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PARTIAL IDENTIFICATION OF DARK $^{14}CO_2$ FIXATION PRODUCTS IN LEAVES OF *CATTLEYA* (ORCHIDACEAE)

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Summary

Following exposure of *Cattleya* leaves to $^{14}CO_2$ in the dark, labelled malate, citrate and an unidentified compound were detected by thin layer chromatography. This is interpreted to suggest the existence in *Cattleya* leaves of a dark CO_2 fixation pathway which may be similar to that found in other plants which can fix large amounts of carbon in the dark.

Introduction

The phenomenon of dark CO_2 fixation with an increase in organic acid levels has been known for over 150 years (Heyne, 1813). A fixation pathway which involves two carboxylations to produce glycerate-3-phosphate from ribulose diphosphate, and oxaloacetate from phosphoenolpyruvate followed by conversion of the oxaloacetate to malate has been suggested (Ranson and Thomas, 1960). Oxaloacetate has also been suggested as being the intermediate leading to malate in beech mycorrhizas (Harley, 1964).

In higher plants, dark fixation of CO_2 although common to all plant organs is more noticeable in plants with thick or succulent leaves (Bradbeer, Ranson and Stiller, 1958; Ranson and Thomas, 1960; Saltman *et al.*, 1956; Warburg, 1886). Certain roots (Ting and Dugger, 1965a, b) and mycorrhizas (Harley, 1964) also fix CO_2. Increased CO_2 uptake and acidity in the dark have been observed in a number of thick-leaved orchids (Warburg, 1886) including *Cattleya* (Nuerenbergk, 1963), *Epidendrum* (Coutinho, 1965) and *Phalaenopsis* (Borriss, 1967). Citric, isocitric and malic acids have been reported in *Epidendrum* and *Cattleya* leaves (Borriss, 1967). Despite this and reports of increased malic acid concentration in the dark (Borriss, 1967), no information is available on the labelling pattern of products following dark $^{14}CO_2$ fixation by orchids and it is not known whether it is similar to that found in other plants. The purpose of this investigation was, therefore, to determine whether the major products of dark $^{14}CO_2$ fixation by orchids are similar to those in other plants and to obtain more information on the physiology of ecological adaptations in the Orchidaceae.

Materials and methods

Leaves, varying in weight between 10 and 12 g, from *Cattleya* White Blossom 'Stardust' × cv. Bob Betts 'Glacier' were used in the experiment. The plants were grown under

12 hour photoperiods at a constant temperature of 23° C before removal of the leaves.

The experimental apparatus (modified after Saltman *et al.*, 1956) consisted of 500 ml Waring blender jars covered with a large, aluminium-reinforced rubber stopper. The stopper was fitted with three tubes, two with stopcocks and the other covered with an ampule cap. One of the jars was painted with black opaque paint.

The $^{14}CO_2$ was released from $Ba^{14}CO_3$ contained in a small beaker suspended from the stopper below the capped tube, with 2N H_2SO_4 injected through the ampule cap. All treatments were given 200 μCi of $^{14}CO_2$, the total amount of carbon being such that the leaves received 0.06 mM carbon hour^{-1} g fresh weight^{-1} (Nuerenbergk, 1963). Experiments were carried out in the afternoon at 18° C and 29° C in both light and dark vessels. Temperatures were maintained by placing the containers in the water bath of a Gilson respirometer equipped with lights, producing a light intensity of 600 ft-candles 10 cm above a 14.5 cm deep water bath. Duplicate experiments were performed. The vessels were equilibrated for 20 minutes before $^{14}CO_2$ release. After 90 minutes the air was evacuated from the chamber through two $Ba(OH)_2$, traps, approximately 100 ml of boiling 80% ethanol were introduced and the leaves homogenized for 90 seconds. The relatively long exposure time was selected due to the slow metabolism of orchids (Arditti, 1967) and delays in CO_2 uptake following the onset of darkness (Nuerenbergk, 1963).

After homogenization, the ethanol extracts were filtered and washed with *n*-pentane until the extracting phase was clear. The *n*-pentane extracts were assayed and found to contain no radioactivity. The resultant, partially clarified, ethanolic extracts were concentrated in a flash evaporator, and dried in a lyophilizer. Each residue was then redissolved in 80% ethanol and brought to a volume of 10 or 20 ml depending on the amount of radioactivity present. Radioactivity was determined in a gas flow counter.

Samples of 25–100 μl were chromatographed (Saltman *et al.*, 1956) in two dimensions on Whatman No. 1 paper (18 in × 22 in). The solvents used were 80% phenol : 20% water (w/w) and *n*-butanol : acetic acid : water (79 : 19 : 50, v/v/v). Authentic samples of amino acids and organic acids were chromatographed in the same manner. Radioactive compounds were located on X-ray film. Amino acids were identified with ninhydrin spray following autoradiography or on separate chromatograms. Organic acids were visualized with 0.3% bromphenol blue and 0.1% methyl red in 95% ethanol.

Additional identification of organic acids was performed by cochromatography with authentic compounds on thin layer plates (modified after Ting and Duger, 1965a). Samples of 10 μl were spotted on silica gel G plates, activated for 30 minutes at 105° C. The plates were developed in water saturated ether : formic acid (7 : 1, v/v). After drying for several hours to evaporate all of the formic acid, the organic acids were visualized with bromphenol blue–methyl red indicator. The acids derived from the leaf extracts were removed from each plate and eluted in 1 ml of 95% ethanol. Their radioactivity was determined in a liquid scintillation counter.

RESULTS AND DISCUSSION

The amounts of CO_2 fixed in the dark by *Cattleya* under our conditions appears to be relatively low, for even the amount fixed at 18° C was only 9.3% of the carbon fixed photosynthetically (Table 1). This is consistent with the lower rates of CO_2 fixation in the dark than in the light reported for *Kalanchoë* (Ranson and Thomas, 1960). However, it should be noted that a high dark CO_2 uptake and a pronounced diurnal acidity rhythm

were reported for *Cattleya* orchids which have fleshy leaves (Nuerenbergk, 1963; Borriss, 1967), whereas no dark fixation of CO_2 into four carbon dicarboxylic acids could be observed in the thin leaved orchid *Cymbidium* (Hatch, Slack and Johnson, 1967).

Paper chromatography and autoradiography indicated labelling of malate and aspartate in the dark; and sucrose, malate, aspartate, serine and glycine in the light.

The labelling pattern in the dark suggests pathways similar to those found in other plants including barley roots and mycorrhizas (Jacobson, 1955; Harley, 1964). The pattern in the light could be due to either photosynthesis by the Calvin pathway superimposed upon this, i.e. a Crassulacean type of metabolism, or by the newly described ^{14}C decarboxylic acid photosynthetic pathway (Hatch and Slack, 1966; Hatch *et al.*, 1967). In the dark, oxalacetate is a common precursor for malate and aspartate, and may be formed by the carboxylation of phosphoenolpyruvate. Phosphoenolpyruvate carboxylase, malic dehydrogenase and malic enzyme which are involved in this pathway are present in the cells of the leaves of *Cattleya* (Knauft, unpublished data).

Three organic acids were labelled during dark fixation. Cochromatography with authentic acids indicates that these are malate, citrate and one unidentified (Table 1).

Table 1. *Light and dark carbon fixation by fleshy orchid leaves during* 90 *minute exposure to* $^{14}CO_2$

	Dark		Light	
	10° C	29° C	18° C	29° C
Total fixation (μM C/g fresh weight)	0.18	0.18	1.93	8.55
Net photosynthesis (μM C/g fresh weight)	–	–	1.75	8.37
Dark as percentage of light	9.3%	2.1%		
Organic acids separated on TLC				
Malate	41%*	61%*		
Citrate	46%*	9%*		
Unidentified	13%	30%		

* Per cent of major organic acids produced during dark $^{14}CO_2$ fixation.

The relative amounts of radioactivity in these acids changed when the temperature was raised from 18° C to 29° C. At 18° C, the major components were malate and citrate, whereas at 29° C malate was the major product. The relative amount of the labelled unidentified acid increases from 13% at 18° C to 30% at 29° C (Table 1). The unknown acid exhibited the same R_f value in our experiments as did pyruvate. However, pyruvate is a volatile acid under reduced pressure which suggests that even if present in the extracts, it probably would have been lost. No appreciable quantities of pyruvate could be recovered in similar procedures (personal communication, I. P. Ting, University of California, Riverside). The unidentified acid could be 2,5-pyrrolidone carboxylic acid (PCA) which has been found in barley roots (Jacobson, 1955) and beech mycorrhizas (Harley, 1964) following dark CO_2 fixation. Labelled PCA apparently arises from glutamic acid derived from 2-ketoglutarate which is in turn produced from ^{14}C-malate via the Krebs cycle (Jacobson, 1955) or during boiling from glutamine (Harley, 1964). However, on chromatograms, pyruvate, the unidentified fraction and isocitric lactone have similar R_f values above malate and below lactate. PCA, on the other hand, appears above citrate and below malate. Separation between all spots is good (personal communication, I. P. Ting). Thus, our unidentified spot is most probably not PCA or pyruvate and may well be isocitric lactone. Its exact identification will be the subject of further work.

It is interesting to note that dark fixation of CO_2 has been generally observed in thick-leaved plants and succulents mostly found under xerophytic conditions. Thick-leaved orchids, on the other hand, are, as a rule, natives of wet, tropical areas and therefore, apparently not xerophytes. Yet, this is not really the case (Nuerenbergk, 1963). Orchids growing as epiphytes or lithophytes in rain forests have exposed roots which are subject to desiccation and dry periods. These plants, although exposed to intense rainfalls at times, are actually growing under modified xerophytic conditions. This is a fact which can be well appreciated following actual orchid collecting expeditions in the jungles of Central and South America. Therefore, open stomata (and uptake of CO_2) during the cooler and moister night periods would constitute an ecological advantage in that it would reduce transpirational water loss. Hence, this particular adaptive character of orchids is the same as that of xerophytes and due to similar ecological pressures.

Another possible benefit of dark CO_2 fixation for an epiphyte growing among actively photosynthesizing leaves of a tree is the advantage of obtaining CO_2 when it is most abundant at night. In the dense vegetation of the rain forest with its relatively enclosed atmosphere, CO_2 concentration could be greatly lowered during the day due to photosynthesis by the plant on which the orchids are growing. Thus, the ability to fix CO_2 in the dark constitutes an adaptation enhancing survival. Recent findings and horticultural practices which show enhanced growth of orchids in a CO_2 enriched atmosphere (Fordyce and Adams, 1967; for a review see Arditti, 1967) and which imply that they can utilize such higher concentrations, also tend to support this view.

In corn roots, the fixation of CO_2 into malate has been suggested as possibly providing a means of transferring reducing power from NAD to NADP (Ting and Dugger, 1965a, b). As malate is formed from oxaloacetate, NADH is oxidized to NAD, and as pyruvate is formed from malate NADP is reduced to NADPH. Since most of the anabolic pathways in organisms require NADPH at a time when it is not being produced photosynthetically this constitutes an important physiological adaptation evolved by these orchids.

One other possible physiological advantage of dark CO_2 fixation may be the replenishment of Krebs cycle intermediates. Because the Krebs cycle is a pool for intermediary metabolism, it is constantly being drained to meet the needs of biosynthesis. Dark fixation of CO_2 replenishes the supply of four-carbon acids at night when need for them may be high since orchids seem to accomplish much of their growth at that time.

A question also arises whether the dark fixation of CO_2 by fleshy leaved orchid represents special adaptation rather than a process common to all plant organs. The answer requires that all aspects of dark fixation by the Orchidaceae be considered. The phenomenon exists, or is at least pronounced only in fleshy leaved orchids (Bendrat, 1929; Nuerenbergk, 1963, Warburg, 1886). It exhibits diurnal acidity rhythm (Borriss, 1967; Coutinho, 1965; Nuerenbergk, 1963). A lag period following onset of darkness is also evident (Nuerenbergk, 1963). The commonly occurring dark fixation of CO_2 would not be expected to exist only in fleshy leaved orchids, be rhythmic or exhibit a lag period. Indeed there is no lag period in *Cymbidium* leaves which are not fleshy (Dueker and Arditti, 1968).

Further, the qualitative increase of acids in *Cattleya* leaves is of a relatively high order of magnitude, reminiscent of fixation by succulent plants. Finally, the relative amount fixed in the dark is much higher than the 0.4% value observed in sugar cane leaves (Hatch and Slack, 1966). Thus, it is possible to conclude from our results and previous findings with orchids and other plants that dark CO_2 fixation by fleshy *Cattleya* leaves

represents a special physiological adaptation rather than the type of dark CO_2 fixation found in all plants. Its relationship to the type of metabolism exhibited by the Crassulaceae requires further study.

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