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Photophosphorylation and Carbon Dioxide Fixation by Chloroplasts Isolated from *Populus deltoides*[1]

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ABSTRACT

A system has been developed for the isolation of photosynthetically active chloroplasts from leaves of *Populus deltoides*. A high proportion of the chloroplasts appeared intact. The maximum rates of different photosynthetic processes were as follows: CO_2 fixation 3.5 micromoles per milligram chlorophyll per hour, noncyclic ATP synthesis 10 micromoles per milligram chlorophyll per hour, and cyclic ATP synthesis 300 micromoles per milligram chlorophyll per hour.

Investigation of photosynthesis using isolated chloroplasts allows the separation of the reactions in the chloroplast compartment from the reactions in the other compartments of the cell. Availability of such active chloroplast preparations is prerequisite for the study of the photosynthetic pathways and control mechanisms at the chloroplast level.

Photophosphorylation and CO_2 fixation by isolated chloroplasts has been studied in many crop plants (3, 7–9). Cell-free preparations isolated from trees have been used to demonstrate the Hill reaction; this included homogenates prepared from cottonwood (5). Tree leaves and needles have not been successfully used as a source of chloroplasts for studies of photophosphorylation and light-dependent CO_2 fixation.

The aim of this study was to devise techniques for the isolation of structurally intact and active chloroplasts from Eastern cottonwood which would be capable of CO_2 fixation and cyclic and noncyclic ATP synthesis.

Except for a few plant species, attempts at isolation of chloroplasts from leaves have encountered considerable difficulty. Two types of problems are present. First, during tissue disruption by the conventional blending technique, many chloroplasts are destroyed by mechanical interaction with tougher surrounding tissue. Second, enzyme inhibitors in high concentrations are released during the grinding process including polyphenols, tannins, and terpenes (10). In some systems it has been possible to avoid loss of activity by the addition of antioxidants (3, 7) or adsorption and precipitation (11) agents in varying concentrations and combinations. No specific explanation is presently acceptable as to why a particular recipe works well in one system, but poorly or not at all in others.

[1] This paper is taken from the Ph.D. thesis of R. Hernández-Gil.

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MATERIALS AND METHODS

A clone of Eastern cottonwood (*Populus deltoides* Bartr.) was grown in a mixture of soil, vermiculite, and perlite in pots in a constant environment chamber. The plants were watered with one-tenth strength Hoagland's nutrient solution. The photoperiod was adjusted to 16 hr of light followed by 8 hr of darkness. Illumination was provided by six 40-w Cool-White fluorescent tubes (Ken-Rad) and four 40-w incandescent bulbs. This gave a light intensity of approximately 7000 lux (38 wm^{-2}). The temperature was maintained at 25 C, with a 5 C night depression, and relative humidity ranging between 60 and 70%.

To reduce the starch content of the chloroplasts before isolation, the plants were kept in darkness for 24 hr followed by a 1 hr light treatment at 30,000 lux (117 wm^{-2}). The dark treatment reduced chloroplast fragmentation substantially.

Preparation of Chloroplasts. Between 8 to 10 g of leaves were freed of midribs and leaf margins, placed in 100 ml of the infiltration and homogenizing medium consisting of 0.35 M sorbitol; 50 mM Tricine, pH 7.6; 2 mM EDTA; 20 mM sodium isoascorbate; and 1.5% (PEG)[3] 4000 (w/v) and infiltrated under vacuum for 30 min at 4 C. After infiltration the leaf pieces and the infiltration buffer were poured into a Monel container and disrupted in a Waring Blendor at full speed for 5 sec. The slurry was then filtered through four layers of nylon cloth, and the resulting liquid was centrifuged for 60 sec at 2445*g*. The supernatant fluid was discarded, and the pellet containing whole chloroplasts was resuspended with a glass rod in a small volume of homogenizing buffer. At this point the chloroplast suspension was stored in an ice box, and the chlorophyll concentration was measured according to Arnon's procedure (2).

The chloroplast suspensions were examined to determine their structural integrity under phase contrast with a Zeiss research model microscope, and with an RCA EMU-3 electron microscope. The chloroplast pellet was found to be free of intact cells. Most of the chloroplasts retained the outer limiting membrane (21).

CO_2 Fixation. This reaction was carried out in small glass centrifuge tubes (22) with rounded ends closed with a "Subaseal" rubber stopper. The reaction mixture contained 192.5 μmoles of sorbitol, 17.5 μmoles of HEPES-NaOH, pH 7.6; 1.7 μmoles pyrophosphate, 0.70 μmole of sodium isoascorbate, and 7.5 mM ^{14}C-bicarbonate (4 μc/μmole) in a final volume of 0.7 ml. Reaction mixtures containing chloroplast suspensions with approximately 30 to 70 μg of chlorophyll were preilluminated for 3 min prior to the injection of ^{14}C-bicarbonate. The tubes were illuminated by four 200-w flood lights

[3] Abbreviations: PEG: polyethylene glycol; PMS: phenazine methosulfate.

Table I. *Definition of Isolation Conditions for CO_2 Fixation by Isolated Cottonwood Chloroplasts*

The isolation medium contained 0.35 M sorbitol; 50 mM Tricine, pH 7.6, and 2 mM EDTA.

Treatment			Rate of CO_2 Fixation
Sodium isoascorbate concn	PEG-4000 concn	Infiltration	
mM	% (*w/v*)		*μmoles/mg chlorophyll·hr*
			0
	1.5	+	0.24
5	1.5	+	0.59
10	1.5	+	0.82
20	0.6	+	0.54
20	1.5	+	1.20
20	1.5		0.36

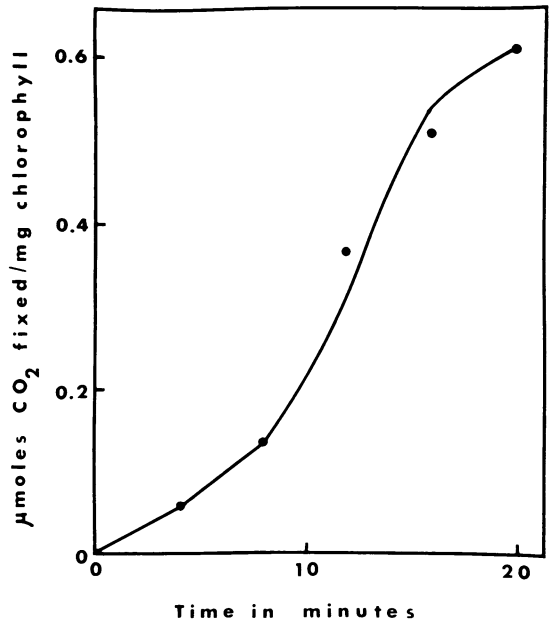

FIG. 1. Progress curve of $^{14}CO_2$ fixation by isolated cottonwood chloroplasts. Light intensity was 20,000 lux; chlorophyll was 43 μg/ml; temperature was 20 C.

Table II. *Cofactor Requirement for Cyclic Photophosphorylation*

The complete reaction mixture contained in a final volume of 1.7 ml, inorganic phosphate (labeled with ^{32}P), 5 μmoles; ADP, 12 μmoles; PMS, 0.1 μmole; $MgCl_2$, 6 μmoles; sodium isoascorbate, 25 μmoles; Tricine-NaOH buffer, pH 8.2, 210 μmoles. Reaction time was 10 min, and the light intensity was 50,000 lux. Chlorophyll was 17 μg/ml, and the temperature was 20 C.

Cofactor	ATP formed
	μmoles/mg chlorophyll·hr
Complete	196
Sodium isoascorbate	79
$MgCl_2$	21
PMS	20
ADP	0

giving approximately 20,000 lux at 20 C. Reactions were stopped by the injection of 2 ml of methanol. Samples of 100 μl were spotted on filter paper (Whatman No. 3 MM), acidified with 0.5 N HCl, dried, and counted in a Packard Tri-Carb scintillation spectrometer. The scintillation medium consisted of 5 g of PPO, 200 mg of POPOP, 6.5 ml of ethanolamine, 500 ml of toluene, plus methanol to 1000 ml. The products of CO_2 fixation by isolation chloroplasts were chromotographed two-dimensionally as described by Pedersen *et al.* (13).

Photophosphorylation. The standard reaction mixture contained 210 μmoles of Tricine, pH 8.2, 12 μmoles of ADP, 0.1 μmole of PMS, 6 μmoles of $MgCl_2$, 25 μmoles of sodium isoascorbate, 5 μmoles of Pi, an adequate amount of ^{32}P (1×10^5–1×10^6 cpm), chloroplasts containing less than 20 μg of chlorophyll, and water to a total volume of 1.7 ml. The reaction mixture was added to small round bottom test tubes and placed in a water bath maintained at 20 C. The reaction was started with light and terminated by addition of 0.3 ml of 20% trichloroacetic acid. Phosphate esterification was measured by the method described by Nielsen and Lehninger (12), as modified by Avron (3).

RESULTS AND DISCUSSION

During extensive tests to increase the photosynthetic performance of cottonwood chloroplasts (Table I), it was found that infiltration with a solution of PEG-4000 and 20 mM sodium isoascorbate gave the highest CO_2 fixation rates. Infiltration (4) of cottonwood leaves with the isolation medium before chloroplast extraction was found to increase the rate of cell-free photosynthesis by 100 to 200%. Infiltration may allow binding of inhibitors before their release during the isolation procedure. Without infiltration, and in the absence of phenol binding agents (4, 11) and antioxidants (11), no activity could be detected (Table I).

The inhibitory substances present in cottonwood leaves have not yet been identified. It is presumed that the beneficial effect of PEG-4000 occurs through the binding of phenols and the stabilization of chloroplast structure (4). The antioxidant sodium isoascorbate has been used widely for obtaining maximal rates of photophosphorylation (1, 3). It can also participate in electron transport. Addition of EDTA to the isolation medium was found to increase the CO_2 fixation rates by 20%.

Attemps were made to replace PEG-4000 and isoascorbate by other phenol binding agents and antioxidants. Polyvinylpyrrolidone-360 and PEG-20,000 had a slight protective effect, but the following treatments were unsuccessful: bovine serum albumin, gelatin, β-mercaptoethanol, glutathione, nitrogen atmosphere.

The rate of CO_2 assimilation by isolated cottonwood chloroplasts varied from day to day with the age of the parent leaf material and with dark-light pretreatments. The highest rate obtained was 3.4 μmoles CO_2 fixed/mg chlorophyll·hr, corresponding to about 10% of the rate in leaf discs of the same species. However, the rates that were consistently obtained were approximately 1.5 μmoles CO_2 fixed/mg chlorophyll·hr.

Figure 1 shows the progress curve for CO_2 fixation by isolated chloroplasts. Dark fixation after 20 min was only 1% of the observed rates in light. An initial lag phase of about 5 min was observed, as has been reported by previous workers for spinach and pea chloroplasts (6, 16). This lag phase could be eliminated in spinach and peas by the addition of certain sugar phosphates (6). The effect of the addition of 7 mM sugar phosphates (glucose-1-P, ribose-5-P, and fructose-1,6-diP) on CO_2 assimilations was tested. Fructose-1,6 diphosphate was found to be the only compound tested to increase CO_2 fixation. The products of the CO_2 assimilation in this experiment consist of four major compounds, the identities of which are presently under investigation.

Photophosphorylation. Maximum rates of ATP synthesis were obtained when the leaf material was infiltrated in the isolation medium containing 1.5% PEG-4000 (w/v) and 10 mM sodium isoascorbate. The optimum pH for cyclic photophosphorylation of cottonwood chloroplasts using Tricine buffer was observed to be at pH 8.2.

The cofactor requirements for cyclic ATP synthesis is shown in Table II. If PMS or Mg^{2+} were absent from the reaction mixture, 11% of the original activity remained. Lack of sodium isoascorbate diminished the activity by 60%. ADP addition was essential for detection of cyclic photophosphorylation.

The rate of ATP synthesis was found to be constant up to 5 min and declined rapidly after 10 min. The rate of cyclic ATP synthesis obtained here was about 300 μmoles ATP/mg chlorophyll·hr. ATP synthesis was absolutely dependent on light.

Rates of noncyclic ATP synthesis measured with NADP as on electron acceptor fluctuated around 10 μmoles/mg chlorophyll·hr. Addition of DCMU is known to poison photosystem II, preventing the noncyclic electron transport of photosynthesis. DCMU in a concentration of 10 μM inhibited completely noncyclic photophosphorylation and cyclic ATP synthesis by 10%.

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