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Changes of Photorespiratory Activity with Leaf Age[1]

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ABSTRACT

We have discovered that younger leaves do not exhibit the same large increase in photosynthesis as do older ones when the oxygen tension is lowered. This phenomenon was observed in various tobacco and citrus species and indicated less photorespiration in young leaves. Activities of glycolate oxidase and of glyoxylate reductase were found to be significantly lower in younger leaves than in more mature ones from the same plant. Assays of glycolate content revealed the same trend. Levels of phosphoenolpyruvate carboxylase were only slightly higher in the younger leaves. This finding lessens the possibility of differing enzymatic pathways for CO_2 fixation. It is concluded that the small photorespiratory activity of young leaves can be attributed to a lower activity of the photorespiratory enzymes and perhaps also to a slightly higher rate of internal recycling of CO_2.

In the light, leaves of many higher plants evolve carbon dioxide and take up oxygen. This apparent reversal of the photosynthetic process is called photorespiration (3, 4, 13). It is easily detectable in, and perhaps restricted to, higher plants which fix carbon dioxide via the enzymes of the Calvin-Benson cycle (13). Recently, Jackson and Volk (13) have published an excellent review concerned with the history of the discovery of photorespiration and the present hypotheses pertaining to its mechanism. In all of these theories, the substrates for photorespiration are considered to be photosynthetically formed glycolate and metabolically related compounds. Photorespiratory enzymes, such as glycolate oxidase and glyoxylate reductase, are located in the leaf peroxisomes (20). However, the observed activity of these enzymes in leaf extracts was not always found to correlate with the measured rates of photorespiration (16).

Zelitch and Day (24) have provided evidence that certain varieties of *Nicotiana tabacum* differ not only in their photosynthetic capacities, but also in respect to their rates of photorespiration. Several workers (2, 6, 15) have shown that different species within one genus vary in their CO_2 compensation points, the magnitude of which is an often used criterion for the relative rate of photorespiratory reactions. Furthermore, El Sharkawy *et al.* (8) demonstrated that age and growth conditions affect photorespiration in *Amaranthus edulis.* Bull (1) has shown similar phenomena in sugarcane.

In this paper we will present data which suggest that the photorespiratory gas exchange of leaves changes during their development. While young leaves were observed to display a high rate of mitochondrial dark respiration, their photorespiration was minimal. Older leaves, in contrast, had much slower rates of dark respiration, but high rates of photorespiration. Our estimation of photorespiratory rates was based on indirect measurements which have been used by other authors as well (1, 5, 13). Since photorespiration leads to a loss of photosynthetically fixed CO_2, and since photorespiration is negligible at low oxygen tensions, the relative difference in the rates of photosynthetic CO_2 fixation in a gas phase with 1% oxygen and with 21% oxygen could be used as a convenient measure of the degree of photorespiratory interference with the process of photosynthetic CO_2 fixation. Determinations of the activity of glycolate oxidase and glyoxylate reductase in leaf extracts as well as assays for the substrate, glycolate, supported the conclusions drawn from our photosynthesis measurements.

MATERIALS AND METHODS

All plants were grown in soil in a greenhouse. The tobacco (*Nicotiana tabacum* L.) varieties used in this study were: John Williams Broadleaf, its aurea mutant Su/su, and a variegated mutant (N.C. 95 Varieg.) whose green leaf sections will be called N.C. green. Corn, *Zea mays* L., was of unknown variety. Different type citrus plants (*C. mitis* L., *C. sinensis* L. var. Navel, *C. limon* L. var. Meyer and Rough) had been purchased from local nurseries.

The age of the leaf was determined arbitrarily and somewhat intuitively. Young tobacco leaves, weighing less than a gram of fresh weight without midribs, were maximally one-fourth the size of more mature leaves. They were generally not yet fully unfolded, had fewer photosynthetic pigments, and a slightly higher dry weight per leaf area than older leaves (Table I). Young citrus leaves had a tender texture with a poorly developed cuticular layer. They contained less chlorophyll and had a lower dry weight per leaf area than older citrus leaves (Table I). Absolute leaf ages are difficult to give because leaves were usually taken from mature plants.

Photosynthetic activity at 23 C was determined by measuring the amount of $^{14}CO_2$ incorporated into leaf slices placed in a sealed, water cooled chamber, which was gassed with a designated CO_2-O_2-N_2 mixture. The procedure was essentially that described by Homann (12). A 750 w tungsten bulb providing approximately 5×10^4 ergs cm^{-2} sec^{-1} white light was used for illumination. This intensity saturated photosynthesis in all leaves with the exception of the aurea mutant Su/su and corn.

Measurements of the dark respiration were done manometrically. Leaf slices were placed over water on small wire coils as described by Schmid (17). The side arm contained 0.2 ml of 20% KOH.

Glycolate oxidase (E.C. 1.1.3.1) activity was determined

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Table I. *Leaf Characteristics of Several Plant Types*

Each value represents the average of two determinations. No significant differences were found.

Plant	Chlorophyll per Leaf Area	Wet Wt. per Leaf Area	Dry Wt. per Leaf Area
	μg/cm²	*mg/cm²*	
N.C. green tobacco (old)	29.8	20.6	2.6
N.C. green tobacco (young)	25.2	21.4	3.3
Su/su tobacco (old)	17.5	22.6	3.6
Su/su tobacco (young)	7.4	25.5	4.3
Rough lemon (old)	51.3	21.7	8.0
Rough lemon (young)	24.3	19.9	4.6
Calamondin (old)	51.5	20.4	7.5
Calamondin (young)	25.9	19.9	5.6

by measuring O_2 uptake with a Clark type oxygen electrode (Yellow Springs Instruments) sealed into a thermoregulated plastic chamber. For enzyme analysis, the crude sonicated extract of leaves was used. Glyoxylate reductase (E.C. 1.1.1.26) in our crude enzyme preparation was assayed using the method of Tolbert *et al.* (20). Since addition of PVP[2] and 2-mercaptoethanol to the grinding medium did not result in any improvements of rates, these protective agents were not used routinely in our assays.

The glycolate content of the various leaves was determined according to Zelitch (23). In order to facilitate entry of HPMS through the waxy surface of citrus leaves, we added 0.03% (v/v) Tween 20 to the solution of the inhibitor. This combination of HPMS and detergent did not significantly inhibit photosynthesis.

Phosphoenolpyruvate carboxylase (E.C. 4.1.1.31) assays were done as described by Slack and Hatch (19), and chlorophyll was estimated according to MacKinney (14).

RESULTS

Photosynthetic CO_2 Fixation at Low and High Oxygen Tensions. The relative differences between photosynthetic CO_2 fixation rates at low and high oxygen tensions are a convenient measure of photorespiration (5). Photorespiration is nearly absent at low oxygen concentrations and increases as the oxygen tension in the gas phase is raised. Furthermore, high CO_2 concentrations have been found to suppress photorespiration (4, 13). However, at the low CO_2 tensions of air, photosynthesis was so slow that the experimental error in the determination of $^{14}CO_2$ fixation with our apparatus became relatively large. We also encountered the problem of significant CO_2 depletion in the sealed chamber. Therefore, as a compromise, we chose to work at 0.15% CO_2. It was, of course, important to ascertain that at this CO_2 concentration photorespiration was still measurable. Table II reveals that significant differences between the photosynthetic CO_2 fixation at high and at low oxygen tensions could be measured in 0.03% as well as 0.15% CO_2.

Table III gives the relative change of photosynthetic CO_2 fixation for a series of representative experiments run at 0.15% CO_2 and a high and a low oxygen concentration. Photosynthesis was affected more in older leaves than in younger ones by a change in the oxygen tension. Corn leaves, which were tested as representatives of tissues with the Hatch and Slack pathway of CO_2 fixation, were found to have nearly identical rates of photosynthesis at low and high oxygen tensions. This result was expected since such C-4 plants are known to lack photorespiratory interference with photosynthetic CO_2 fixation (2, 10, 13). It did come as a surprise, however, that younger leaves from C-3 plants behaved like corn leaves, *i.e.*, appeared to have no photorespiration.

The differences in the stimulation of photosynthesis in old and young leaves at lowered O_2 concentrations might have been confounded by differences in epidermal and mesophyll resistances, as well as by the number of stomata and their rate and degree of opening. Therefore, assays of enzymes involved in photorespiration were undertaken using extracts from various old and young leaves. Furthermore, the glycolate content of the leaves was compared.

Glycolate Content and Enzymes of Glycolate Metabolism. Glycolate oxidase and glyoxylate reductase (22, 23) are two peroxisomal enzymes which have been postulated to participate in the photorespiratory metabolism of photosynthetically formed glycolate. Recent studies by Tolbert *et al.* (21) have indicated, however, that the main function of glyoxylate

Table II. *Variation of Photosynthetic $^{14}CO_2$ Fixation at Different CO_2 and O_2 Concentrations in the Gas Phase*

Variations are given as standard deviations from the mean.

CO_2 Concn	Plant	Averaged No. of Determinations	Rate	
			21% O_2	1% O_2
%			*μmoles $^{14}CO_2$ fixed/mg chl·hr*	
0.03	N.C. green tobacco (old)	8	31 ± 4	53 ± 5
	N.C. green tobacco (young)	3	66 ± 6	69 ± 6
	Navel orange (old)	5	20 ± 5	32 ± 9
	Navel orange (young)	3	52 ± 4	52 ± 7
	Corn	6	73 ± 6	75 ± 8
0.15	N.C. green tobacco (old)	4	148 ± 8	210 ± 13
	N.C. green tobacco (young)	8	140 ± 20	150 ± 13
	Navel orange (old)	3	66 ± 4	90 ± 16
	Navel orange (young)	3	58 ± 5	60 ± 8
	Corn	9	150 ± 30	140 ± 24

Table III. *Changes of Photosynthetic CO_2 Fixation Due to Photorespiration*

Data are from at least five determinations with variations given as the standard deviation from the mean. Relative rates of photorespiration were calculated as the ratio of the average rates of photosynthesis at 1% O_2 over that at 21% O_2. The CO_2 concentration was 0.15%.

Plant	Rate of Photosynthesis		Relative Rate of Photo-respiration
	21% O_2	1% O_2	
	μmoles $^{14}CO_2$ fixed/mg chl·hr		
J.W.B. tobacco (old)	88 ± 9	123 ± 13	1.4
J.W.B. tobacco (young)	150 ± 17	157 ± 21	1.0
N.C. green tobacco (old)	148 ± 8	210 ± 13	1.4
N.C. green tobacco (young)	140 ± 20	150 ± 13	1.0
Su/su tobacco (old)	250 ± 10	400 ± 40	1.6
Su/su tobacco (young)	185 ± 29	188 ± 34	1.0
Navel orange (old)	28 ± 7	44 ± 8	1.6
Navel orange (young)	58 ± 5	60 ± 8	1.0
Rough lemon (old)	60 ± 11	96 ± 17	1.6
Rough lemon (young)	58 ± 7	64 ± 7	1.1
Corn	150 ± 30	153 ± 24	1.0

[2] Abbreviations: HPMS: hydroxypyridinemethanesulfonic acid; PEP: phosphoenolpyruvate; PVP: polyvinylpyrrolidone.

reductase may not be the reduction of glyoxylate to glycolate, but rather the conversion of hydroxypyruvate to glycerate.

The data presented in Tables IV and V clearly show that the activity of glycolate oxidase and of glyoxylate reductase was higher in old leaves of C-3 plants than in their young counterparts. This trend was evident when rates were based on a chlorophyll or on a protein basis. Similar findings of a reduced glycolate oxidase activity in young leaves have been reported in the literature (7, 16). Our data in Tables IV and V corroborate the conclusions drawn from the photosynthesis measurements, namely, that photorespiratory processes in young leaves are slow.

In the course of our measurements with various types of chlorophyllous tissue, we made the interesting observation that a relatively high glycolate oxidase activity was present also in the peel of a calamondin (*Citrus mitis* L.) fruit. In fact, the poor photosynthetic rates of such a tissue could be increased by as much as 75% when the oxygen tension in the gas phase was lowered from 21% to 1%. It follows that even a fruit peel photorespires.

From the data presented above we may conclude that photorespiration in young leaves might be limited by the low activity of peroxisomal enzymes. The question may be posed whether this limitation extends to the mechanism involved in the formation of the substrate of photorespiration, *i.e.*, glycolate.

The steady state concentration of glycolate in a leaf depends on the rates of its production and oxidation. Our data on glycolate oxidase had revealed a higher activity in old leaves. Consequently, the steady state level of glycolate in old leaves might be expected to be quite low even if the rate of glycolate production is high. Addition of an inhibitor of glycolate oxidase (HPMS) would prevent utilization of glycolate and result in its accumulation. On the other hand, we might suspect that the low activity of glycolate oxidase in young leaves leads to a great abundance of glycolate in this tissue under steady state conditions unless glycolate formation is very slow. An addition of HPMS should not significantly affect this level, however, whether it is low or high. The data in Table VI represent measurements of the glycolate concen-

Table IV. *Glycolate Oxidase Activity*

Glycolate oxidase was assayed in a Clark type oxygen electrode after sonicating leaf extracts prepared in 0.1 M potassium phosphate buffer, pH 7.8. The assay medium contained 50 mM potassium phosphate buffer, pH 7.8; 400 units of catalase (1 unit decomposes 1 μmole H_2O_2 per min at pH 7.0); 4% ethanol; and 50 mM glycolate.

Plant	No. of Determinations	Activity: Uptake of O_2 (μmoles per hr)					
		Old	Young	Old/young	Old	Young	Old/young
		per mg chlorophyll			*per mg protein*		
N.C. green tobacco	5	33	13	2.5	17	3	5.7
Su/su tobacco	2	108	70	1.5	19	10	1.9
Rough lemon	2	33	6	5.5	7	1	7.0
Calamondin, fruit peel	1	47					

Table V. *Glyoxylate Reductase Activity*

Leaf extracts prepared in 0.4 M sucrose and 20 mM glycylglycine buffer, pH 7.5, were assayed in a medium containing 24 mM glycylglycine, pH 5.8; 0.01% (v/v) Triton X-100; 0.3 mM NADH; and 100 mM glyoxylate. Data are from one representative experiment.

Plant	Activity: μmoles NADH oxidized per hr					
	Old	Young	Old/young	Old	Young	Old/young
	per mg chlorophyll			*per mg protein*		
N.C. green tobacco	310	200	1.6	140	68	2.1
Su/su tobacco	600	505	1.2	90	41	2.2
Rough lemon	455	165	2.8	92	20	4.6
Calamondin, leaves	505	420	1.2	83	30	2.8

Table VI. *Glycolate Content of Leaf Extracts*

Glycolate was determined in an extract from leaf sections which had been floated in the light for 20 min in water or on a solution of 10 mM HPMS.

Plant	Concn after Incubation: Water	HPMS
	μmoles per mg chl	
N.C. green tobacco (old)	0.3	7.0
N.C. green tobacco (young)	0.5	0.6
Rough lemon (old)	0.5	5.3
Rough lemon (young)	0.5	0.4
Corn (old)		0.9

Table VII. *PEP Carboxylase Activity*

PEP carboxylase was assayed after sonicating leaf extracts prepared in 0.1 M tris, pH 7.8, containing 10 mM 2-mercaptoethanol. The assay medium contained tris buffer, pH 8.2; 2.5 mM $NaHCO_3$; 3 mM $MgCl_2$; 3 mM dithiothreitol; 0.3 mM NADH; 40 units of malic dehydrogenase (Calbiochem); and 2.5 mM phosphoenolpyruvate. Values reported are from one representative experiment.

Plant	Activity: Old	Young	Old/young
	μmoles NADH oxidized/mg chl·hr		
J.W.B. tobacco	27	67	0.4
Su/su tobacco	35	112	0.3
Rough lemon	38	58	0.6
Corn	1,024		

Table VIII. *Dark Respiration*

Respiration was measured manometrically under air in the dark. The data represent the averages of two determinations which differed only slightly from one another.

Plant	Respiration Rate (μmoles per hr): *per mg chlorophyll*	*per cm² leaf area*
J.W.B. tobacco (young)	14	0.5
J.W.B. tobacco (old)	9	0.3
Su/su tobacco (young)	55	0.5
Su/su tobacco (old)	14	0.2
Navel orange (young)	27	0.8
Navel orange (old)	8	0.4
Meyer lemon (young)	29	0.8
Meyer lemon (old)	6	0.4

tration in young and old leaves during normal photosynthesis in the absence or presence of glycolate oxidase action. It can be seen that the glycolate content in young leaves is low under both conditions, whereas in old leaves large amounts of glycolate are accumulated, provided that glycolate oxidase had been inhibited. These findings support our earlier measurement on the age-dependent variation of glycolate oxidase activity in leaves. In addition, they allow the conclusion that the slow photorespiratory rates in young leaves are reflected not only by low activities of peroxisomal enzymes, but also by a sluggish formation of glycolate. It should be noted that leaves which one would classify as middle aged exhibited intermediate rates of glycolate production (not shown).

Phosphoenolpyruvate Carboxylase and Mitochondrial Respiration. From the foregoing it appeared that, in respect to their photorespiratory characteristics, young leaves behaved like nonphotorespiring C-4 plants which form oxaloacetate as the first detectable carboxylation product in photosynthesis. For this reason we analyzed the leaves for the activity of phosphoenolpyruvate carboxylase.

Table VII shows that the activity of this enzyme was indeed two to three times higher in extracts from young leaves than in the corresponding extract from old leaves. However, a comparison with the data obtained with a typical C-4 plant like corn reveals that the enzyme activity in young leaves from C-3 plants was not high enough to warrant the inference of the C-4 pathway as the major mechanism for CO_2 fixation in such tissue. Nevertheless, the higher levels of the PEP carboxylase may make young leaves more efficient than older leaves in the utilization of carbon dioxide. This may be of physiological significance because losses of CO_2 produced by mitochondrial respiration would be kept at a minimum. In fact, our young leaves respired more rapidly than the old leaves (Table VIII).

DISCUSSION

Our data reveal that photorespiratory activity in leaves of C-3 plants increases from a very low level in young leaves to a very high one in old, fully expanded leaves. Such high activity of photorespiration is of the magnitude generally reported in the literature and considered to be normal for leaves which fix CO_2 via the Calvin-Benson cycle (C-3 pathway). Not only was the activity of peroxisomal enzymes low in young leaves, but also their capability to produce glycolate was poorly developed.

Why will CO_2 fixation via the Calvin-Benson cycle, under certain conditions, give rise to the formation of glycolate in older leaves, but not in younger ones? The most obvious characteristic of young leaves is their need for production and conversion of metabolites needed in growth. This demand is reflected in their high rates of mitochondrial respiration (Table VIII). While the metabolism of older leaves may be directed more to the accumulation of storage products for subsequent hydrolysis and translocation, young leaves may be expected to drain off continuously various intermediates of the Calvin-Benson cycle before they are transformed into starch. Thus, the reaction sequences involved in the metabolism of photosynthetic CO_2 fixation products may indeed be quite different at various stages of leaf development.

Obviously, more experiments are needed to understand the interaction between environmental factors and physiological characteristics of a given chlorophyllous cell system. From a purely rational point of view it makes sense, of course, that young tissue with high metabolic demands ought to keep losses of photosynthetically fixed CO_2 via peroxisomal photorespiration at a minimum. Similar losses might be better tolerated by older cells. The observed differences between young and old leaves may also explain why, under high light intensities, the aurea tobacco mutant Su/su of Schmid and Gaffron (18) grows as fast as its green ancestor during the first weeks after germination, but shows a markedly slower growth later (17, 24), due to the appearance of an exceptionally strong photorespiratory activity.

At this point, our data do not directly provide a new insight into the physiological significance of photorespiration in C-3 plants. The questions which our results pose, and the attractive possibility of working with photorespiring and nonphotorespiring tissue from a single plant, may nevertheless be very helpful in future investigations. At the very least, our data make it imperative for future publications in photorespiration to state the approximate age of the plant material.

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