

Photosynthetic Carbon Assimilation in a Shootless Orchid, *Chiloschista usneoides* (DON) LDL

A VARIANT ON CRASSULACEAN ACID METABOLISM

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ABSTRACT

Photosynthetic carbon assimilation in the roots of a shootless orchid *Chiloschista usneoides* (DON) LDL involves the synthesis and accumulation of malic acid from CO₂ in darkness. Malic acid is consumed in the light.

The roots do not possess stomata or any means of diurnally regulating the diffusive conductance of the pathway between the internal gas phase of the plant and the atmosphere. Regulation of internal CO₂ concentration near to atmospheric levels avoids a large net loss of CO₂ to the atmosphere during malic acid consumption in the light.

The water-absorbing function of the velamen conflicts with the photosynthetic function of the roots. Plants with water-saturated velamina do not acquire CO₂ from the atmosphere at night.

Photosynthesis in many epiphytic orchids involves the nocturnal acquisition of CO₂ and accumulation of malic acid followed the next day by the release of CO₂ from malic acid and its utilization in the reductive pentose phosphate pathway. Stomatal resistance is high at night and low during the day. Clearly these plants exhibit CAM (1).

Another commonly observed feature of such orchids is the possession of Chl-containing aerial roots and it has recently been shown that these roots, like leaves, exhibit the diurnal fluctuation in acid content and CO₂ exchange pattern characteristic of CAM (6). The aim of the present work is to extend information on the photosynthetic metabolism of orchid roots.

The experimental material used in this study is *Chiloschista usneoides*, a member of the group known as shootless orchids which has been eloquently described by Benzing *et al.* (2). In the mature vegetative state, *C. usneoides* consists of Chl containing velamentous roots which encircle and adhere to the substrate tree branch. The shoot is represented only by a very short structure from which the roots are produced. Leaves are usually absent although small leaves may occasionally be present, particularly during the early stages of development of the plant. Virtually all photosynthetic carbon assimilation takes place in the roots of *C. usneoides* and the use of this plant avoids problems associated with the possibility of translocation of photosynthetic products between leaves and roots encountered in plants which possess both of these organs.

MATERIALS AND METHODS

Plant Material. The shootless orchid *Chiloschista usneoides* (DON) LDL was grown outdoors or in a controlled environment

cabinet with: 12 h photoperiod (200 μmol m⁻² s⁻¹ PAR); day temperature 26 to 30°C; night temperature 25°C; RH > 70% day and night.

Plants were thoroughly soaked by spraying with water once each morning. When fully engorged, the velamen, which appears silvery when dry, becomes transparent and the roots appear dark green in color. In some plants, the velamen was heavily colonized by green algae. Such plants were not used for experimental work.

Photoperiod. In all experiments, unless specifically noted, experimental photoperiods coincided as much as possible with the natural photoperiod (7–19 h).

Measurement of Acid Content. Duplicate samples of roots weighing approximately 1 g were boiled in distilled H₂O for 15 min and the extracts were then titrated to the phenolphthalein end-point with 0.01 N NaOH and the average acid content calculated. Because the extracts contained varying proportions of a mixture of acids, the data are expressed as μeq of acid.

CO₂ Exchange. The CO₂ concentration in air which had passed at a rate of 300 ml⁻¹ min⁻¹ through a glass cuvette maintained at 25°C containing a whole plant was estimated by open-system, differential analysis using a Beckman model 865 IR gas analyzer. Water vapor was removed from the usually saturated gas stream by absorption in silica gel prior to analysis. Calibration of the system utilized standard CO₂ mixtures. Plants were illuminated by a 700 w Philips mercury lamp and PAR was measured using a LI-COR quantum meter with a LI-190 SB sensor. Experiments were repeated at least four times.

Products of Dark ¹⁴CO₂ Fixation. Radioactive CO₂ (50 μCi in 1 μmol) generated from NaHCO₃ by the addition of lactic acid was injected through a serum bottle stopper into darkened 50-ml flasks containing intact plants. After incubation at 25°C for 14 h, the plants were killed and extracted in boiling ethyl alcohol.

The ethanol extract was analyzed by paper chromatography and autoradiography using propan-1-ol, ammonia, water, and phenol; water as solvents. The identity of radioactive compounds from two experiments was confirmed by co-chromatography and relative amounts of radioactivity measured by Geiger Müller counting of the chromatograms.

Internal CO₂ Concentration. Using a microsyringe, 10-μl samples of gas were removed from the internal atmosphere of plants under the conditions detailed in the results section and analyzed by GC on Porapak Q at 100°C. Carrier gas was N₂ at 15 ml/min. Following separation, CO₂ was hydrogenated to produce CH₄ (Pye Unicam catalytic converter) and then detected and quantified using a flame ionization detector. Standard gas mixtures were used to calibrate the method. Since there are open pathways between the internal gas phase of the root and the atmosphere, the withdrawal of samples from the internal atmosphere by syringe will inevitably result in 'contamination' of the

sample by atmospheric air and will yield minimal estimates of differences between the internal and external atmospheres.

Scanning Electron Microscopy. Short sections of root were frozen in liquid N_2 , freeze-dried, then coated with gold and examined using an International Scientific Instruments model 60 scanning electron microscope.

RESULTS

Acid Fluctuations. In plants with dry, silvery velamina, acid accumulated during the night period from a minimum of around $40 \mu\text{eq g}^{-1}$ fresh weight at the end of the light period to a maximum of around $115 \mu\text{eq g}^{-1}$ fresh weight just after dawn the following morning—a fluctuation of more than $70 \mu\text{eq g}^{-1}$ fresh weight (Fig. 1). Subsequent measurements made at more widely spaced intervals (also shown in Fig. 1) confirmed the substantial changes in titratable acidity. These experiments used plants growing in the natural environment and while the first 2 d were bright and cloudless ($900 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR at the plant surface at noon) the final day was cloudy (variable PAR

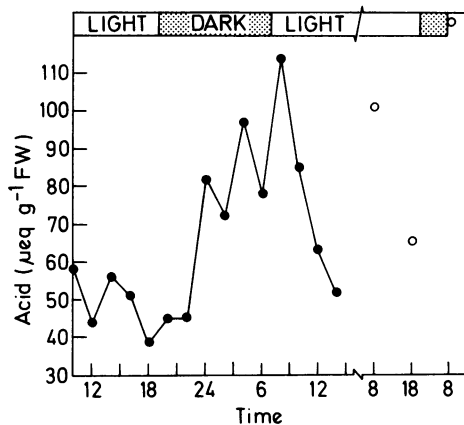


FIG. 1. Changes in acid content of roots of *C. usneoides* during the diurnal cycle. (●), Means of replicate samples taken at 2-h intervals. (○), Samples taken at the end of acid-accumulating and acid-consuming phases on subsequent days (a 3-fold decrease in scale follows the break in the x axis).

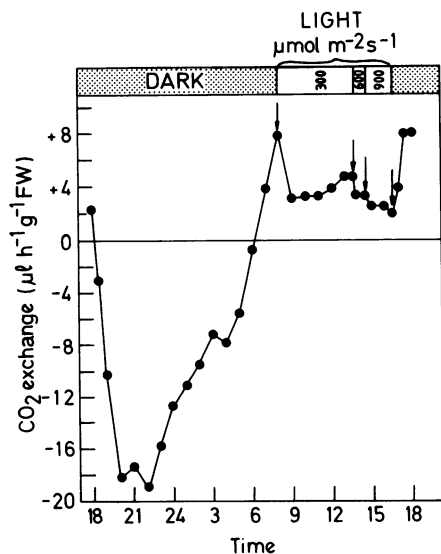


FIG. 2. CO_2 exchange in dark and light in dry *C. usneoides*. Following incubation in darkness at 25°C for 15 h, the plant was illuminated at 300, 600, and $900 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR. Finally, the plant was returned to darkness. (Negative CO_2 exchange = uptake).

with approximately $400 \mu\text{mol m}^{-2} \text{s}^{-1}$ at the plant surface at noon). The level of acid at the end of the cloudy day was $65 \mu\text{eq g}^{-1}$ fresh weight compared with less than 40 at the end of the bright days.

Acid fluctuations were also studied in plants which had water-saturated velamina throughout a 24-h cycle. Because the velamen absorbs a great deal of water (approximately 40% of the weight of a root with a dry velamen), corrections must be made when using a fresh weight basis to compare the amounts of acid accumulated by dry and saturated roots. Accordingly, the data for saturated roots have been corrected on the basis of differences in weights of saturated roots and the weights of the same roots dried in air for 5 h.

In plants with fully saturated velamina, acid content rose from 20 to $41 \mu\text{eq g}^{-1}$ fresh weight during the dark phase and decreased to 16 during the subsequent light phase. In a second experiment, acid content increased from 12 to $21 \mu\text{eq g}^{-1}$ fresh weight during the dark phase.

CO_2 Exchange. The data shown in Figure 2 were obtained in an experiment which gave results representative of those obtained in four similar experiments using plants which had not been watered for several hours and in which the velamen was dry and silvery in appearance. CO_2 uptake commenced within 1 h after the plants entered the dark phase and the rate of uptake increased to a maximum of around $19 \mu\text{l h}^{-1} \text{g}^{-1}$ fresh weight after 4 h in darkness, then declined until CO_2 evolution occurred around the subjective dawn (Fig. 2). Illumination at $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ resulted in a decline in the rate of CO_2 evolution to $3 \mu\text{l h}^{-1} \text{g}^{-1}$ fresh weight from a rate of $8 \mu\text{l h}^{-1} \text{g}^{-1}$ fresh weight which had occurred in the preceding dark period. CO_2 evolution at this lower rate was maintained for 5 h. Illumination at 600 and $900 \mu\text{mol m}^{-2} \text{s}^{-1}$ caused further decreases in the rate of CO_2 evolution. At all light levels investigated, including $900 \mu\text{mol m}^{-2} \text{s}^{-1}$ which approximates the upper levels of irradiation experienced by the plants in the natural environment, net CO_2 evolution was always observed. Transition from light to darkness was followed by a rapid increase in CO_2 evolution.

Effects of Water on CO_2 Exchange. Figure 3 illustrates data representative of four separate experiments carried out on plants in which the velamen was fully engorged with water. CO_2 evolution occurred in both dark and light. Illumination at $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ resulted in a transient decrease in the rate of CO_2 evolution from 8 to $4 \mu\text{l CO}_2 \text{ h}^{-1} \text{g}^{-1}$ fresh weight followed by a slow increase to $6 \mu\text{l CO}_2 \text{ h}^{-1} \text{g}^{-1}$ fresh weight at the end of the experiment.

CO_2 exchange was followed in a plant with a dry, silvery velamen until it was clear that dark CO_2 fixation was in progress (Fig. 4). The plant was then sprayed with distilled H_2O until the velamen was soaked. Following this treatment, CO_2 evolution occurred through the remaining 9 h of the dark period. The rate

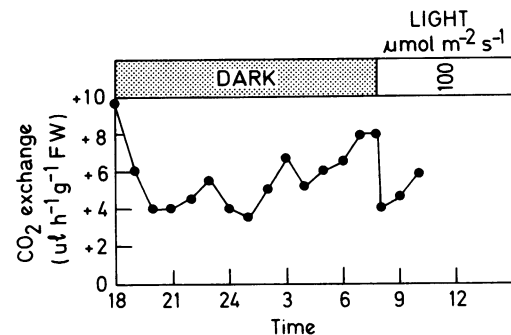


FIG. 3. CO_2 exchange in *C. usneoides* with water-saturated velamen. Incubation of the plant at 25°C for 15 h was followed by illumination at $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR. The velamen was saturated with water throughout the experiment.

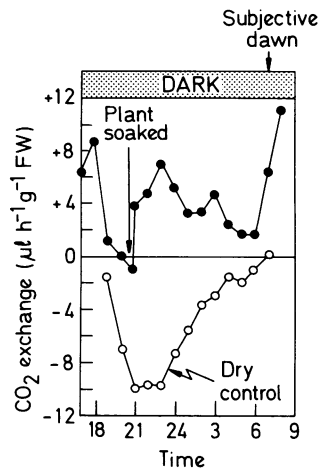


FIG. 4. Effect of saturating the velamen on the progress of CO_2 exchange in darkness. CO_2 exchange was followed at 25°C until it was established that a normal CO_2 exchange pattern was developing. At the time indicated, the plant was sprayed with distilled H_2O until the velamen was saturated. (O), CO_2 exchange pattern of the same individual plant when the velamen remained dry throughout the dark period. (Negative CO_2 exchange = uptake).

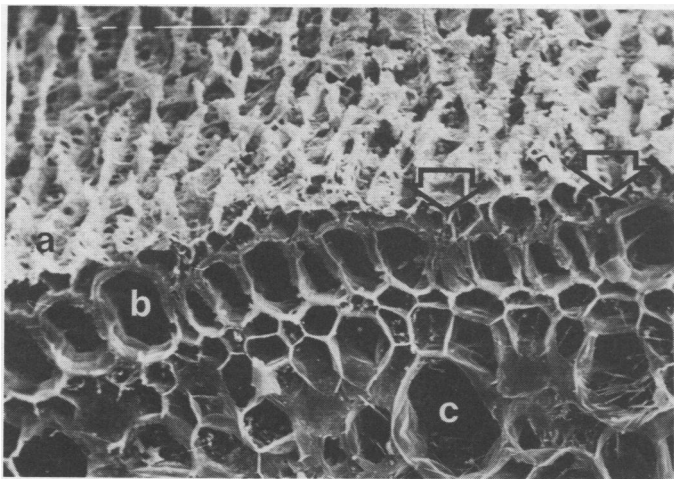


FIG. 5. Scanning electron micrograph of surface and section of cortex of photosynthetic root of *C. usneoides*. Long scale bar = $100\ \mu\text{m}$. Arrows indicate apparent aeration pathways through the velamen (a) and exodermis (b) leading to the photosynthetic cortex (c).

of release increased from 2 to $11\ \mu\text{l h}^{-1}$ as the subjective dawn approached. Also plotted on Figure 4 for comparative purposes is the progress curve of CO_2 exchange in the same individual when the velamen was dry throughout the experiment.

Products of Dark Fixation of $^{14}\text{CO}_2$. Autoradiography of extracts of small intact plants incubated with $^{14}\text{CO}_2$ in darkness for 14 h illustrated that malic acid contained the majority of the radioisotope. In one experiment in which the plant weighed 1.7 g, 96% of the activity was located in malic acid, citric acid contained 3%, and the remainder was accounted for by two unidentified components.

Anatomy of Roots. Figure 5 shows a scanning electron micrograph of the surface and a section across part of a root. Pathways through the velamen and exodermis can be seen (marked by arrow) which apparently link the atmosphere with the internal gas spaces of the root. There is no evidence of any structures which might exert diurnal control over the diffusive resistance between the atmosphere and the internal gas spaces.

Internal CO_2 Concentration. The concentration of CO_2 in

samples taken from plants which were 5 h into their photoperiod (acid consuming phase) was $315 \pm 16\ \text{vpm}^1$ (mean of three independent determinations). The air of the cabinet in which they were growing contained $305 \pm 24\ \text{vpm}$ (mean of three independent determinations).

The concentration of CO_2 in samples taken from roots which had experienced 4 h of darkness (acid accumulating phase) was $269 \pm 79\ \text{vpm}$ (mean of three independent determinations) when the air in the cabinet in which they were grown was $429 \pm 23\ \text{vpm}$ (mean of three independent determinations).

DISCUSSION

The CO_2 exchange data (Figs. 2 and 4) clearly demonstrate that dry roots of *C. usneoides* acquire CO_2 from the atmosphere in darkness. The large diurnal fluctuations in acidity measured in the present work substantiate earlier findings (1, 12). Comparison of the kinetics of CO_2 fixation and the kinetics of acid accumulation (Figs. 1 and 2) support the contention that CO_2 fixation leads to acid synthesis and accumulation. Malic acid is the major product of dark $^{14}\text{CO}_2$ fixation and, in conjunction with the low $\delta^{13}\text{C}$ values reported by Winter *et al.* (12), this indicates that the nocturnal uptake of CO_2 is via PEP carboxylase.

The acquisition of CO_2 from the atmosphere by gaseous diffusion in land plants has two conflicting aspects, the inward diffusion of CO_2 , which there is advantage in maximizing, and the inevitably associated outward diffusion of water vapor which there is advantage in minimizing. C_3 , C_4 , and most CAM plants utilize the control of gaseous diffusion afforded by stomata to achieve the necessary balance. In CAM plants, apart from the shootless orchids, the stomata allow adequate diffusion of CO_2 into the plant at night and, when closed during the light decarboxylation phase, reduce the outward diffusion of water and CO_2 . Since this is not possible in *Chiloschista*, the question arises as to how this shootless orchid exhibits CAM in the absence of stomata.

Although the roots possess structures specialized to allow gaseous exchange between the atmosphere and the internal gas phase of the plant (*i.e.* pneumatophodes and the special cortical cells described by Benzing and Ott [3] and Benzing *et al.* [2]), there does not appear to be any possibility for the diurnal regulation of gaseous diffusion as is effected by stomata in the leaves and shoots of CAM plants (Fig. 5). In these photosynthetic roots, gaseous diffusion between internal and external atmosphere takes place across a diffusion barrier over which the plant has no control. The proportion of exchange which occurs through the pneumatophodes and the proportion which occurs through the surface of the velamen and underlying tissues remains to be determined. However, regardless of the details of the diffusive pathway, it is certain that the diurnal changes in diffusive resistance which are an essential component of CAM in leaves and stems are not a feature of the photosynthesis of this shootless orchid.

In CAM plants with stomata, leakage to the atmosphere during the light phase is small (10). Despite the absence of stomata in *Chiloschista* roots, the rate of loss of CO_2 to the atmosphere in the light decarboxylation phase is also low (Fig. 2). The near atmospheric values measured for internal CO_2 concentration in the light in *Chiloschista* suggest that the slow loss to the atmosphere may be the result of the magnitude of the diffusion gradient of CO_2 between the internal and external atmosphere. It appears that in place of the control of CO_2 loss by control of diffusion resistance found in CAM plants with functional stomata, the loss of CO_2 from *Chiloschista* in the light phase is minimized by the balance between decarboxylation and photosynthetic CO_2 fixation being regulated such that the level of CO_2 within the roots

¹ Abbreviation: vpm, volumes per million volumes.

is maintained around atmospheric concentration. Thus, unlike the situation in other CAM plants (4, 11), the externally directed diffusion gradient in the light is not great. The biochemical mechanism which achieves this balance between CO₂ release and consumption remains to be elucidated.

In addition to being high enough to limit water loss to a level which can be tolerated by the plant, the diffusion resistance of the roots must obviously also be low enough to allow acquisition of an adequate supply of CO₂. The utilization of PEP carboxylase in CAM may be important in this context because (unlike ribulose-1,5-bisphosphate carboxylase/oxygenase) it can operate effectively at low (virtually zero) CO₂ concentrations in air (5) and therefore has the potential to maximize the inwardly directed CO₂ diffusion gradient into the roots.

Figures 3 and 4 demonstrate that saturation of the velamen with water has a dramatic deleterious effect on the CO₂ exchange of the plant. The curves show that the amplitude of acid fluctuation is reduced by a factor of about 5 and dark fixation of CO₂ is abolished. A superficial explanation would be that saturation of the velamen increases diffusion resistance—the liquid water layer acting as a diffusion barrier—thereby reducing inward diffusion of CO₂ and associated acid accumulation. However, although this may well be part of the answer, the finding that plants with saturated velamina evolve CO₂ in darkness rather than simply taking up less CO₂ indicates that the situation is complicated. A similar response of CO₂ exchange to wetting in the bromeliad CAM plant *Tillandsia usneoides* has been reported by Martin and Siedow (9). Further work is required on this phenomenon.

The photosynthetic metabolism of shootless orchids represents an addition to the presently recognized mechanisms by which plants acquire atmospheric CO₂, namely C₃, C₄, CAM, and the recently discovered aquatic acid metabolism photosynthesis (7, 8). Although the metabolism of *C. usneoides* is clearly derived from CAM, we consider that the absence of stomatal control of diffusional resistance represents a significant physiological distinction and we propose the term astomatal CAM for this variant

of photosynthetic carbon metabolism.

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