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Short Communication

Large Effects of Small Water Deficits on Chlorophyll Accumulation and Ribonucleic Acid Synthesis in Etiolated Leaves of Jack Bean (*Canavalia ensiformis* [L.] DC.)[1]

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In the course of investigating RNA metabolism in greening plastids of unexpanded leaves of jack bean (*Canavalia ensiformis* [L.] DC.) seedlings, it was found that relative humidity had to be controlled in order to obtain reproducible rates of chlorophyll accumulation and ^{3}H-uracil incorporation into newly synthesized RNA. Under high relative humidity conditions, chlorophyll accumulation occurred rapidly in the light while low relative humidity conditions were associated with slow accumulation and much reduced incorporation of radioactive uracil into RNA.

MATERIALS AND METHODS

Jack bean seeds were germinated and grown in the dark under high humidity (80–90%) conditions at 25 C. In the greening experiments etiolated shoot samples (8–10 days old) were placed in small tubes or vials containing distilled water or one-fifth strength Hoagland's solution with or without addition of 1% sucrose. Use was made of single leaves with petioles, shoots excised just above the cotyledons, and shoots excised several inches below a single remaining cotyledon. Effects were noted of humidity, nutrient supply, and organ excision on chlorophyll accumulation. Chlorophyll was determined by Arnon's (1) method.

RNA was extracted from the primary leaves by the phenol procedure of Bourque (2) and Bourque and Naylor (3) and will be described in detail elsewhere. An aliquot was sampled for RNA content, and the remaining RNA was precipitated with an equal volume of cold 10% trichloroacetic acid. The precipitate was filtered through glass fiber filters and washed with equal volumes of 5% trichloroacetic acid, 100% ethanol, and petroleum ether (boiling point 65–110 C). The radioactivity bound on the dried filters was determined with a liquid scintillation spectrometer.

RESULTS

Table I shows that in the dry chamber when a single cotyledon was present on the shoot axis there was little leaf weight loss, and chlorophyll accumulated to only half the level found in leaves on shoot axes in the damp chamber. It is known (18) that respiratory weight losses in excised wheat leaves may amount to 6% in 24 hr and greening can be sustained only by the addition of glucose or sucrose (17). Excised etiolated leaves of *Phaseolus vulgaris* also quickly exhaust their food supplies during greening (14, 19), and chlorophyll synthesis has been found to be sustained only by the addition of glucose and sucrose. In our plants, however, exogenous nutrients were ineffective in restoring chlorophyll-accumulating capacity in the absence of cotyledons. Indeed, regardless of the humidity conditions or excision treatments, neither water uptake nor dye translocation from the bathing solution was observed even after 48 hr in the light in samples run simultaneously with the experiments shown in Table 1. However, all the leaves on shoots lacking an intact cotyledon were visibly wilted after 24 hr in the light. It, therefore, was postulated that the cotyledons supplied water as well as nutrients to the greening leaves which allowed maximal chlorophyll production by the leaves in the damp chamber.

In order to learn the optimal humidity conditions for greening, experiments were carried out in growth chambers maintained at 25 C. Light was supplied by Cool White fluorescent tubes supplemented by incandescent lamps yielding 4.5×10^4 ergs cm^{-2} sec^{-1} at the leaf surface. Relative humidity was adjusted at 85, 50, and 25% in three comparable runs of the experiment. Uniform, dark-grown seedlings 18 to 25 cm tall and 8 to 10 days old with leaves that had not yet emerged from between the cotyledons were used as experimental material.

Figure 1 shows that at 85% relative humidity etiolated jack bean leaves accumulated chlorophyll rapidly with little or no lag phase in synthesis. The rate of chlorophyll accumulation was essentially linear during 48 hr of greening. At 50% relative humidity (measured value, 47%), the rate of chlorophyll accumulation was somewhat retarded, only half as much having accumulated per leaf after a 12-hr period as in the 85% relative humidity chamber. Subsequently, however, metabolic adaptation seemed to occur and accumulation was so rapid that after 48 hr the level of chlorophyll was 90% of that found in the 85% relative humidity chamber. When the leaves were allowed to green at 25% relative humidity, very slow chlorophyll accumulation occurred reaching only one-quarter of that found in the 85% relative humidity chamber after 12 hr. Also, little or no metabolic adaptation occurred, and the amount of chlorophyll after 48 hr was only 36% of that accumulated at 85% relative humidity. In the absence of a cotyledonary food supply, greening was negligible even after 48 hr under high relative humidity conditions.

In addition, ^{3}H-uracil incorporation into newly-synthesized

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Table I. *Effects of Humidity, Nutritional Status, and Excision on Chlorophyll Accumulation after 26 Hr of Greening*

Chamber temperature was 23 C and no supplemental incandescent lighting was supplied in this experiment. Light intensity at the leaf surface was not measured. One leaf pair (6 days old) was used for each measurement. The experimental setup was essentially the same as in Figure 1 except as noted.

Excision[1] Method	Nutrient Solution	Fresh Wt. per Leaf	Chlorophyll per Leaf	Maximum Observed Chlorophyll Accumulation
		mg	*μg*	%
Damp Chamber (85% relative humidity				
Cut below cotyledon[2]	Distilled H_2O	66	37	100[3]
Cut above cotyledons	Distilled H_2O	67	12	32
	1/5 Hoagland's	56	8	22
	1/5 Hoagland's + 1% sucrose	51	5	14
Single leaf with petiole	Distilled H_2O	60	5	14
	1/5 Hoagland's + 1% sucrose	59	8	22
Dry Chamber (50% relative humidity)				
Cut below cotyledon[2]	Distilled H_2O	60	18	49
Cut above cotyledon	Distilled H_2O	58	1	3
	1/5 Hoagland's	36	3	8
	1/5 Hoagland's + 1% sucrose	46	1	3
Single leaf with petiole	Distilled H_2O	39	4	11
	1/5 Hoagland's	45	3	8
	1/5 Hoagland's + 1% sucrose	23	1	3

[1] The cut end of the excised organ was placed in vials in contact with the bathing solution in all treatments.

[2] One cotyledon remaining.

[3] The chlorophyll accumulated in the presence of one cotyledon at 85% relative humidity was assigned a value of 100%.

RNA (Table II) was markedly inhibited by the absence of cotyledons when etiolated primary leaves were allowed to green at 85% relative humidity.

In contrast, 9.4% of the total radioactive uracil applied in the presence of one cotyledon was incorporated into RNA in this experiment. It appears that lack of appreciable transport of externally supplied water through the cut hypocotyl may account for the low incorporation of labeled uracil into RNA in the primary leaves. Attempts to carry out greening experiments with detached leaves floating on a nutrient medium (1% sucrose, 10 μg/ml lysozyme, and one-fifth strength Hoagland's solution) were unsuccessful in replacing the cotyledon as the nutrient supply to the developing chloroplasts. In the floating leaf experiments, ^{14}C-uracil incorporation was barely detectable after 4 hr incubation (about 1–2 cpm/μg RNA). Chlorophyll accumulation proceeded slowly and was markedly uneven among leaves within the same experimental

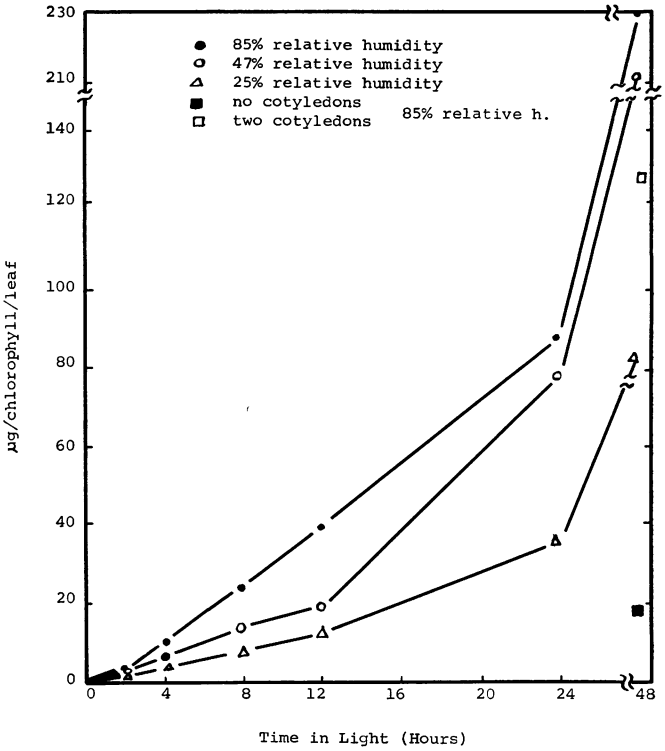

FIG. 1. Chlorophyll accumulation in greening leaves under different relative humidity conditions. All three curves were obtained from greening experiments carried out using shoots excised several inches below the cotyledons. One cotyledon was removed to expose the still folded primary leaves and the cut shoot was bathed in distilled water in small vials which were held in test tube racks. Light exposure for all leaf pairs was equivalent at a constant distance from the light source. Data for chlorophyll accumulation in the presence and absence of both cotyledons after 24 hr in the light (data displaced on the abscissa for clarity) are shown for comparison. All data points represent chlorophyll determinations for three pairs of leaves pooled before pigment extraction. The plants used in this experiment were 8 to 10 days old and were comparable in pigment content (qualitatively) to 9-day-old barley seedlings (8) as judged by absorption spectra *in vivo* of intact leaves.

Table II. *^3H-Uracil Incorporation into Total RNA of Jack Bean Leaves during the 15th to 19th Hr of Greening at 85% Relative Humidity*

Cut shoots as in Figure 1 were used except as noted.

Experiment No.	Treatment	Specific Radioactivity[1]
		dpm/μg RNA
1	One cotyledon present[2]	303 ± 33
2	No cotyledons present[2]	116 ± 46
3	No cotyledons, ^3H-uracil in bathing medium	less than 1.0
4	As in 3, leaf tips cut	less than 1.0

[1] Radioactivity determined as trichloracetic acid insoluble counts and based on RNA absorbance of 1.0 A = 45 μg RNA; dpm values expressed after correction for counting efficiency. Values are averages for three experiments and variance is average deviation from the mean.

[2] Two and one-half microcuries 5-^3H-uracil (26 μc/μmole) in 5 μl was applied directly to the leaf surface with a Hamilton microsyringe and gently distributed over the exposed half of one leaf of the pair with the needle barrel.

group. In contrast to the plants studied by other investigators (14, 17, 18), jack bean primary leaves appear to require something from their own cotyledonary nutrient supply to permit rapid chlorophyll accumulation and RNA synthesis to take place during the greening phase of chloroplast development.

Because of the possibility that slow accumulation of chlorophyll under low humidity conditions was due to previously unsuspected water stress in the leaves measurements were made of the water status of the unexpanded leaves. In this investigation leaves were allowed to green for 4 hr under three humidity regimes (25, 50, and 85%), and their water potential (Ψ_{leaf}) was determined with a Richards and Ogata type thermocouple psychrometer (16). One leaf was unfolded and placed in the bottom of each chamber; psychrometer equilibration time was 6 to 8 hours at 27 ± 0.001 C. At 85% relative humidity (Table III), the leaf water potential was −4.8 bars indicating that, even when the humidity was high, cuticular transpiration was occurring and the leaves were under some water stress. At 50% relative humidity and 25% relative humidity, the leaf water potential was about −8 bars. Thus a difference in water potential of −3 bars was sufficient to result in 45% less chlorophyll accumulation than otherwise. Presumably, the water deficit of the young leaves did not go beyond −8 bars because an equilibrium was established at that point between loss of water from the leaves and its supply from other parts of the seedling. Boyer (4) found that, even in leaves from several plant species supplied with pure water through their petioles, the leaf water potential never rose above about −1.5 to −2.5 bars. Although Boyer (5) observed large inhibitions of leaf enlargement at leaf water potentials in the range of −4 bars, leaf unfolding and expansion appeared to be little affected at any of the humidity regimes tested in our experiments.

Correlated with the rates of chlorophyll production under the different humidity regimes were striking differences in thylakoid development in the etioplasts (2). While details relevant to the ultrastructural changes will be given elsewhere, the electron micrographs clearly show that water stress interferes with the physical mechanisms of prolamellar body dispersal and grana formation.

Jack bean leaves greened in a dry chamber (50% relative humidity) had markedly inhibited chloroplast development (2). Partial prolamellar body transformation occurred within 2 hr in the light. Little further dispersal was noted after 4 hr and no further vesicle dispersal had occurred after 8- and 12-hr exposure. A prolamellar center still existed after 24 hr in the light and it was only then that the first signs of membrane pairing were observed. At the 24-hr stage, grana formation was roughly equivalent to that found in leaves greened for 2 hr in the damp chamber. Thus, complete dispersal of the prolamellar body, which occurs in less than 2 hr in the damp chamber, is greatly delayed in the dry chamber. In many respects the sequence of ultrastructural changes observed in jack bean leaves under low humidity conditions was similar to those described in salt-stressed greening bean leaves (15).

Table III. *Water Potential and Chlorophyll Accumulation after 4 Hr Greening at Different Humidities*

Cut shoots with one remaining cotyledon were used as in Figure 1.

Relative Humidity	No. of Measurements	Ψ_{leaf}	Chlorophyll per Leaf
%		*bars*	*μg*
85	3	−4.8 ± 0.3[1]	11
50	3	−7.7 ± 0.4	6
25	2	−7.8 ± 0.3	3

[1] Variance is average deviation from the mean. Maximal deviation from the mean was less than 1.0 bars in all treatments.

DISCUSSION

The cause or causes of delayed greening under dry air conditions are not known. Several chemical activities of chloroplasts are known however to be sensitive to the water status of the leaf (6, 11–13, 15, 18). A light-induced membrane-bound ATPase has been found in chloroplasts (13), and chloroplasts from water-stressed Swiss chard leaves have consistently shown enhanced phosphatase activity (11). Packer and Marchant (13) have concluded that the membrane-bound ATPase plays a controlling role in chloroplast membrane conformation. This may account in part for Nir and Poljakoff-Mayber's (12) observation that chloroplasts from leaves of Swiss chard hydrated approximately 10% below full turgidity were only two-thirds as efficient in carrying out cyclic photophosphorylation and photoreduction as chloroplasts from fully hydrated leaves.

In addition, reduced rates of oxygen evolution by chloroplasts isolated from water-stressed leaves have recently been demonstrated (6).

In contrast to the results of Kessler (9) and Gates and Bonner (7), who found no reduction of radioisotope incorporation into RNA in water-stressed plants, greening jack bean leaves under mild water stress conditions exhibit reduced rates of chlorophyll and RNA synthesis which are probably due primarily to suppressed transport of cotyledonary nutrient supply rather than as a result of a direct effect of water stress on the early events in chloroplast development from etioplasts. In the case of greening jack bean leaves, water stress-induced hydrolysis and turnover of preexisting RNA as suggested by Kessler (9) appears unlikely to be able to provide an adequate nucleotide pool to permit normal chloroplast development in the absence of the usual translocational influx of energy sources and nucleotides from the cotyledons. The possibility cannot be ruled out, however, that synthesis of G-C rich RNA (18) may inhibit the uptake of the radioactive uracil in our experiments when chlorophyll accumulation is suppressed by conditions of mild water stress.

Virgin's (18) observation that the rate of synthesis of protochlorophyll was markedly affected by small changes in water potential in excised wheat leaves together with the results shown in Figure 1 may be interpreted to mean the principal block in chlorophyll synthesis occurs in the metabolic pathway to protochlorophyll. In greening jack bean leaves the enzymic step or steps most sensitive to water stress appears to be capable of adapting, in time, to the water stress conditions unless the relative humidity drops below the 50% level.

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