BRIEF COMMUNICATION

Diel acid fluctuations in C_4 amphibious grasses

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Abstract

Orcuttieae is a small tribe of C_4 grasses endemic to seasonal pools in the southwestern U.S., comprising the basal genus *Neostapfia, Tuctoria,* and the most derived group, *Orcuttia.* Growth is initiated underwater, and when pools dry, species undergo a metamorphosis replacing aquatic foliage with terrestrial foliage. *O. californica* and *O. viscida* exhibit CAM-like diel fluctuations in acidity in the aquatic foliage. Pulse-chase studies showed that although CO_2 was fixed into malic acid in the dark, an overnight chase in the dark revealed that most label was not retained in organic acids, indicating a role other than CAM. Terrestrial foliage exhibited a very different diel fluctuation; acids accumulated during the day, and diminished overnight. Malic acid predominated and was secreted on the surface of the leaf in a manner similar to another arid land species. This terrestrial daytime acid accumulation may not be related to photosynthetic pathway but may play an anti-herbivore function. No acid fluctuations were observed in either *N. colusana* or *T. greenei.*

Additional key words: aquatic and terrestrial leaves; ^{14}C labelling; chlorophyll; malic acid; *Neostapfia colusana; Orcuttia californica* and *O. viscida*; titratable acidity; *Tuctoria greenei.*

Winter rains, coupled with mild temperatures in mediterranean-climate California create conditions conducive to a unique ecosystem known as vernal pools, with many endemic species (Holland and Jain 1977). These systems have a unique flora, including a tribe of nine annual grass species endemic to such seasonal pools. *Orcuttieae* comprises three genera, the monotypic *Neostapfia, Tuctoria* with three, and *Orcuttia* with five species (Reeder 1982). Placement within the *Poaceae* has

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been problematical due to the lack of clear alliances and the unique synapomorphy of eligulate leaves (Reeder 1965). All species in the tribe have C_4 photosynthesis (Keeley, unpublished), consistent with their placement in the *Chloridoideae*, a subfamily of approximately 1400 species, apparently all but one having C_4 photosynthesis (Watson and Dallwitz 1992).

Germination occurs in spring while seeds are submerged, and the hypoxic conditions resulting from submergence are a pre-requisite for germination of some species (Griggs 1976, Keeley 1988). In low rainfall years seeds remain dormant, sometimes for years in a row (Crampton 1959, Holland 1980, Reeder and Reeder 1982). Although all species in the tribe produce an aquatic foliage distinct from the terrestrial foliage, *Orcuttia* species are far more specialized to the aquatic environment (Keeley, unpublished). Apomorphies found in these latter species include large lacunae, absence of stomata, and floating leaves. In all species, the aquatic foliage is replaced by terrestrial foliage when the pools dry in mid to late spring, and both are C_4.

The presence of these C_4 grasses in California vernal pools is interesting, because adjacent grasslands are depauperate in C_4 plants (Teeri and Stowe 1976); only 9 % of the grasses in grasslands are C_4 compared to 63 % of the vernal pool grass flora, and if weighted by biomass the difference would be substantially greater (Keeley, unpublished). The paucity of C_4 in California grasslands is likely due to the low growing-season temperatures in the winter-rain summer-drought mediterranean-climate (Ehleringer and Monson 1993). However, vernal pool basins retain moisture much longer into the spring and summer, and thus greatly increase the growing season temperature relative to surrounding uplands. These vernal pool grasses persist well into the summer drought, often reaching anthesis in June on the dry, cracked substrates in the pool basins.

All *Orcuttieae* spend a significant part of their life cycle as submerged-aquatic plants, an unusual habitat for C_4 plants (Ehleringer and Monson 1993). In light of the fact that these grasses coexist with aquatic CAM plants (Keeley 1995) and the prior report of CAM in a C_4 plant (Koch and Kennedy 1980), diel changes in titratable acidity and malic acid were investigated (Table 1). No significant diel changes were observed for either *Neostapfia* or *Tuctoria*, however, aquatic leaves of both *O. viscida* and *O. californica* showed significant overnight increases in acidity.

Aquatic leaves: Overnight acid accumulation in *Orcuttia* species was an order of magnitude lower than is typical of associated aquatic CAM species such as *Isoetes howellii and Crassula aquatica* (Keeley 1995). Several lines of evidence suggest that acid fluctuation in *Orcuttia* species is not indicative of the CAM pathway. Although both species accumulated acids overnight, the extent of malic acid accumulation was highly variable (Table 1). On some sampling dates malic acid accumulated and on other occasions it did not, although on average *O. viscida* had no significant accumulation but *O. californica* did.

[14]C fixation studies in the dark showed that both *Orcuttia* species fixed carbon into malic acid, however, pulse-chase studies in the dark showed turnover into other products. This is illustrated by the fact that following a 3 h dark pulse and 9 h dark

Table 1. Diel changes in titratable acidity (to pH 7.0) [mmol(H$^+$) kg^{-1}(fr.m.)], and malic acid and chlorophyll (Chl) concentrations [mmol kg^{-1}(fr.m.)] in aquatic and terrestrial leaves of *Orcuttieae*. Leaves were ground in distilled H$_2$O and titrated to pH 7.0 with CO$_2$-free 0.01 M NaOH. An aliquot was taken for Chl determination (Šesták *et al.* 1971), and another deproteinized and assayed for malate (Gutman and Wahlefeld 1974). AM = morning hours within 1 h following sunrise, PM = afternoon hours within 1 h before sunset. NS = $p > 0.05$, $^* = p < 0.05$, $^{**} = p < 0.01$ ($n = 6$) with two-tailed t-test.

Species		Titratable acidity			Malic acid			Chl
		AM	PM	p	AM	PM	p	
Neostaphia colusana	aquatic	16	17	NS	8	13	NS	0.81
	terrestrial	13	12	NS	23	17	NS	1.47
Tuctoria greenei	aquatic	3	2	NS	6	11	NS	0.70
	terrestrial	17	14	NS	16	21	NS	2.00
Orcuttia viscida	aquatic	23	7	**	12	10	NS	1.40
	floating	37	8	**	7	15	NS	1.72
	terrestrial	55	119	**	37	75	**	1.61
Orcuttia california	aquatic	12	1	**	16	4	**	0.63
	floating	11	5	**	24	16	*	1.96
	terrestrial	67	128	**	73	106	**	1.92

chase, less than 10 % of the dark-fixed carbon remained in malic acid (Fig. 1). This pattern is inconsistent with the CAM pathway; *e.g.*, when similar studies are done with aquatic CAM plants such as *Isoetes howellii*, nearly all of the dark-fixed label remains in malic acid throughout the 12 h dark period (Keeley 1995).
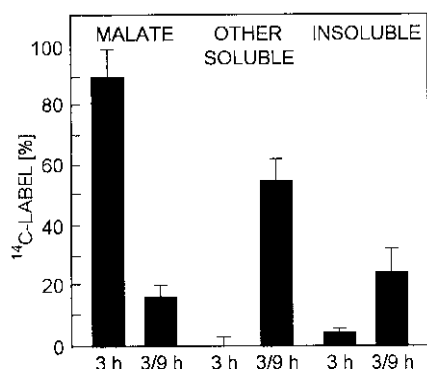

Fig. 1. Distribution of dark-fixed ^{14}C label after steady-state labeling for 3 h and after labeling for 3 h followed by a 9 h chase in ^{14}C-free medium for aquatic leaves of *Orcuttia viscida*. According to methods in Keeley and Sandquist (1991).

Thus, although having diel acid fluctuations, aquatic *Orcuttia* do not have CAM photosynthesis. As demonstrated for algae, dark fixation may play an anaplerotic role, perhaps for balancing intracellular reducing power (Turpin *et al.* 1990), to generate carbon substrates for nighttime nitrogen assimilation (Amory *et al.* 1991, Turpin *et al.* 1991) or recycling P$_i$ (Theodorou *et al.* 1991), as well as other functions.

Terrestrial leaves: Particularly surprising was the daytime increase in acidity and malic acid in terrestrial leaves of both *Orcuttia* species (Table 1). Incidental "taste-tests" of leaves revealed the surfaces to be acidic, and measurements on leaves with

surfaces lightly wiped with ethanol prior to testing showed no diel acidity fluctuation. These leaves possess multicellular glands where the acids appear to accumulate. Pulse-chase studies in the light showed that after 5 min chase, 3 % of the radiocarbon in acid-stable products was in surface secretions, and with chases of 1, 3, and 6 h this increased to 7, 7, and 9 %, respectively, indicating a fraction of the current photosynthate was transferred into these glands during the day.

This daytime acid accumulation on the leaf surface is not likely related to photosynthetic pathway but its role is far from clear. A similar pattern of daytime malate accumulation has been reported for desert plants (Willert *et al* 1984), although at substantially lower levels than reported here. The plants studied by Willert *et al.* (1984) were all C_3, and no mention was made of malic acid secretion on the leaf surface.

Chickpea (*Cicer arietinum*), a C_3 plant native to the Mid-East, on the other hand, has an external exudate of malic acid (van der Maesen 1972), but apparently no daytime acid accumulation. Santakumari *et al.* (1979) reported nighttime acid accumulation, but Winter (1981) found no acid accumulation, day or night. The malic acid exudate does not affect leaf reflectance (Koundal and Sinha 1981), but it does increase with water stress (Winter 1981).

Several authors have suggested that such secretions in chickpea are inhibitors to insect predation, although malic acid is not normally included in reviews of anti-herbivore plant secretions. This is a worthwhile hypothesis to account for these acids accumulating on terrestrial *Orcuttia* leaves. The arid conditions during normal *Orcuttia* development are highly conducive to herbivorous insect outbreaks (Mattson and Haack 1980), particularly when vernal pool green plants are surrounded by dried upland vegetation, greatly increasing their apparence to herbivores (Feeny 1976). The hypothesis that malic acid secretion may have an anti-herbivore role is supported by two independent reports of extensive grasshopper predation on *Tuctoria greenei* (which lack daytime acid accumulation and secretion, Table 1) while nearby *Orcuttia* was left untouched (Griggs 1980, Stone *et al.* 1988).

References

Amory, A.M., Vanlerberghe, G.C., Turpin, D.H.: Demonstration of both a photosynthetic and a nonphotosynthetic CO_2 requirement for NH_4^+ assimilation in the green alga *Selenastrum minutum*. - Plant Physiol. **95**: 192-196, 1991.

Crampton, B.: The grass genera *Orcuttia* and *Neostapfia*: A study in habitat and morphological specialization. - Madroño **15**: 97-110, 1959.

Ehleringer, J.R., Monson, R.K.: Evolutionary and ecological aspects of photosynthetic pathway variation. - Annu. Rev. Ecol. Syst. **24**: 411-439, 1993.

Feeny, P.P.: Plant apparency and chemical defense. - Rec. Adv. Phytochem. **10**: 1-40, 1976.

Griggs, F.T.: Life history strategies of the genus *Orcuttia* (*Gramineae*). - In: Jain, S.K. (ed.): Vernal Pools, Their Ecology and Conservation. Pp. 57-63. University of California, Davis 1976.

Griggs, F.T.: Population studies in the genus *Orcuttia* (*Poaceae*). - Ph.D. Thesis. University of California, Davis 1980.

Gutmann, I. , Wahlefeld, A.W.: L-malate: determination with malate dehydrogenase and NAD. - In: Bergmeyer, H.U. (ed.): Methods of Enzymatic Analysis. Vol. 4. Pp. 1585-1589. Academic Press,

New York 1974.

Holland, R.F.: What constitutes a good year for an annual plant? Two examples from the Orcuttieae. - In: Elias, T.S. (ed.): Proceedings of a California Conference on the Conservation and Management of Rare and Endangered Plants. Pp. 329-333. California Native Plant Society, Sacramento 1980.

Holland, R.F., Jain, S.K.: Vernal pools. - In: Barbour, M.B., Major, J. (ed.): Terrestrial Vegetation of California. Pp. 515-533. John Wiley & Sons, New York 1977.

Keeley, J.E.: Anaerobiosis and fungi in the germination of two vernal pool grasses. - Amer. J. Bot. **75**: 1086-1089, 1988.

Keeley, J.E.: Aquatic CAM photosynthesis. - In: Winter, K., Smith, J.A.C. (ed.): Crassulacean Acid Metabolism, Biochemistry, Ecophysiology and Evolution. Pp. 281-295. Springer-Verlag, New York 1995.

Keeley, J.E., Sandquist, D.R.: Diurnal photosynthesis cycle in CAM and non-CAM seasonal-pool aquatic macrophytes. - Ecology **72**: 716-727, 1991.

Koch, K., Kennedy, R.A.: Characteristics of Crassulacean acid metabolism in the succulent C$_4$ dicot, *Portulaca oleracea* L. - Plant Physiol. **65**: 193-197, 1980.

Koundal, K.K., Sinha, S.K.: Malic acid exudation and photosynthetic characteristics in *Cicer arietinum*. - Phytochemistry **20**: 1251-1252, 1981.

Mattson, W.J., Haack, R.A.: The role of drought in outbreaks of plant-eating insects. - BioScience **37**: 110-118, 1980.

Reeder, J.R.: The tribe *Orcuttieae* and the subtribes of the *Pappophoreae* (*Gramineae*). - Madroño **18**: 18-28, 1965.

Reeder, J.R.: Systematics of the tribe *Orcuttieae* (*Gramineae*) and the description of a new segregate genus, *Tuctoria*. - Amer. J. Bot. **69**: 1082-1095, 1982.

Reeder, J.R., Reeder, C.G.: Rediscovery of *Orcuttia fragilis* (*Gramineae*). - Phytologia **46**: 341-343, 1982.

Santakumari, M., Reddy, C.S., Reddy, A.R.C., Das, V.S.R.: CAM behavior in a grain legume, chickpea. - Naturwissenschaften **66**: 54-55, 1979.

Šesták, Z., Čatský, J., Jarvis, P.G. (ed.): Plant Photosynthetic Production. Manual of Methods. - Dr W. Junk, The Hague 1971.

Stone, R.D., Davilla, W.B., Taylor, D.W., Clifton, G.L., Stebbins, J.C.: Status survey of the grass tribe *Orcuttieae* and *Chamaesyce hooveri* (*Euphorbiaceae*) in the Central Valley of California. - Technical Report. U.S. Fish and Wildlife Service, Office of Endangered Species, Sacramento 1988.

Teeri, J.A., Stowe, L.G.: Climatic patterns and the distribution of C$_4$ grasses in North America. - Oecologia **23**: 1-12, 1976.

Theodorou, M.E., Elrifi, I.R., Turpin, D.H., Plaxton, W.C.: Effects of phosphorus limitation on respiratory metabolism in the green alga *Selenastrum minutum*. - Plant Physiol. **95**: 1089-1095, 1991.

Turpin, D.H., Vanlerberghe, G.C., Amory, A.M., Guy, R.D.: The inorganic carbon requirements for nitrogen assimilation. - Can. J. Bot. **69**: 1139-1145, 1991.

Turpin, D.H., Weger, H.G., Smith, R.G., Plaxton, W.C., Lin, M.: Interactions between respiration and nitrogen assimilation during photosynthesis. - In: Ullrich, W.R., Rigano, C., Fuggi, A., Aparicio, P.J. (ed.): Inorganic Nitrogen in Plants and Microorganisms. Pp. 124-130. Springer-Verlag, New York 1990.

van der Maesen, L.J.G.: *Cicer* L., a Monograph of the Genus, with Special Reference to the Chickpea (*Cicer arietinum* L.), its Ecology and Cultivation. - H. Veenman & Zonen, Wageningen 1972.

Watson, L., Dallwitz, M.J.: The Grass Genera of the World. - CAB International, Wallingford 1992.

Willert, D.J. von, Brinckmann, E., Eller, B.M., Scheitler, B.: Water loss and malate fluctuations during the day for plants in the southern Namib desert. - Oecologia **61**: 393-397, 1984.

Winter, K.: CO$_2$ and water vapour exchange, malate content and δ^{13}C value in *Cicer arietinum* grown under two water regimes. - Z. Pflanzenphysiol. **101**: 421-430, 1981.