FUNCTIONAL STOMATA OF THE ATMOSPHERIC EPIPHYTE TILLANDSIA USNEOIDES L.[1]

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Although stomatal opening in *Tillandsia usneoides* is not detectable by light microscopy, the guard cells swell in the dark and in the light in buffers known to stimulate stomatal opening. Abscisic acid or phenylmercuric acetate substantially reduced subsequent nocturnal CO_2 uptake. Potassium ions were more concentrated in the guard cells in the dark than in the light. The stomata of this CAM epiphyte appear functional: open at night and closed throughout most of the day.

Introduction

Lack of functional stomata in *Tillandsia usneoides* was first reported by MEZ (1896). BILLINGS (1904) subsequently supported this claim with the following evidence: (1) purported immobility of the guard cells as a result of their extremely thick walls, (2) failure to detect visibly open stomata, and (3) failure of attempts to induce stomatal opening (BILLINGS 1904). Conversely, TOMLINSON (1969) suggested that guard cells of this epiphyte might be permanently open as a hygromorphic feature, supposedly to promote an increase in transpiration and subsequent uptake of elemental nutrients. DOWNS (1974) also discussed the possibility that the stomata of *T. usneoides* are permanently open. Evidence for the total absence of stomata in other atmospheric bromeliads was provided by LINSBAUER (1911).

MARTIN (1982) reported that the stomata of *T. usneoides* were nonfunctional: (1) with light microscopy, open stomata were not seen in live material, which had been treated to stimulate stomatal opening; (2) high concentrations of CO_2 did not inhibit the rates of nighttime $^{14}CO_2$ uptake in this CAM plant; (3) ABA did not reduce nocturnal net CO_2 uptake; and (4) gas diffusion resistance analyses demonstrated that CO_2 and H_2O vapor were diffusing along different pathways. COCKBURN (1983) viewed these findings as evidence for a potentially novel variation on the CAM theme.

The only evidence for functional stomata in atmospheric bromeliads was found in studies of CO_2 exchange patterns. In the two species studied, a typical CAM CO_2 exchange pattern was observed: stomata open at night and close throughout most of the day (KLUGE et al. 1973; LANGE and MEDINA

1979; MARTIN et al. 1981; MARTIN and SIEDOW 1981). Also, nocturnal CO_2 exchange rates were sharply diminished by abrupt decreases in atmospheric RH in *T. recurvata* (LANGE and MEDINA 1979) and in *T. usneoides* (MARTIN and SIEDOW 1981). Such responses have been attributed to rapid stomatal closure in other CAM and non-CAM species (LANGE et al. 1971; OSMOND et al. 1979; BUNCE 1981).

Given the conflicting evidence regarding the functionality of stomata in atmospheric bromeliads, particularly *T. usneoides,* we wished to determine whether the stomata of *T. usneoides* are functional. Our results indicate that the stomata of this epiphyte function normally.

Material and methods

Tillandsia usneoides was collected from several trees ca. 50 km north of Corpus Christi, Texas, in June 1982, transported to Kansas, and hung from wood and wire in a greenhouse at the University of Kansas. Plants were watered several times each week and occasionally sprayed with a dilute nutrient solution. Environmental conditions in the greenhouse in the winter and spring of 1983 when this study was undertaken were: maximum PPFD from above, ca. 500 $\mu mol \cdot m^{-2} \cdot s^{-1}$; maximum D/N air temperatures, 35/20 C; and RH, ca. 40%/80%. Photoperiod was extended 5 h with fluorescent lamps.

Two *T. bryoides* plants were purchased from Tropical Imports (Coarsegold, Calif.) in June 1983. They were collected in June 1982 in Argentina.

MICROSCOPIC OBSERVATIONS

To facilitate observation of the stomata, trichome shields were removed from the leaves by the repeated application and removal of adhesive tape. In the late afternoon or early evening, leaves were detached and soaked in one of the following solutions: (1) 0.01 M citrate, 0.02 M Na_2PO_4, pH 3 (JINNO and KURAISHI 1982), (2) 0.01 M PIPES, 0.0176 M KCl, 10^{-4} M kinetin (JEWER and INCOLL 1981), or (3) 0.08 M KCl, 0.025 M MES, pH 6.3 (ZEIGER et al. 1983). All leaves were soaked for 2 h under either room light or in the dark. Controls
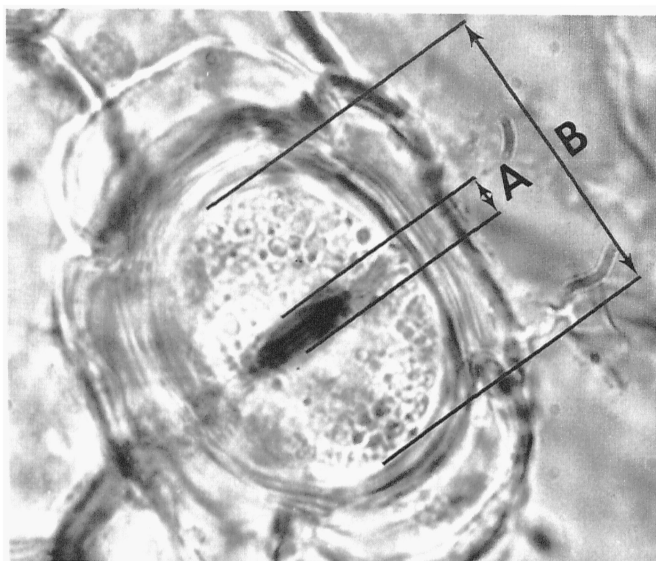
FIG. 1.—Dimensions of stomatal complex measured on the surface of *Tillandsia usneoides* leaves after various treatments designed to open and close the stomata. Whole leaves were mounted in water after removal of trichomes. The stomate is ca. 50 μm wide.

were soaked in deionized water.

After soaking, random stomata on each leaf were measured at ×400 using an ocular scale on a Leitz compound microscope. As an indicator of stomatal opening, the distance between the guard cell inner walls (*A* in fig. 1) was divided by the width of the entire stomatal complex (distance between guard cell outer walls, *B* in fig. 1).

For anatomical observations, leaves of *T. usneoides* were fixed in formalin-acetic acid, dehydrated in a graded series of water-ethanol, embedded in paraffin, sectioned, and stained with safranin and fast green.

GAS EXCHANGE EXPERIMENTS

An open, differential infrared gas analysis system monitored CO_2 and H_2O exchange, and leaf

and air temperatures (MARTIN and ZEE 1983). The plant chamber was constructed of polycarbonate, which has lower CO_2 and H_2O absorption characteristics than does Plexiglas (MODERN PLASTICS ENCYCLOPEDIA 1980, pp. 535, 547). Air mixing within the chamber was provided by a Micronel U.S. fan (Model V241L; Fallbrook, Calif.), which does not alter the gas composition of the chamber (BINGHAM and COYNE 1977). The fan created air turbulence within the clump of *T. usneoides* at rates up to $0.15 \text{ m} \cdot \text{s}^{-1}$. Clumps of *T. usneoides* (ca. 2 g DW) were completely sealed in this chamber (0.8-liter volume). Although this bulk of plant material in the chamber resulted in considerable self-shading and thick boundary layers, large samples were necessary to obtain reasonable rates of CO_2 exchange because of the slow rates typical of this CAM

TABLE 1

CHANGES IN STOMATAL DIMENSIONS FOR LEAVES OF TILLANDSIA USNEOIDES AFTER SOAKING 2 h IN THE SOLUTION SHOWN

Treatment	Stomatal width (μm)	"Pore" width (μm)	Ratio	No. stomata	No. leaves
H_2O—dark	34.7 ± 2.4	7.4 ± 1.2	.22 ± .03**	22	3
Control (H_2O—light)	32.0 ± 2.8	5.6 ± 1.3	.18 ± .04	26	3
KCl, MES—light	37.6 ± 3.1	10.4 ± 1.8	.27 ± .04***	27	3
Control (H_2O—light)	32.0 ± 2.8	5.6 ± 1.3	.18 ± .04	26	3
Citrate, Na_2PO_4—light	49.5 ± 6.1	8.9 ± 1.8	.18 ± .03***	41	5
Control (H_2O—light)	47.2 ± 5.1	7.3 ± 1.5	.15 ± .02	31	5
PIPES, KCl, kinetin—light	51.7 ± 4.9	8.4 ± 1.4	.16 ± .02*	40	4
Control (H_2O—light)	50.7 ± 3.6	7.7 ± 1.1	.15 ± .02	40	4

NOTE.—Ratios are "pore" widths divided by stomatal widths (see fig. 1). Levels of significance are results of *t*-tests comparing the mean ratios of each treatment and its control.

$*P < .05.$

$**P < .01.$

$***P < .001.$

epiphyte (KLUGE et al. 1973; MARTIN and SIEDOW 1981). In spite of this, rates of H_2O exchange (loss from plant during day, uptake at night) were barely detectable and not quantifiable. In all gas exchange experiments, chamber environmental conditions were: (1) PPFD inside the chamber ranged from 1,100 to 1,800 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (12-h photoperiod), (2) D/N air temperatures were 25/15 C, (3) daytime leaf temperatures were 4–5 C above air temperature, and (4) D/N air RH were 50%/90% (MARTIN and ZEE 1983).

Attempts to observe the effects of high CO_2 concentrations on rates of nocturnal CO_2 uptake as an indication of stomatal closure in *T. usneoides* were thwarted for the following reasons: (1) no decrease in CO_2 uptake was noted, probably because the steep gradient of external to internal CO_2 concentration resulted in CO_2 uptake rates large enough to compensate for any stomatal narrowing; and (2) it was not possible to utilize the H_2O exchange data because the rates were low and the bulk of H_2O exchange probably represented hydration or dehydration of the very hygroscopic trichome shields.

For the ABA and PMA experiments, a clump of *T. usneoides* was wetted with a control solution of deionized water containing 0.01% (vol/vol) Triton X-100 and 0.2% (vol/vol) ethanol or DMSO. The clump was surface dried and inserted into the gas exchange chamber immediately before darkness. Following the nighttime period of CO_2 uptake and the subsequent daytime period of little or no CO_2 exchange, the clump was removed from the chamber, wetted with 10^{-4} M (±) cis-trans ABA (Sigma
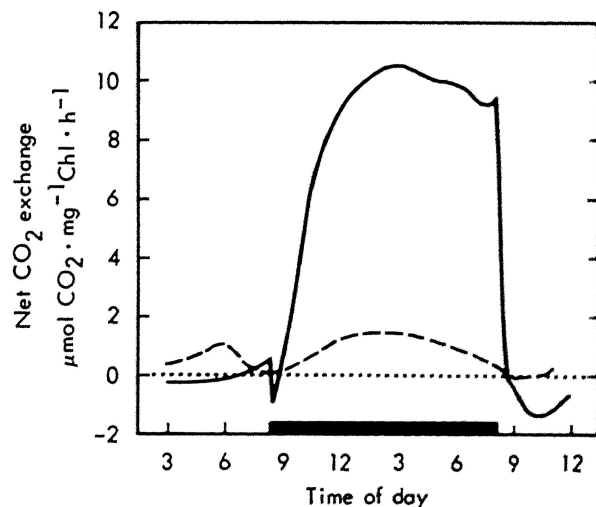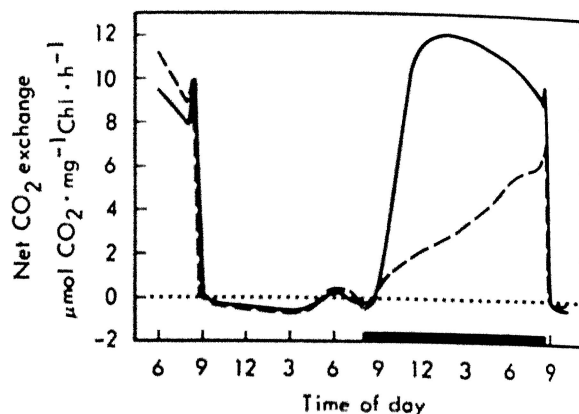

FIG. 3.—Effects of PMA on nocturnal net CO_2 exchange in *Tillandsia usneoides*. After the control solution was applied at 8 P.M., CO_2 exchange (solid line) was monitored for 24 h. At 8 P.M. of the second day, the clump was wetted with 10^{-1} M PMA, and its CO_2 exchange (dashed line) was subsequently monitored. Curves represent mean CO_2 exchange rates taken at 15-min intervals with three different clumps of plant material. Standard deviations ranged from 0.01 to 2.56 (no. = 184 intervals). The black bar indicates darkness.

Chemical, St. Louis) in 0.2% (vol/vol) ethanol or 10^{-3} M PMA (Sigma) in 0.2% (vol/vol) DMSO and 0.01% (vol/vol) Triton X-100, air-dried, and placed back into the chamber. Gas exchange was then monitored a second night. All procedures were carried out three times for each experimental solution.

Following a gas exchange experiment, the clump of *T. usneoides* was removed, ground in 80% aqueous acetone in a Waring Blendor for 1 min, extracted 2–3 h, and vacuum filtered. The extract was analyzed spectrophotometrically for Chl content (ARNON 1949).

POTASSIUM LOCALIZATION

Potassium ions were localized by precipitation as potassium cobaltinitrite (HUMBLE and HSIAO 1970) and subsequently viewed microscopically. Leaves were stained after 3 h in the light or in the dark in late afternoon or early evening. This was repeated three times.

Results

Although open stomata of *Tillandsia usneoides* were never observed in fresh tissue under the light microscope, the shape of the stomatal complex enlarged in the dark and in the light when treated with buffers known to open stomata (table 1), relative to the controls in deionized water in the light. Variability in the dimensions of the stomata reflect stomatal size differences between strands of Spanish moss.

Daytime applications of either 10^{-4} M ABA (fig. 2) or 10^{-3} M PMA (fig. 3) resulted in a substantial reduction of CO_2 uptake rates the following night.

FIG. 2.—Effects of ABA on nocturnal net CO_2 exchange in *Tillandsia usneoides*. After the control solution was applied at 8 P.M., CO_2 exchange (solid line) was monitored for 24 h. At 8 P.M. of the second day, the clump was wetted with 10^{-4} M ABA, and its CO_2 exchange (dashed line) was subsequently monitored. Curves represent mean CO_2 exchange rates taken at 15-min intervals with three different clumps of plant material. Standard deviations ranged from 0.06 to 2.85 (no. = 204 intervals). The black bar indicates darkness.
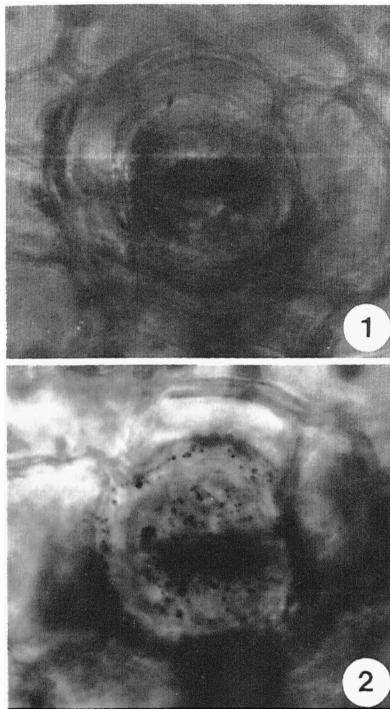
Fig. 4.—Potassium cobaltinitrite (black precipitate) localization in leaf epidermal cells of *Tillandsia usneoides* after 3 h in the light (*1*) or in the dark (2). The stomate is ca. 50 μm wide.

Only the ABA-treated plants exhibited some recovery in CO_2 uptake during the later part of the dark period.

Accumulation of the K^+ precipitation complex in the guard cells of *T. usneoides* occurred in the dark but not in the light (fig. 4). In the light, stain was not found in the stomata or in other epidermal cells; however, large quantities of stain were deposited in the trichome stalk cells (not shown), indicating that the staining technique worked properly.

Discussion

Although each experiment may not conclusively prove that the stomata of *Tillandsia usneoides* function normally, all of the evidence together strongly indicates normal CAM functioning. The guard cells enlarge when leaves are placed in the dark or in buffers that stimulate stomatal opening in other plants (JEWER and INCOLL 1981; JINNO and KURAISHI 1982; ZEIGER et al. 1983). Nocturnal CO_2 exchange is inhibited by applications of ABA or PMA, which cause stomatal closure in other plants (SCHULT and DÖRFFLING 1981; ZEIGER 1983). Finally, the guard cells accumulate large amounts of potassium during the night but not during the day. The lack of stain in closed stomata is not unusual and has been found in several C_3 species after dark treatment (DAYANANDAN and KAUFMAN 1975). Thus, contrary to previous reports, these results in-

dicate that the stomata of this atmospheric epiphyte are functional.

The absence of open stomata in this and previous studies (BILLINGS 1904; MARTIN 1982) does not preclude the possibility that the stomata open at night (though the stomatal pore is not perceptible by light microscopy) and close during the day, thus regulating gas exchange in typical CAM fashion. Visualizing stomatal opening may be hindered by the large antechambers characteristic of Spanish moss (fig. 5), as suggested by WEYERS and TRAVIS (1981). Furthermore, thick guard cell walls do not necessarily restrict movement of these cells, as proposed by BILLINGS (1904), since many taxa with functional stomata also exhibit very thick guard cell walls (WU and SHARPE 1980).

Most of the preliminary data obtained by MARTIN (1982) are compatible with our explanation, in spite of his tentative conclusion that the stomata of *T. usneoides* are nonfunctional. His observation, from gas resistance analysis, that CO_2 and H_2O vapor follow pathways of different resistances can be explained by the following: CO_2 is taken up through the stomata at night while most of the H_2O vapor is absorbed by the desiccated trichomes (PENFOUND and DEILER 1947; VIRZO DE SANTO et al. 1976). Thus, water loss through the stomata would be obscured by this water vapor uptake. The results of the ABA and PMA experiments in the current study contradict the lack of an ABA effect on nocturnal CO_2 exchange found by MARTIN (1982). This discrepancy may be attributed to the forms of ABA utilized in the two studies: MARTIN (1982) used the "mixed isomers" form, which is less active than the "(±) cis-trans" form (MARTIN, personal observation).
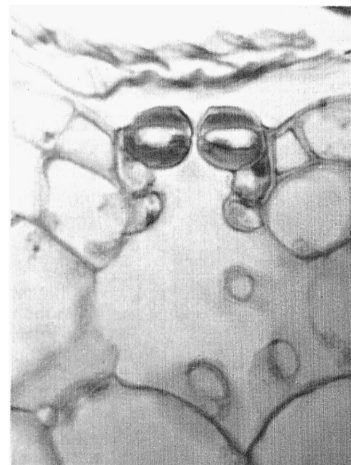
ABA and PMA may have inhibitory effects on

Fig. 5.—Cross section of a leaf of *Tillandsia usneoides* showing the guard cells with extremely thick cell walls and the large antechamber beneath the stomate. Large mesophyll cells surround the antechamber. Remnants of trichomes are visible above the stomate, which is ca. 50 μm wide.

leaf physiology other than causing stomatal clo-
sure. Exogenously applied ABA enhances leaf se-
nescence (ZEIGER 1983); however, given the in-
creasing rates of nocturnal CO_2 uptake following
ABA treatment in this study, it seems more likely
that ABA directly affected the stomata, and that it
was catabolized throughout the night (MILBORROW
1983), allowing stomatal opening. PMA may also
disrupt chloroplast membranes (SCHULT and
DÖRFFLING 1981), which cannot be discounted in
our study.

Reports (LINSBAUER 1911; TOMLINSON 1969) of
Tillandsia species, including T. bryoides, with few
or no stomata supported the possibility that func-
tional stomata are not required for regulating gas
exchange in atmospheric bromeliads. Such reports
probably encouraged the unusual hypotheses con-
cerning the control of gas exchange in these epi-
phytes (TOMLINSON 1969; DOWNS 1974; MARTIN
1982; COCKBURN 1983). The presence of stomata

on T. bryoides leaves was confirmed by the current
study as well as by Dr. WERNER RAUH (personal
communication) and contradicts the previous stud-
ies. This finding, in conjunction with the observed
sensitivity of nocturnal CO_2 uptake to changes in
RH (MARTIN and SIEDOW 1981) and the results of
the current study indicating that the stomata of the
atmospheric epiphyte T. usneoides are indeed
functional, should eliminate further speculation re-
garding the control of gas exchange in such epi-
phytes.

Acknowledgments

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ERRATUM

CRAIG E. MARTIN, ANNE E. LUBBERS, and JAMES A. TEERI. Variability in Crassulacean acid metabolism: a survey of North American succulent species. Published December 1982, pages 491–497.

One of the species we examined on montane rock ledges, *Sedum nevii*, was incorrectly identified. Dr. CHARLES H. UHL recently collected specimens from our study site and examined our vouchers. He concluded that this species is *S. ternatum*. In the rock-ledge population, we found a small but significant acid fluctuation without nighttime $^{14}CO_2$ uptake, indicative of CAM cycling (sensu STERNBERG et al. 1984); yet we found no acid fluctuation in the forest stream-bank population. With this nomenclatural correction, all species of *Sedum* thus far investigated exhibit nocturnal accumulations of titratable acid, with (CAM) or without (CAM cycling) concomitant CO_2 uptake (TEERI 1982).

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