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C_3 PHOTOSYNTHESIS IN THE GAMETOPHYTE OF THE EPIPHYTIC CAM FERN *PYRROSIA LONGIFOLIA* (POLYPODIACEAE)[1]

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Sporophytes of some epiphytic species in the fern genus *Pyrrosia* exhibit Crassulacean acid metabolism (CAM), generally considered to be a derived physiological response to xeric habitats. Because these species alternate between independent sporophytic and gametophytic generations yet only the sporophyte has been characterized physiologically, experiments were conducted to determine the photosynthetic pathways present in mature sporophytes, immature sporophytes, and gametophytes of *Pyrrosia longifolia*. Diurnal CO_2 exchange and malic acid fluctuations demonstrated that although the mature sporophytes exhibited CAM, only C_3 photosynthesis occurred in the gametophytes and young sporophytes. Consideration of the above results and those from previous studies, as well as the life cycle of ferns, indicates that the induction of CAM probably occurs at a certain developmental stage of the sporophyte and/or following exposure to stress. Elucidation of the precise mechanisms underlying this C_3-CAM transition awaits further research.

By restricting stomatal opening and CO_2 uptake to the nighttime, plants with Crassulacean acid metabolism (CAM) have extremely high water-use efficiencies (CO_2 uptake per H_2O lost) and are capable of colonizing arid habitats (Kluge and Ting, 1978; Winter, 1985; Lüttge, 1987). The absorption of atmospheric CO_2 at night results in production of malic acid, which is subsequently stored in the massive vacuoles characteristic of CAM plants. The malic acid content of the vacuole increases throughout the night, effecting a considerable reduction in pH of the photosynthetic tissue, which is easily observed with tissue homogenates or extracts. In the morning, light triggers the release of malic acid from the vacuoles, whereupon the resultant malate is decarboxylated. The released CO_2 accumulates in the tissue, causing stomatal closure, and is eventually reduced to carbohydrate in the photosynthetic carbon reduction cycle. The large diurnal amplitude of tissue malic acid concentration is diagnostic of CAM; tissue acidity does not change appreciably on a daily basis in photosynthetic tissues of C_3 and C_4 plants.

Given that CAM constitutes a complex adaptation that conserves large amounts of water, the finding of CAM in tropical and subtropical epiphytes was at first surprising. Plant desiccation between rainfalls, however, can apparently result in severe drought stress (Osmond, Winter, and Ziegler, 1982; Sinclair, 1983; Kluge et al., 1989; Kluge, Avadhani, and Goh, 1989; Martin, 1994); epiphytes with CAM presumably minimize the degree of such stress. By far the great majority of CAM epiphytes are bromeliads and orchids (Winter, 1985; Martin, 1994). In the mid-1970s, however, Wong and Hew (1976) reported CAM in the epiphytic ferns *Pyrrosia longifolia* and *P. piloselloides*. This report of CAM in pteridophytes was followed by several more (Winter et al., 1983; Hew, 1984; Ong, Kluge, and Friemert, 1986; Winter, Osmond, and Hubick, 1986). Currently, five species of ferns, all in the genus *Pyrrosia* (species previously recognized as *Drymoglossum* were transferred to *Pyrrosia* by Ravensberg and Hennipman [1986]), have been reported as CAM plants, although not all species of *Pyrrosia* exhibit CAM (Winter et al., 1983; Kluge, Avadhani, and Goh, 1989). This genus of ferns is a member of the Polypodiaceae, a group considered by most authorities (e.g., Holttum, 1949; Tryon and Tryon, 1982) to contain a high percentage of derived character states. Thus, the observation that all ferns having CAM, a physiological feature generally considered to be derived relative to C_3 photosynthesis (Lüttge, 1987), are members of the Polypodiaceae follows this general evolutionary trend.

Because ferns have two independent, autotrophic life forms—the sporophyte and gametophyte—the presence of CAM in *Pyrrosia* raises an interesting question. Are the photosynthetic pathways of the two life forms identical? Photosynthesis in the gametophyte, which lacks structural complexity and has half the chromosome complement of the sporophyte, has not been examined in ferns with CAM. The presence of CAM in the gametophytes would be surprising given their lack of stomata, extremely thin cuticles, and affinity for deep shade. In contrast, the lack of CAM in these gametophytes would be of interest in light of the development and regulation of CAM (Winter, 1985; Lüttge, 1987). Therefore, the objective of this study was to determine the photosynthetic pathway of the gametophytes of the CAM epiphyte *P. longifolia*.

MATERIALS AND METHODS

Plant material—Whole plants of *Pyrrosia longifolia* (N. L. Burm.) Morton (Polypodiaceae) with mature sporangia were obtained from greenhouse stock at the Institut für

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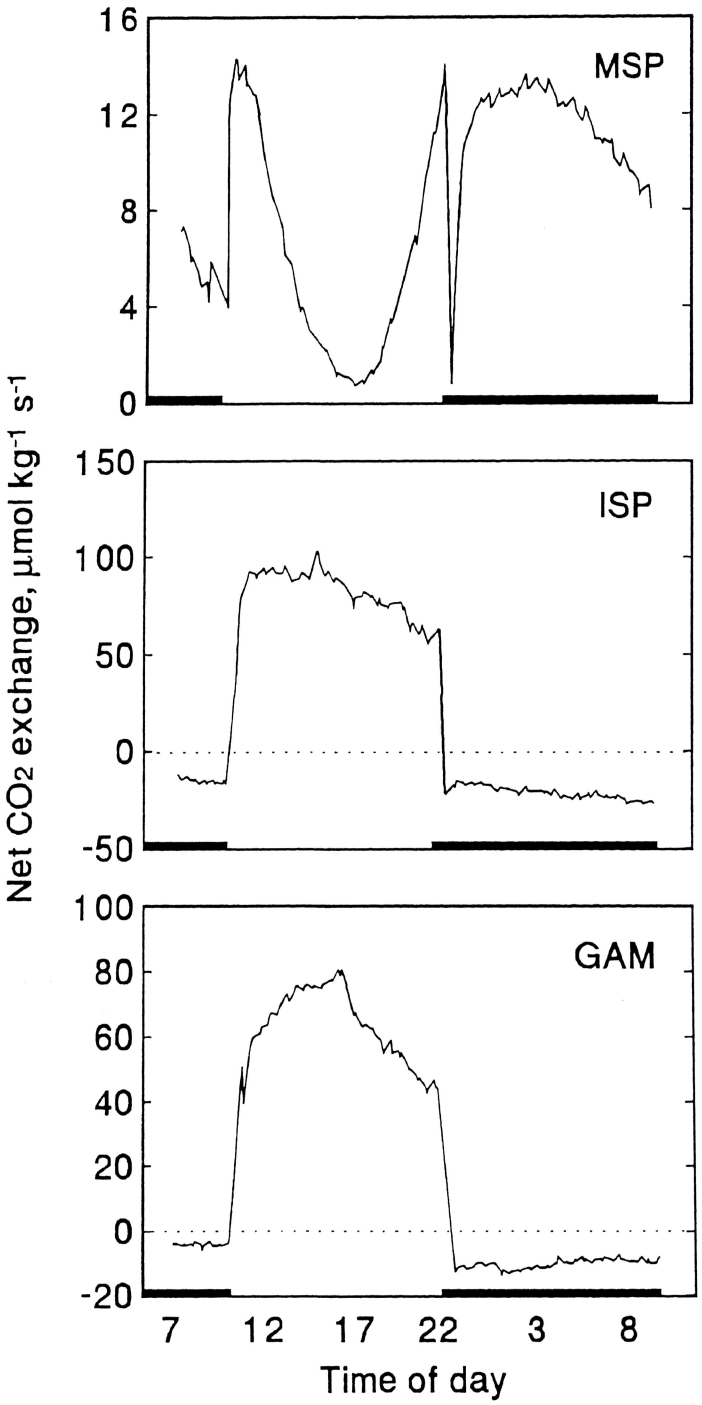

Fig. 1. Net CO_2 exchange (negative values indicate CO_2 loss) throughout a 24-hour period for a mature sporophyte (MSP), a group of immature sporophytes (ISP), and a group of gametophytes (GAM) of *Pyrrosia longifolia*. Data are expressed on a dry mass basis. Means of cumulative day and night CO_2 exchange for four individuals or groups of plants are provided in Table 1.

Botanik, Technische Hochschule Darmstadt, Darmstadt, Germany. The plants were originally collected in Singapore. Rhizomes were planted in standard greenhouse soil (7:2:1:1 [v/v] mixture of clay loam, peat moss, Perlite, and vermiculite) and kept moist in the University of Kansas greenhouse for several months before use. Environmental conditions in the greenhouse were approximately 1,000 μmol m^{-2} sec^{-1} maximum photosynthetic photon flux density (PPFD), although typical daytime values were much lower, 27–36/15–26 C day/night air temperature ranges, and 1.3–3.0/0.7–1.6 kPa day/night vapor pressure deficit (vpd) ranges. Plants were fertilized weekly with a dilute solution of 18% of each of total N, P_2O_5, and K_2O, including trace elements.

Spores were sown on sterile agar medium (supplemented with Thompson's macroelements and Parker's microelements as in Klekowski [1969]), and gametophytes were cultured in glass petri dishes at room temperature until used as described below. The gametophytes received approximately 125 μmol m^{-2} sec^{-1} PPFD for 12 hours each day. Sections of agar containing dense populations of gametophytes were removed from the petri dishes and used for the experiments after 6 months of growth. Several petri dishes were used after 6 more months; the latter contained a mixture of gametophytes and young sporophytes (hereafter referred to as "immature sporophytes") which were approximately 0.5 cm long. These sporophytes were gently removed with forceps, placed on fresh agar, and immediately used for the gas exchange experiments.

Gas exchange—Gas exchange was measured with an open-flow, differential infrared gas analyzer system as described in Harris and Martin (1991) and Gravatt and Martin (1992). Environmental conditions within the cuvettes were: 250–300 μmol m^{-2} sec^{-1} PPFD for 12 hours, $^{20.0}/_{18.8}$ C day/night air temperatures, and $^{0.6}/_{0.4}$ kPa day/night vpd (but see below).

Plant material was sealed into the gas exchange cuvettes for 3 days: plants were acclimated to cuvette conditions on day 1; gas exchange data were recorded on day 2; and malic acid decarboxylation was measured on day 3. Single attached fronds (approximately 30 cm long, 3 cm wide) of the sporophytes were sealed into the cuvettes; the remainder of the plant, its roots, and soil remained outside. Pots were kept well watered throughout the measurements.

Gametophytes and immature sporophytes with their supporting agar media were sealed into the cuvettes intact. The wet agar resulted in condensation of water vapor in the cuvettes; however, net CO_2 exchange of similar amounts of agar lacking plant material was zero throughout the day and night in spite of this condensation.

At lights-on of the third day, half the plant material in the cuvette was removed and frozen at −65 C. Similarly, at lights-out of the same day, the remaining plant material was removed and frozen until malate analysis.

Malate analyses—Sporophyte frond sections were thawed and sliced; gametophytes and immature sporophytes were removed from the agar using forceps. In all cases, tissue liquid was removed by centrifugation as in Smith and Lüttge (1985). The remaining tissue was dried at 65 C for subsequent determination of dry mass. Agar contamination of the gametophyte and immature sporophyte mass determinations was minimal but undoubtedly contributed to the high variability of the CO_2 exchange data (see Table 1). Malate concentration of the tissue liquid was determined using the enzymatic/spectrophotometric method of Gutmann and Wahlefeld (1974)

TABLE 1. Cumulative day and night CO_2 exchange and malic acid fluctuation (mature sporophytes only; morning minus evening values; see Fig. 2) in mature sporophytes, immature sporophytes, and gametophytes of *Pyrrosia longifolia*. Values are means (standard deviations in parentheses) of four individuals (mature sporophytes) or groups of plants (immature sporophytes and gametophytes) and are expressed on a dry mass basis. Representative CO_2 exchange curves are shown in Fig. 1.

Plant material	Cumulative CO_2 exchange, mmol kg^{-1}				Malic acid fluctuation mmol kg^{-1}
	Day		Night		
	Uptake	Loss	Uptake	Loss	
Mature sporophyte	413 (342)	0	420 (211)	0	557 (247)
Immature sporophyte	2,680 (1,058)	0	0	800 (260)	—
Gametophyte	2,904 (825)	0	0	458 (507)	—

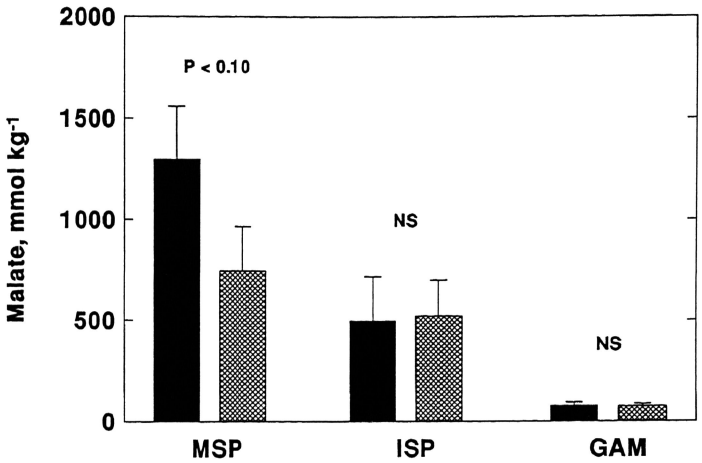

Fig. 2. Malate concentrations of tissue collected in the morning (solid bars) and evening (hatched bars) for mature sporophytes (MSP), immature sporophytes (ISP), and gametophytes (GAM) of *Pyrrosia longifolia*. Values are means and SD (error bars) of four individuals of mature sporophytes and four groups of immature sporophytes and gametophytes and are expressed on a dry mass basis. These are the same plants or groups of plants used in the gas exchange experiments (see Fig. 1, Table 1). Results of the Mann-Whitney *U*-test for significant differences between each pair of means are shown; NS = not significant ($P > 0.20$).

by comparing the data with results using known malate concentrations. Given the high pH of the buffer used in this assay, most malic acid is converted to malate when analyzed. Thus, although malate concentrations were measured, discussion of tissue malic acid concentration is often more accurate.

Statistical analysis—Because sample sizes were small, pairs of means were compared with the nonparametric Mann-Whitney *U*-test (Sokal and Rohlf, 1981; Potvin and Roff, 1993). In all comparisons, variances of the means were not significantly different.

RESULTS AND DISCUSSION

Leaves of the mature sporophytes of *Pyrrosia longifolia* exhibited CAM; CO_2 uptake occurred at night (Fig. 1), and the malic acid concentration in the morning was higher than that in the evening (Fig. 2). Although the observed difference in tissue malic acid concentrations was of marginal statistical significance ($P < 0.10$), the malic acid concentration in the morning was substantially greater than that in the evening in all four plants investigated. High variability among the plants precluded a greater degree of statistical significance. Substantial amounts of morning (Phase II, see Osmond, 1978) and afternoon (Phase IV) CO_2 uptake were also observed in the mature sporophytes, which is not uncommon in some CAM species. As expected in obligate CAM plants under well-watered conditions, the amount of malic acid decarboxylated during the day (presumed equal to the amount accumulated during the previous night) was not significantly different ($P > 0.20$) from the amount of CO_2 absorbed throughout the night (Table 1). After appropriate conversion of the units, similar rates of CO_2 uptake and diel fluctuations in tissue acidity were reported for mature sporophytes of *P. longifolia* in the field in Australia by Winter, Osmond, and Hubick (1986).

Unlike the sporophytes, the gametophytes and immature sporophytes lacked CAM; no nocturnal CO_2 uptake was observed (Fig. 1), nor were morning malic acid concentrations different from those in the evening (Fig. 2). The results indicate that the gametophytes and immature sporophytes exhibited C_3 photosynthesis. The extremely high rates of CO_2 uptake observed in the immature sporophytes and gametophytes, relative to the mature sporophytes, probably reflect the dry mass basis of the data. The fronds of the mature sporophytes included much more nonphotosynthetic, e.g., structural, tissue, relative to the undifferentiated tissue of the gametophytes, as well as the undeveloped immature sporophytes. Consideration of the dry mass basis of the malic acid data underscores the relatively large increase in tissue malic acid content associated with the development of CAM in the mature sporophytes. Reasons for the greater malic acid concentrations in the immature sporophytes vs. the gametophytes are unclear; however, this difference might reflect greater phospho*enol*pyruvate (PEP) carboxylase activity in the immature sporophytes, possibly a precursor to the onset of CAM.

There are several potential explanations for the lack of CAM in the gametophytes of *P. longifolia*. These include: 1) minimal structural complexity of the tissue; 2) a developmental stage premature for CAM; 3) growth under environmental conditions unfavorable for CAM induction; 4) lack of a full chromosomal complement; and/or 5) lack of a molecular "signal." Regarding tissue complexity as a prerequisite for CAM, the results of Brulfert et al. (1987), who examined the nature of photosynthesis in undifferentiated callus tissue of the CAM plant *Kalanchoe blossfeldiana*, are particularly interesting. Although net CO_2 exchange was always negative, i.e., CO_2 was always lost by the tissue, day/night CO_2 exchange patterns were CAM-like: CO_2 losses were much less at night than during the day. In addition, tissue malic acid concentrations increased at night in the callus. Thus, these results indicate that development of tissue complexity per se may not be necessary for the induction of CAM. In support of this, the changeover from C_3 photosynthesis to CAM

at a certain developmental stage is not necessarily accompanied by major morphological changes in *Mesembryanthemum crystallinum* (Cushman, Michalowski, and Bohnert, 1990; DeRocher and Bohnert, 1993; Lüttge, 1993).

Given that both gametophytes and mature sporophytes of *P. longifolia* in the current study were grown under constantly moist conditions and similar, although not identical, irradiances, temperatures, and vpd, it seems unlikely that minor differences in environmental conditions during growth would have resulted in such radically different modes of photosynthesis. Exposure of the mature sporophytes to stress prior to this study, however, could possibly have induced irreversible CAM in the sporophytes. Finally, the lack of any indication of CAM in the immature sporophytes might suggest that the development of CAM in *P. longifolia* is not solely related to an increase in ploidal level. These results are consistent with others that indicated little or no correlation between ploidal level and expression of traits specific to gametophyte or sporophyte generations (Sheffield and Bell, 1987).

Regardless of the trigger mechanism, the switch from C_3 to CAM accompanying the transition from gametophyte to mature sporophyte in *P. longifolia* is presumably the result of regulation at the molecular level. As has been found in *M. crystallinum* (Cushman, Michalowski, and Bohnert, 1990; Schmitt and Piepenbrock, 1992; DeRocher and Bohnert, 1993; Lüttge, 1993), an enhanced production of PEP carboxylase RNA transcripts may precede the onset of CAM in this fern. The results of the current study indicate that the C_3-CAM transition most likely occurs as the sporophyte matures, either at a certain stage of development and/or after exposure to stress. Further work is required before the mechanism of CAM induction in *P. longifolia* is understood.

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