in stressed leaves (Chl *a/b* = 4.5) was even more enriched in Chl *a* than that found in nonstressed leaves (Chl *a/b* = 3.0) (1); the CPII complex from fully green leaves typically contains equimolar concentrations of Chl *a* and *b* (24). The particularly high Chl *a* enrichment in the isolated complex is likely to be a consequence of the lag in Chl *b* accumulation in stressed tissue.

Table I. Percentage of Total Lamellar Chlorophyll Associated with CPII

CPII was determined from gel scans of SDS-extracted membranes prepared from stressed and nonstressed leaves after various periods of illumination.

Light Exposure <i>hr</i>	Total Chl in CPII	
	Stressed	Nonstressed
0	ND	ND
2	21	44
4	28	58
6	40	65
8	50	55
12	48	49

The appearance of CPII was not affected by the lag phase resulting from small water deficits during greening. CPII was first detected on gels after 6 hr of greening in both stressed and nonstressed leaves, and could be isolated by hydroxylapatite chromatography and shown to contain photooxidizable P700. The concomitant appearance of CPII and photochemically active P700 has been reported previously (1, 2).

## DISCUSSION

There is general agreement in the literature that atmospheric water stress which results from low RH has distinct consequences on plant growth (30). Little is known about the specific biochemical events which are sensitive to RH. Bourque and co-workers (7, 8) showed previously that low RH and consequently water stress in the range of  $-5$  to  $-8$  bars has rather dramatic effects on chloroplast development. In the present study, reduction in Chl accumulation resulting in a 3- to 4-hr lag was characteristic of etiolated tissue greening in low RH or subjected to small root water deficits. The effects of these small water stresses were manifested primarily during early chloroplast development and are not detectable in more mature chloroplasts (*cf.* ref. 15; E. L. Fiscus and R. S. Alberte, in preparation). However, leaves greening in high RH showed no lag in Chl accumulation. The latter situation represents the first demonstration that the lag phase in chloroplast development previously thought to be inherent, can be eliminated without light pretreatment or addition of exogenous substance to etiolated tissue.

The lag in Chl accumulation was also reflected in the leaf Chl *a/b* ratios. Leaf material subjected to water stress (whether atmospheric or applied to the roots) showed a lag in Chl *b* accumulation compared to nonstressed leaves (Fig. 1). This lag phase in the lowering of the leaf Chl *a/b* ratio in early greening (between 2 and 4 hr) is found concomitant with a 3- to 4-hr lag in Chl accumulation as observed elsewhere (6, 29). These effects on Chl accumulation had pronounced consequences on the production rate of the major light-harvesting Chl *a/b* protein (CPII) during early greening (Table I). This complex typically contains equimolar amounts of Chl *a* and *b*, accounts for about 50% of the total lamellar Chl, and contains essentially all the Chl *b* present in the membranes (5, 36). Therefore, a much reduced rate of formation of this complex can explain not only the depressed rate of total Chl accumulation but also the lag in Chl *b* accumulation found in stressed tissue (Fig. 1). It cannot be ascertained at this time whether the primary effect of the water deficit is on Chl biosynthesis or on assembly of the protein moiety of CPII, or both.

The observation that assembly and appearance of the photosystem I P700-Chl *a* protein (CPI) were unaltered during greening of stressed tissue may suggest that the machinery necessary for the formation of this complex is less sensitive to water stress than that for CPII. However, during the period of greening preceding the detection of CPI, leaf water potentials were not as low as those preceding formation of CPII (see Fig. 2); therefore, the sensitivity of CPI formation to small water deficits cannot be determined here. Other studies have shown that both P700 activity (16) and CPI (R. S. Alberte and E. L. Fiscus, in preparation) are very stable in tissues subjected to large water deficits.

The lag phase in chloroplast development under low RH was attributable to water deficits in the etiolated tissue (37). This observation was substantiated in seedlings subjected to small root water stresses (*cf.* ref. 15) (Fig. 2). The sharp increase in leaf water potentials between 0 and 4 hr of illumination (from -8 bar to -5 bar) coincided with the end of the lag phase in total Chl accumulation, with end of the delay in reduction of leaf chlorophyll *a/b* ratio (Figs. 1 and 2), and with the reduced rate of CPII formation. These observations demonstrate clearly that the lag phase is caused primarily by environmental conditions prevailing during development and greening of etiolated tissue and is not indicative of a programmed event in chloroplast development.

The rapid increases in leaf water potentials in all leaves during the first 4 hr of greening might be explained by two important aspects of leaf surface morphology. Etiolated jack bean leaves are essentially devoid of any cuticle (R. S. Alberte, unpublished observations; 26), subjecting the full leaf surface to transpirational water loss. Low RH conditions place a greater evaporative demand on tissue than high RH conditions; therefore, it is reasonable to expect greater leaf water deficits to develop in tissue growing under low RH. Upon illumination of etiolated tissue rapid deposition of cutin is initiated (26) resulting in greatly increased resistance of the evaporative surface to water loss. Consequently, leaf water deficits are reduced. Concomitant with the initiation of cutin deposition upon illumination of dark-grown tissue is the commencement of functional development of the leaf stomatal complexes (17, 25, 32). Studies on greening jack bean leaves reveal that the stomates do not become functional until after about 8 hr of greening, shortly after which the leaves reach CO<sub>2</sub> compensation (net CO<sub>2</sub> exchange = 0) (R. S. Alberte, S. B. Fox, and A. W. Naylor, in preparation). The rapid increase in resistance of the evaporative surface to water loss and the lack of stomatal water loss during early greening can account for the increased leaf water potentials between 0 and 4 to 6 hr of illumination. The decrease in leaf water potentials observed after 24 hr (Fig. 2) are quite likely attributable to much increased stomatal transpiration.

Another important point with respect to the functioning of guard cells during greening is the correlation between the onset of stomatal opening with the formation of the photosystem I reaction center complex, the P700-containing CPI complex. It is well established that potassium flux provides the driving force for stomatal activity (3, 21). Furthermore, it is apparent that photosystem I cyclic photophosphorylation is the primary energy source for active potassium transport into guard cells (31). Therefore, it is reasonable to expect the initiation of stomatal activity to follow the development of the photosystem I reaction center (after 6 hr of greening) in greening leaves.

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