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The effect of leaf age on gas exchange and malate accumulation in C_3-CAM plant *Marrubium frivaldszkyanum* (*Lamiaceae*)

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Abstract

For the first time the expression of C_3 and CAM in the leaves of different age of *Marrubium frivaldszkyanum* Boiss. is reported. With increasing leaf age a typical C_3 photosynthesis pattern and high transpiration rate were found. In older leaves a shift to CAM occurred and the 24-h transpiration water loss decreased. A correlation was established between leaf area and accumulation of malate. Water loss at early stages of leaf expansion may be connected with the shift to CAM and the water economy of the whole plant.

Additional key words: C_3-CAM shift; Crassulacean Acid Metabolism; leaf expansion; net photosynthetic rate; transpiration rate; water use efficiency.

Introduction

The Crassulacean Acid Metabolism (CAM) is usually regarded as a metabolic adaptation of very specialised plants to long-term drought periods (Kluge and Ting 1978). CAM is widespread among plants from very different habitats, including submerged water plants (Keely 1996), and covers large diversity of gas exchange patterns (Griffiths 1988, Smith and Winter 1996). These plants optimise their carbon metabolism and gas exchange very flexibly in response to changes in environment and development (Wagner and Larcher 1981, Zima and Šesták 1985, Lee and Griffiths 1987, Schmitt *et al.* 1988, Herppich *et al.* 1992). Thus, the mode of photosynthesis can be changed from approximately that of a C_3 plant to an exclusive night-time CO_2 uptake with or without greatly affecting nocturnal accumulation of malic acid (Winter and Smith 1996).

One of the species showing a high plasticity of CAM is *Marrubium frivaldszkyanum* (*Lamiaceae*). In this family only few CAM species have been noted (Kluge and Ting 1978, Herppich and Herppich 1996) and they all belong to the genus *Plectranthus*. In *M. frivaldszkyanum*, which is Bulgarian local endemic, patterns of CAM and diurnal gas exchange respond very flexibly to environmental and developmental signals (Markovska *et al.*, unpublished). This species survived as a tertiary relict on the Balkan Peninsula. It is a typical chasmophyte that inhabits sun exposed chiefly limestone slopes up to an altitude of more than 1 800 m. Its natural habitats are characterised by high mean annual temperatures (about 20 °C), frequent mist and dew fall in the early morning and in the evening, and high periodic rainfalls (annual mean 600 mm). Episodic droughts and high temperatures and irradiance may be the major environmental constraints affecting gas exchange pattern.

There is no information about photosynthetic performance and CAM in the genus *Marrubium*. Therefore, controlled environment experiments with high mountain C_3-CAM intermediate *M. frivaldszkyanum* were done to elucidate the relationships between leaf expansion, expression of CAM, and water economy of the whole plant.

Materials and methods

Fifteen plants of *Marrubium frivaldszkyanum* Boiss., collected from the region of Batchkovo Monastery in the biosphere reserve "Cervenata stena" (Bulgaria), were propagated from cuttings in plastic pots containing mixture of forest soil and gravel and watered every 3 d in the greenhouse. Four weeks prior to experiments the pots

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Abbreviations: CAM – Crassulacean Acid Metabolism; DM – dry mass; *E* – transpiration rate; LA – leaf age class; P_N – net photosynthetic rate; WC – leaf water content; WCA – water content per leaf area; WUE – water use efficiency, Δmal – amount of accumulated malate.

with the plants were transferred to a climate chamber with a 12-h photoperiod (from 08:00 to 20:00), day/night temperature 32/28 °C, relative humidity 60/65 %, photon irradiance ~400 µmol m^{-2} s^{-1}. The growing conditions in the chamber corresponded to those in the natural habitat in the middle of vegetation period of the plants.

Net photosynthetic (P_N) and transpiration (E) rates at different distances from the meristem, representing several leaf ages (24-30 replicates), were measured with a portable photosynthetic system *LI-6000* (*LI-COR*, Lincoln, USA). The studies were accomplished in three consecutive years (1997-1999). P_N and E were integrated for 12 h to obtain values for both the light and dark periods. Water use efficiency (WUE) was calculated as P_N/E.

Leaf malic acid content and water status: Twelve leaf ages from 15 equally treated plants, *ca.* 1 and 10 h after irradiation, were immediately copied onto paper, and their fresh and dry masses (80 °C to constant mass) were determined. Leaf area was measured with a planimeter on leaf copies. Leaf water content (WC) was calculated from the difference between fresh and dry masses, and related to leaf area (WCA). Using hot water extracts of the dried leaves, malic acid contents were determined enzymatically (Mollering 1974). Accumulated malate (Δmal) was calculated as the difference between values at the beginning and the end of the photoperiod. CO_2 recycling was calculated indirectly as the difference between Δmal and the integrated night-time P_N (Borland and Griffiths 1989). The amount of water potentially saved through recycling of respiratory CO_2 was estimated by dividing CO_2 fixed at night by daytime WUE and was expressed as a percentage of daytime transpiration water loss (Martin *et al.* 1988).

Results

The leaves of different ages were characterised by a steady increase in leaf area and thickness (Fig. 1*A*). Leaf area was chosen as appropriate for expressing the actual status of leaf expansion and thus maturity. The water content (WC) was practically independent on leaf age class (LA, Fig. 1*B*). WCA and DM/leaf area increased markedly with the maturation of the leaves (Fig. 1*B*). WCA is more appropriate to characterise plant water status than the water content.

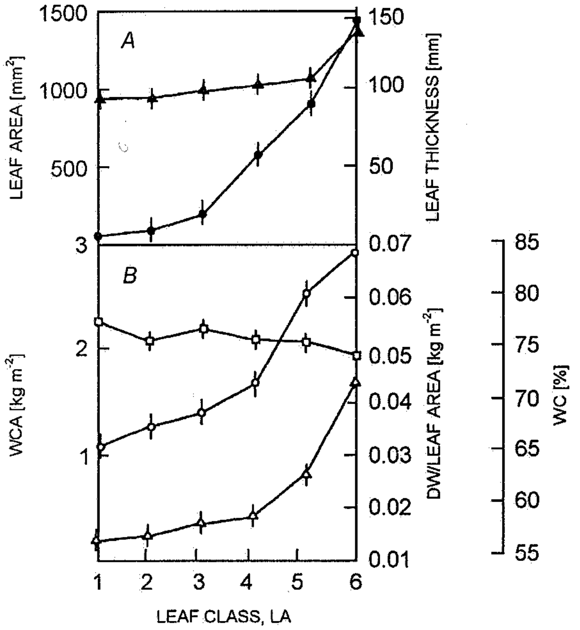

Fig. 1. Area (●) and thickness (▲) (*A*), water content (WCA, ○), dry mass per leaf area (DM, Δ), and water content (WC, □) (*B*) of different leaf age classes of *M. frivaldszkyanum*. Means ± S.E., $n = 24\text{-}30$.

In the youngest leaves, marked net CO_2 uptake was found during light period (Fig. 2*A*); only towards the end of the dark period a small CO_2 dark fixation occurred. The leaves of age classes 2, 3, and 4 showed different P_N during the light and dark periods (LA2, LA3, LA4, Fig. 2*B,C,D*). In the older classes, a shift of the gas exchange pattern from predominantly C_3 photosynthesis to CO_2 dark fixation and thus to a progressive expression of a typical CAM was observed (LA5, LA6, Fig. 2*E,F*). The changes in CO_2 exchange pattern resulted in an increase of the daily carbon gain with the leaf age (Fig. 3*A*). Concomitant with the enhancement of CO_2 dark fixation during leaf expansion, the 24-h transpiration water loss decreased (LA6, Fig. 3*B*). As a consequence, WUE was improved substantially in leaves of age class 6 (Fig. 3*C*). The nocturnal accumulation of malate (Δmal), occurring as a result of recycling of respiratory CO_2 (CAM-cycling), was higher in the leaves of the same class (Table 1). With increasing leaf age the percentage of recycled CO_2 decreased while potential conservation of water increased from 10 to 141 % of total daytime E (Table 1).

Discussion

P_N (Fig. 2*A,F*) and malate accumulation (Table 1) proved that inducible CAM (C_3-CAM, Griffiths 1988) operates in the leaves of older classes of *M. frivaldszkyanum*. For many CAM plants the induction of CAM depends upon leaf tissue maturity (Jones 1975, Winter *et al.* 1978, Amagasa 1982, Eller *et al.* 1988). We found three different stages in the expression of the CO_2 exchange pattern as associated with leaf maturation. In the first phase (LA1, Fig. 2*A*) a weak CO_2 dark fixation indicates that in very young leaves the enzymatic complement necessary for CAM is functional (unpublished values). The nocturnal malate accumulation in the young leaves of *M. frivaldszkyanum* was low (Table 1). Presumably a limiting factor for the activity of CAM could be the smaller storage capacity for malate of the cells in young leaves. A highest proportion of respiratory CO_2 is recycled during the night as a result of the operation of CAM (CAM-cycling). Water potentially saved through recycling amounted to a mean of 10 % of daytime water loss (Table 1). The youngest leaves are covered with dense trichomes on both surfaces (Stojanov *et al.* 1967). This also could contribute to the reduction of the water loss by increasing boundary layer resistance (Wuenscher 1970).

Table 1. Amount of malate accumulated overnight, Δmal [mol m^{-2} d^{-1}], percentage of recycled CO_2, water saved [mol m^{-2} d^{-1}], and percentage of daytime water loss of the leaf age classes of *M. frivaldszkyanum*. See Materials and methods for details.

Leaf age	Δmal	Recycling	Water saved	Water loss
LA1	0.088	77.3	4.4	10.4
LA2	0.099	69.7	8.6	20.1
LA3	0.111	70.3	6.3	20.2
LA4	0.112	61.6	9.3	33.8
LA5	0.114	56.1	18.5	68.8
LA6	0.218	22.5	17.8	141.3

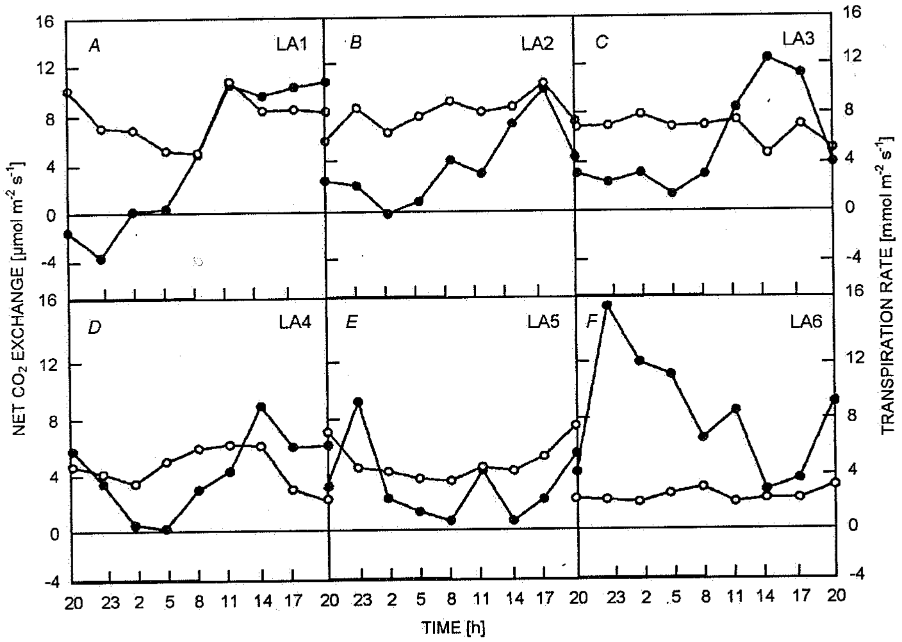

Fig. 2. Diurnal changes in the net CO_2 exchange (●) and transpiration (○) rates of different leaf age classes of *M. frivaldszkyanum*. Each point is the mean of 24-30 replicates with S.E.<10 % of mean (not shown). The solid bar on the x-axis indicates the period of darkness.

In the second phase of leaf development (LA2, LA3, LA4) the contribution of dark CO_2 fixation to the carbon balance increased slightly (Fig. 3*A*, columns *2*, *3*, *4*). Dark fixation in LA4 represented only 25 % of the carbon acquired during 24 h. Compared to constitutive CAM plants, such as cacti, in which up to 99 % of the carbon is acquired through dark fixation (Nobel 1988), CAM obviously is not an important pathway of biomass accumulation in the second phase of leaf development. However, since DM/area is not affected significantly (Fig. 1*B*), CAM may have contributed to the maintenance of biomass.

In the third phase of development, the WUE increased reaching a maximum daytime value of 9.5 mmol(CO_2) mol^{-1}(H_2O) for LA6. For CAM plants, WUE values range between 6-20 mmol(CO_2) mol^{-1}(H_2O) (Eller *et al.* 1988). During the dark period the WUE is as high as 13.8 mmol(CO_2) mol^{-1}(H_2O) due to the high CO_2 dark fixation of 0.17 mol m^{-2}. Hence, in the course of development, the CO_2 dark fixation capacity reaches its maximal value soon after the leaves are fully expanded. The percentage of recycled CO_2 decreased with increasing leaf age, but potential conservation of water varied from 10 to 141 % of total daytime transpiration (Table 1).

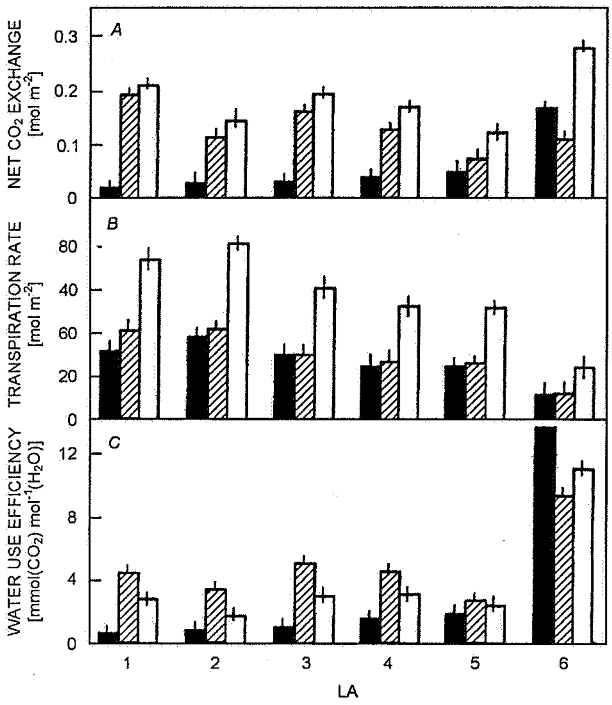

Fig. 3. Integrated total net CO_2 exchange (*A*), integrated transpiration rate (*B*), and water use efficiency (*C*) during daytime (*hatched columns*), night-time (*dark columns*), and 24 h period (*open columns*) of different leaf age classes of *M. frivaldszkyanum.*

The shift from C_3 photosynthesis to CAM is induced by environmental conditions and/or is a function of leaf maturity (Willert *et al.* 1976, Winter *et al.* 1978, Holthe *et al.* 1987). We propose that at early stages of leaf development the increase of transpiration water loss induces in *M. frivaldszkyanum* the shift from C_3 photosynthesis to CAM. Transpiration water loss per leaf (Fig. 4) reached 0.45 g leaf^{-1} which coincides with the shift from nocturnal CO_2 to fixation of CO_2 (Fig. 2*E*) and increases of the WCA in phase III (Fig. 1*B*). Through restriction of transpiration water loss per leaf to a low level, the leaf can further increase its water storage capacity (Fig. 3, LA3 to LA6). This can be achieved at an early stage of leaf expansion though high recycling of respiratory CO_2. Our findings agree with the suggestion of Martin *et al.* (1988) that CAM-cycling is a much more efficient mechanism of saving water than CAM. Its functioning is of primary importance for the survival of the youngest leaves during sudden drought since it is directly associated with their water balance. As drought frequently occurs in the *M. frivaldszkyanum* habitat, the creation of a potential reserve of water is the only chance of avoiding lethal water stress and might help in the preservation of physiological activity.

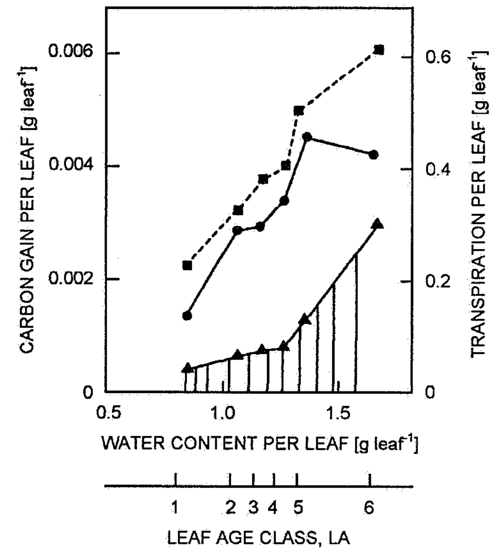

Fig. 4. Transpiration water loss (*solid line*) and carbon gain (*dashed line*) per 24 h and per leaf, and nocturnal carbon gain (*hatched area*) per leaf in relation to leaf age class and water content per leaf of *M. frivaldszkyanum.*

References

Amagasa, T.: The influence of leaf age on the diurnal changes of malate and starch in the CAM plant *Kalanchoe daigremontiana* Hamet et Perr. – Z. Pflanzenphysiol. **108**: 93-96, 1982.

Borland, A.M., Griffiths, H.: The regulation of citric acid accumulation and carbon recycling during CAM in *Ananas comosus*. – J. exp. Bot. **40**: 53-60, 1989.

Eller, B.M., Ruess, B.R., Sharma, S.: Carbon gain, water conservation and, expression of CAM during leaf development of *Senecio medley-woodii*. – J. Plant Physiol. **133**: 304-309, 1988.

Griffiths, H.: Crassulacean acid metabolism: A re-appraisal of physiological plasticity in form and function. – Adv. bot. Res. **15**: 43-92, 1988.

Herppich, W.B., Herppich, M.: Ecophysiological investigations on plants of the genus *Plecthranthus* (fam. *Lamiaceae*) native to Yemen and southern Africa. – Flora **191**: 401-408, 1996.

Herppich, W.B., Herppich, M., Willert, D.J. von: The irreversible C_3 to CAM shift in well-watered and salt-stressed plants of *Mesembryanthemum crystallinum* is under strict ontogenetic control. – Bot. Acta **105**: 34-40, 1992.

Holthe, P.A., Sternberg, L. da S.L., Ting, I.P.: Developmental control of CAM in *Peperomia scandens*. – Plant Physiol. **84**: 743-747, 1987.

Jones, M.B.: The effect of leaf age on leaf resistance and CO_2 exchange of the CAM plant *Bryophyllum fedtschenkoi*. – Planta **123**: 91-96, 1975.

Keely, 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, Berlin – Heidelberg - New York 1996.

Kluge, M., Ting, I.P.: Crassulacean Acid Metabolism: Analysis of an Ecological Adaptation. – Springer-Verlag, Berlin – Heidelberg – New York 1978.

Lee, H.S.J., Griffiths, H.: Induction and repression of CAM in *Sedum telephium* L. in response to photoperiod and water stress. – J. exp. Bot. **38**: 834-841, 1987.

Martin, C.E., Higley, M., Wang, W.-Z.: Ecophysiological significance of CO_2 recycling via Crassulacean acid metabolism in *Tallinum calycinum* Engelm. (*Portulacaceae*). – Plant Physiol. **86**: 562-568, 1988.

Mollering, H.: Malatbestimmung. – In: Bergmeyer, H.U. (ed.): Methoden der enzymatischen Analyse. Vol. 2. Pp. 1636-1639. Verlag Chemie, Weinheim 1974.

Nobel, P.S.: Environmental Biology of Agaves and Cacti. – Pp. 171-172. Cambridge University Press, Cambridge – New York – New Rochelle – Melbourne – Sydney 1988.

Schmitt, A.K., Lee, H.J.S., Lüttge, U.: The response of the C_3-CAM tree *Clusia rosea* to light and water stress. I. Gas exchange characteristics. – J. exp. Bot. **39**: 1581-1590, 1988.

Smith, J.A.C., Winter, K.: Taxonomic distribution of CAM. – In: Winter, K., Smith, J.A.C. (ed.): Crassulacean Acid Metabolism: Biochemistry, Ecophysiology and Evolution. Pp. 427-436. Springer-Verlag, Berlin – Heidelberg – New York 1996.

Stojanov, N., Stefanov, B., Kitanov, B.: Flora na Bulgaria II. [Bulgarian Flora II.] – Pp. 356-357. Nauka Izkustvo, Sofia 1967. [In Bulg.]

Wagner, J., Larcher, W.: Dependence of CO_2 gas exchange and acid metabolism of the alpine CAM plant *Sempervivum montanum* on temperature and light. – Oecologia **50**: 88-93, 1981.

Willert, D.J. von, Kirst, G.O., Treichel, S., Willert, K. von: The effect of leaf age and salt stress on malate accumulation and phosphoenolpyruvate carboxylase activity in *Mesembryanthemum crystallinum*. – Plant Sci. Lett. **7**: 341-346, 1976.

Winter, K., Lüttge, U., Winter, E., Troughton, J.H.: Seasonal shift from C_3 photosynthesis to Crassulacean acid metabolism in *Mesembryanthemum crystallinum* growing in its natural environment. – Oecologia **34**: 225-237, 1978.

Winter, K., Smith, J.A.C.: An introduction to Crassulacean Acid Metabolism. Biochemical principles and ecological diversity. – In: Winter, K., Smith, J.A.C. (ed.): Crassulacean Acid Metabolism: Biochemistry, Ecophysiology and Evolution. Pp. 1-13. Springer-Verlag, Berlin – Heidelberg – New York 1996.

Wuenscher, J.E.: The effect of leaf hairs of *Verbascum thapsus* on leaf energy exchange. – New Phytol. **69**: 65-73, 1970.

Zima, J., Šesták, Z.: Carbon fixation pathways, their enzymes and products during leaf ontogeny. – In: Šesták, Z. (ed.): Photosynthesis during Leaf Development. Pp. 145-156. Academia, Praha; Dr W. Junk Publ., Dordrecht – Boston – Lancaster 1985.