Capacity of Enzymes of the Euphorbiacea *Aleurites montana* Involved in CO_2-Fixation, Compared to Plants Having C_3-, C_4- and *Crassulacean* Acid Metabolism

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Z. Naturforrsch. **55c**, 383–391 (2000); received March 2/April 7, 2000

Dedicated to Professor Wilhelm Menke on the occasion of his 90th birthday

Aleurites montana, Phosphoenolpyruvate Carboxylase, Malic Enzyme, Malate Dehydrogenase, Ribulose Bisphosphate Carboxylase

Capacities of phosphoenolpyruvate carboxylase (PEP-Co), ribulose bisphosphate carboxylase (Rubisco), $NADP^+$ malic enzyme (ME) and of malate dehydrogenase (MDH) were measured in the Euphorbiacea *Aleurites montana*, grown under 700 ppm CO_2 for four weeks prior to enzyme extraction. For comparison *Bryophyllum daigremontiana* (CAM), *Saccharum officinarum* (C_4) and *Capsicum frutescens* (C_3) were treated in the same way. PEP-Co capacity of *Aleurites* was in the range of 12-, that of *Capsicum* approx. 26 nmol × min^{-1} × mg $protein^{-1}$, without significant influence of the light period or CO_2-treatment. In contrast, the activity of the enzyme from *Saccharum* was, depending on the duration of light, 160- respectively 96 times higher than that of the tung-oil tree. In *Bryophyllum* a rather low activity in the morning was increased during the day to approx. 230 nmol × min^{-1} × mg $protein^{-1}$ in plants grown in the greenhouse and to approx. 115 nmol × min^{-1} × mg $protein^{-1}$ in those from the growth chamber. Malate was hardly detectable in extracts of *Aleurites*, whereas it was high in *Bryophyllum*, depending on the light period. The ratio of average PEP-Co to Rub-Co capacity was high for the CAM-plant (20:1), somewhat lower for sugar cane (10:1), but almost at equality for *Aleurites* (0.9:1) and chilli (0.8:1). For the $NADP^+$ malic enzyme, low capacity (20 to 28 nmol × min^{-1} × mg $protein^{-1}$) was found for *Aleurites* and for *Capsicum*, whereas it was 10 to 17 times higher in *Saccharum*. In *Bryophyllum*, the activity was up to 80 nmol × min^{-1} × mg $protein^{-1}$, dependent on light period. MDH capacity was extremely high in all plants investigated. Highest rates (10–20 μmol × min^{-1} × mg $protein^{-1}$), were obtained for *Bryophyllum*, followed by sugar cane and *Capsicum* with 5–8 μmol × min^{-1} × mg $protein^{-1}$. Again, the lowest capacity was found in extracts of *Aleurites* with approx. 1.3 to 1.6 μmol × min^{-1} × m $protein^{-1}$. Thus, in *Aleurites montana* no indication for C_4- or *Crassulacean* acid metabolism was obtained. Therefore, the earlier observed very efficient uptake of CO_2 cannot be explained by a high expression of the PEP-Co protein, known to occur in CAM- and C_4-plants.

Introduction

The Chinese tung-oil tree represents an important crop for the production of seed-oil for the Chinese lacquer industry (Fang and Que, 1981). Increasing contents of CO_2 and of SO_2, resulting from the burning of fossil fuels by the expanding industries and the increased private energy demands (Grayson, 1989), endanger the particularly sensitive cultures of the tree in China and thereby the supply of the country with the important seed-oil of a highly specific composition (Hopkins and Chisholm, 1962; Fang *et al.*, 1985; Radunz *et al.*, 1998).

In earlier investigations of our group, predicted changes in the composition of the atmosphere were simulated by growing young trees in SO_2- or CO_2-enriched air, respectively. It has been shown

Abbreviations: EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, [ethylenebis (oxy-ethylenenitrilo)] tetraacetic acid; PEG 8000, polyethylene glycol; DTT, 1,4-dithiothreitol; PEP-Co, phosphoenolpyruvate carboxylase; Rubisco, ribulose bisphosphate carboxylase; MDH, malate dehydrogenase; TRIS, tris-(hydroxymethyl)-aminomethane; LDH, lactate dehydrogenase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ME, malic enzyme; CAM, *Crassulacean* acid metabolism; PVP, polyvinylpyrrolidone; PMSF, phenylmethylsulfonyl fluoride.

0939–5075/2000/0500–0383 $ 06.00 D

that not only the total oil production is changed under the influence of the air pollutants and the increased CO_2-content, but also the composition of the plasmalemma and of other membranes, such as tonoplast, endoplasmatic reticulum, mitochondrial – and thylakoid membranes (He *et al.*, 1996 a; He *et al.*, 1997). The growth rate was substantially better (40%) at 700 ppm CO_2, while the content of soluble protein, soluble sugars and total chlorophyll was decreased (He *et al.*, 1996b).

Gas exchange measurements by mass spectrometry for photosynthetic $^{16}O_2$-evolution and for total $^{18}O_2$-uptake of *Aleurites* leaves in the light, show low rates of an oxygen uptake during photosynthesis in plants that have been grown in normal air (350 ppm), which is totally suppressed in CO_2-treated plants if the measurement of the same plants is carried out at high CO_2-content in air (700 ppm) (He *et al.*, 1998). Hence, photorespiration is low but present. The ratio of O_2 evolved to CO_2 fixed in the light in normal air, gave a value of 0.5, in contrast to approx. 1, usually found in C_3-plants like tobacco. Elevated CO_2-concentrations during plant growth even led to a further decline in the O_2-/CO_2-ratio and may partially explain the stimulatory effect of CO_2 on growth of this plant (He *et al.*, 1995). From this a highly efficient CO_2-uptake system could be expected for the tree. Although belonging to the family of Euphorbiaceae, *Aleurites* does not look like a typical succulent plant, as many members of this group. Assuming, that part of the CO_2 fixed is channelled into a metabolic pathway without concomitant O_2-evolution, the tung-oil tree was suspected to perform some type of CAM- or C_4-photosynthesis. Also it appeared reasonable to assume a connection between CO_2-fixation and biosynthesis of fatty acids via the malate metabolism, which has been demonstrated in *Ricinus communis*, another Euphorbiacean plant (Sangwan *et al.*, 1992; Smith *et al.*, 1992) and in the C_4-plant *Zea mays* (Preis *et al.*, 1994).

In the present study we therefore followed the fate of CO_2, by investigating enzyme capacities, known to be affected by the respective type of the CO_2-fixation pathway (e.g. Kluge and Ting, 1978; Osmond, 1978; Dittrich, 1979; Chollet *et al.*, 1996). Together with the tung-oil tree, typical, well known members of the different CO_2-fixation types of plants, such as *Bryophyllum daigremontiana* (CAM), sugar cane *Saccharum officinarum* (C_4) and chilli *Capsicum frutescens* (C_3), were tested for capacities of phosphoenolpyruvate carboxylase (PEP-Co), ribulose bisphosphate carboxylase (Rubisco), malate dehydrogenase (MDH) and malic enzyme (ME) in plants grown in normal air and in an atmosphere containing an increased CO_2-content of 700 ppm. As additional indicators for the assumed *Crassulacean* acid metabolism, the malate content and the pH of protein free cell extracts were determined.

Material and Methods

Growth conditions

Aleurites montana, *Bryophyllum daigremontiana*, *Saccharum officinarum* and *Capsicum frutescens* were grown in a greenhouse under natural light and temperature conditions during summer 1999 and additionally in climatized growth chambers. *Aleurites* seeds were obtained from the Central South Forestry University, Zhuzhou, Hunan. *Capsicum* plants were obtained from the local market, *Bryophyllum* and *Saccharum* originated from the collection of the institute (Bielefeld University). For enzymatic analysis plants were transferred to an automatic fully climatized growth chamber, with a light/dark cycle of 14/10 hours at a day temperature of 26 °C, a night temperature of 22 °C and 60% relative humidity. Light periods started at 7 a.m. and ended at 9 p.m.. In order to obtain reliable data for Rubisco capacity, plants had to be exposed to the light for at least 2 hours, to overcome the dark induced binding of the inhibitor 2-carboxy arabinitol-1-phosphate (Besford, 1984; Gutteridge *et al.*, 1986; Besford *et al.*, 1990). For this reason, samples were collected after 2 hours (9 a.m.) and after 7 hours (2 p.m.) of illumination. Comparable material was used for gas exchange measurements (He *et al.*, 1998).

In order to study the influence of elevated CO_2, plants were grown for four weeks in glass compartments in the same growth chamber and gassed with air containing 700 ppm CO_2, using the set-up described by Schmid *et al.* (1981) and by Ishii and Schmid (1982). Control plants were grown in normal air during the same time period in the same growth chamber but for comparison also in a greenhouse.

Preparation of plants for enzymatic analysis

Discs of 2 cm in diameter were cut from leaves of the middle region of the plants and extracted under cooling with liquid nitrogen by grinding with sand in a mortar. The extraction buffer used was prepared as described for *Ricinus communis* with slight modifications according to Sangwan *et al.* (1992). For each preparation 3 leaf discs were homogenized in 3 ml of ice-cold 25 mM K-phosphate buffer pH 7.0, containing 1 mM EDTA, 1 mM EGTA, 25 mM NaF, 5 mM thiourea, 5 mM malate, 0.1% (v/v) Triton X-100, 20% (v/v) glycerol, 10 mM $MgCl_2$, 4% (w/v) PEG-8000, 1% (w/v) polyvinylpyrrolidone (PVP) (Roth, Karlsruhe, Germany), PMSF and dithiothreitol (DTT) (2 mM each) were added from stock solutions directly before extraction. Other buffers tested yielded lesser enzyme activities, due to unidentified inhibitory factors or precipitations in the extracts.

The homogenates were centrifuged for 20 min at 20000×*g* (4 °C, Sorvall RC-5 Superspeed Refrigerated Centrifuge). The resulting supernatants (crude extracts) were used for determination of the enzyme activities.

Enzyme assays

All enzyme assays were conducted at saturating substrate and cofactor concentrations. Maximum *in vitro* activity (was taken as capacity) was proportional to the amount of extract added and remained linear with respect to time.

PEP-Co (EC 4.1.1.31) was measured in a coupled test (Meyer *et al.*, 1988; Grotjohann and Hippe, 1993), by following the oxidation of NADH by MDH, resulting from the reduction of oxaloacetate produced by PEP-Co. In order to detect pyruvate resulting from decarboxylated oxaloacetate in the test system, lactate dehydrogenase was added. The assay mixture contained TRIS-HCl buffer pH 8.5, $NaHCO_3$ 33.4 mM, $MgCl_2$ 6.7 mM, NADH 0.2 mM, MDH 3.5 U/ml test, LDH 8 U/ml test, crude extract 15–250 µg protein/ml test (depending on the plant species). The reaction was started with 5.4 mM phosphoenolpyruvate.

Rubisco (EC 4.1.1.39) activity was measured according Besford (1984), using a method based on Lilley and Walker (1975), in which the reduction of newly formed 3-phosphoglycerate was coupled to NADH oxidation and monitored at 334 nm (25 °C). The reaction mixture contained: 50 mM HEPES buffer pH 8.0 containing 20 mM $MgCl_2$, 25 mM $KHCO_3$ and 0.2 mM EDTA, 5 mM DTT, 0.35 mM ATP, 0.2 mM NADH, 3.5 mM phosphocreatine, phosphoglycerate kinase 6.5 U/ml test, creatine kinase 10 U/ml test, glyceraldehyde 3-phosphate dehydrogenase 5 U/ml test, crude extract corresponding to 150–250 µg protein/ml test (depending on the plant species). The reaction was started with 0.114 mM ribulose 1,5-phosphate.

Activity of ME (EC 1.1.1.39) was monitored by measuring the reduction of $NADP^+$ at 334 nm (25 °C) in a test system according to Colombo *et al.* (1997), containing 50 mM TRIS-HCl pH 7.0, $NADP^+$ 0.5 mM, $MgCl_2$ 10 mM, crude extract corresponding to 150–250 µg protein/ml test (depending on the plant species). The reaction was started with 8 mM malate.

MDH (EC 1.1.1.37) was measured according to Bergmeyer (1974), by following the oxidation of NADH resulting from the reduction of oxaloacetate by the enzyme. The assay mixture contained 100 mM K-Na phosphate buffer pH 7.5, NADH 0.2 mM, crude extract corresponding to 7.5–12.5 µg protein/ml test. The reaction was started with 0.5 mM oxaloacetate.

All auxiliary enzymes were purchased from Roche Diagnostics GmbH (Mannheim).

Determination of malate content and of pH in leaf extracts

For each determination leaves corresponding to 1.5 g fresh weight (fw) were cut and transferred immediately into glass tubes containing 10 ml of boiling water. After disruption for 60 sec. in a warring blender and extraction in a boiling water bath for 5 min, the homogenate was filtered through a Büchner funnel and filled up with distilled water to a total volume of 25 ml. These extracts were used for the determination of the pH and of the malate content (Boehringer test: TC L-Malic Acid no. 139068), by following the reduction of NAD^+ by MDH.

Soluble protein

Soluble protein in crude extracts was determined according to Lowry *et al.* (1951) using bovine serum albumine as reference.

Results and Discussion

Phosphoenolpyruvate carboxylase

PEP-Co activity was determined in extracts of the tung-oil tree, *Bryophyllum daigremontiana*, *Saccharum officinarum* and of *Capsicum frutescens*, grown in a greenhouse during summer, under natural conditions (Table I). From these plants, – having in all probability different CO_2-fixation pathways –, *Aleurites* showed low enzyme activity in the morning (12.7 nmol × min^{-1} × mg $protein^{-1}$), but also in the middle of the light period (13.6 nmol × min^{-1} × mg $protein^{-1}$). In contrast, PEP-Co activity in extracts of *Saccharum* (C_4), was about 160 times higher than in *Aleurites* at 9 a.m. and still approx. 96 time higher after 7 hours in the light. The values for the CAM-plant *Bryophyllum* were surprisingly low with 8.4 nmol × min^{-1} × mg $protein^{-1}$ in the morning but reached the value of 230.9 in the middle of the light period. This low capacity at the beginning of the daily light period and the comparatively high rate after 7 hours in the light, might rather be explained by different states of phosphorylation (Nimmo *et al.*, 1986; Jiao and Chollet, 1988, 1992), than by feed back inhibition of the enzyme by a high malate content in the morning. This has been shown to occur in *Bryophyllum* and *other* CAM-plants, under certain conditions (Kluge and Osmond, 1972; Willert *et al.*, 1979; Jones *et al.*, 1981; Winter, 1981). In order to preserve the highest possible PEP-Co capacity in our preparations, malate (5 mM) was required in the extraction buffer used, as has been shown for the enzyme of *Ricinus communis* by Sangwan *et al.* (1992). Moreover, it must be borne in mind, that the PEP-Co capacity in CAM-plants besides phosphorylation and the metabolic feedback inhibition mentioned above, is influenced by circadian rhythms (Morel and Queiroz, 1974; Queiroz and Morel, 1974).

PEP-Co activity in extracts of *Capsicum* is almost twice as high as in the tung-oil tree, but as has been shown for the latter, there is no significant influence of the light period (Table I). Summerizing, it can be stated that the PEP-Co capacities of all plant species grown in the greenhouse confirm the expected high level of the enzyme in the C_4- and the CAM-plant, whereas the C_3-type plant *Capsicum* shows low CO_2-fixation via this enzyme. The lowest activity of all was found for *Aleurites*, with only half the rate determined for chilli plants.

Experiments in an automatic fully climatized growth chamber, confirm the results obtained for the greenhouse plants. For *Aleurites* and *Capsicum* practically no significant changes of the comparably low PEP-Co activity could be detected; neither the light period, nor the CO_2-treatment had any influence. The activity of the enzyme in *Bryophyllum* after 2 hours in light is almost the same as in the greenhouse (Table I). Only plants grown in air with 700 ppm CO_2 yield in the morning 69.2, in comparison to 8.5 nmol × min^{-1} × mg $protein^{-1}$ in the control plants. After 5 additional hours of light, PEP-Co activity is approx. 50% of that found in the comparable greenhouse plants, which is probably due to the lower light intensity and the thereby lower demand for CO_2. There is no other significant influence of an elevated CO_2-content in the atmosphere. In the afternoon, the *Bryophyllum* enzyme is 10 times higher in activity, than in extracts from the tung-oil tree. For technical reasons, sugar cane plants were not investigated in growth chamber experiments.

The obtained data for *Aleurites* PEP-Co do not support the presence of a *Crassulacean* type me-

Table I. Activity of phosphoenolpyruvate carboxylase in nmol × min^{-1} × mg $protein^{-1}$ for *Aleurites montana*, *Bryophyllum daigremontiana*, *Saccharum officinarum* and *Capsicum frutescens* at 9 a.m. (beginning of light period) and at 2p.m. (middle of light period).

Organism	Greenhouse		Growth chamber normal air		Growth chamber 700 ppm CO_2	
	9 a.m.	2 p.m.	9 a.m.	2 p.m.	9 a.m.	2 p.m.
Aleurites	12.7 ± 0.8	13.6 ± 0.9	9.2 ± 0.1	11.2 ± 0.2	10.8 ± 1.3	11.6 ± 1.3
Bryophyllum	8.4 ± 0.4	230.9 ± 8.4	8.5 ± 0.1	122.9 ± 6.2	69.2 ± 3.3	109.2 ± 5.1
Saccharum	2116 ± 185	1306 ± 48	Not determined			
Capsicum	23.6 ± 1.4	25.5 ± 2.7	25.8 ± 3.1	27.6 ± 2.1	24.4 ± 0.5	28.7 ± 2.0

Data are averages of 4–8 independent measurements (CO_2-content in air 350 ppm).

tabolism in this plant, as has been assumed, recently by us (He *et al.*, 1998). From gas exchange measurements, which showed a very efficient CO_2-uptake, in comparison to other plants, a high level of PEP-Co capacity, like in CAM- or C_4-plants, had been expected. The increased CO_2-fixation rate observed when plants had been grown in CO_2-enriched (700 ppm) atmosphere, characterized by a low O_2-evolution/CO_2-fixation ratio, as detected by mass spectrometry, can therefore not be explained, by an enhanced PEP-Co capacity. It should also be admitted that in extracts of *Aleurites*, no phosphoenolpyruvate carboxykinase activity could be detected, an enzyme, known to be involved in malate metabolism in some CAM plants (Dittrich, 1979).

The determination of the pH and of the malate content in hot water extracts of *Aleurites* and *Bryophyllum*, support the above conclusion, namely that of no CAM type CO_2-fixation in the tung-oil tree (Table II). In extracts of *Aleurites* malate was hardly detectable after 2 hours in the light, being still negligible after 5 additional hours in the light, while the pH was more or less constant. In contrast, the malate content in extracts of the CAM plant *Bryophyllum* was high in the morning and decreased during the day, whereas the pH was low in the morning and high in the afternoon (Table II). The Δ-malate (9.6 mg × g fw^{-1}) and the Δ-pH (1.44), calculated between 9 a.m. and 14 p.m., were biggest in extracts of CAM-plants from the greenhouse, probably due to higher light intensity there. The values are followed by those plants grown in an CO_2-enriched atmosphere in the growth chamber.

Ribulose bisphosphate carboxylase

In extracts of *Aleurites* the Rubisco capacity was in the range of 12–14 nmol × min^{-1} × mg $protein^{-1}$ in the morning (Table III). After 5 hours of light it dropped to 55% in plants from the greenhouse – probably due to the inhibitory light intensity –, whereas an increase of approx. 20% in plants from the growth chamber was observed. At 9 a.m. the Rubisco capacity of *Bryophyllum* was rather low (3.2–5.7 nmol × min^{-1} × mg $protein^{-1}$), but was enhanced to approx. 100% in plants from the greenhouse and to approx. 60% in those from the growth chamber, after 5 hours of light. The increase in Rubisco in the light seems to be the consequence of higher level of total Rubisco-protein, as well as of an enhanced specific activity, resulting from changed levels of inhibitory substances (e.g. Besford, 1984). No influence of the light period could be seen on the enzyme of *Saccharum* and of *Capsicum* (Table III).

Under our test conditions no influence of the elevated CO_2-atmosphere on Rubisco capacities of the different plant species studied, could be found. In long term experiments with high CO_2, an increase in growth rate (e.g. Downton *et al.*, 1980), but also a decrease in Rubisco capacity, as well as a decreased Rubisco protein content, has been described for several plant species (e.g. Wong, 1979; Besford *et al.*, 1990; Faria *et al.*, 1996). For *Aleurites* a CO_2-content of 700 ppm in air resulted in better growth and lower amounts of Rubisco protein (He *et al.*, 1996). However, if calculated on the basis of total crude extract protein, the capacity of the enzyme remained unchanged. It should be mentioned, that calculated on the basis of leaf area, both carboxylase capacities (PEP-Co and Rub-Co) taken together, closely match the CO_2-fixation rates determined by mass spectrometry (He *et al.*, 1998). It is obvious that the *in vitro* data of the enzymes do not necessarily lead to unequivocal conclusions with respect to the respective significances of the various enzymes *in vivo*.

Table II. Malate content and pH of hot water extracts from *Aleurites montana* and *Bryophyllum daigremontiana* leaf discs, at 9 a.m. (beginning of light period) and at 2 p.m. (middle of light period).

Malate [mg × g fw^{-1}]	Greenhouse 9 a.m.	Greenhouse 2 p.m.	Growth chamber normal air 9 a.m.	Growth chamber normal air 2 p.m.	Growth chamber 700 ppm CO_2 9 a.m.	Growth chamber 700 ppm CO_2 2 p.m.
Aleurites	0.18 ± 0.01	0.26 ± 0.02	0	0.43 ± 0.03	0	0.55 ± 0.05
Bryophyllum	13.7 ± 1.60	4.1 ± 0.56	7.93 ± 0.3	6.05 ± 0.05	8.13 ± 0.40	5.32 ± 0.28
pH of extract						
Aleurites	5.74 ± 0.16	5.76 ± 0.24	4.97 ± 0.04	5.13 ± 0.07	5.03 ± 0.03	5.24 ± 0.01
Bryophyllum	4.46 ± 0.15	5.90 ± 0.09	4.04 ± 0.01	4.68 ± 0.02	3.98 ± 0.07	5.03 ± 0.01

Data are averages of 4–5 independent measurements (CO_2-content in air 350 ppm).

Table III. Activity of ribulose bisphosphate carboxylase in nmol × min^{-1} × mg $protein^{-1}$ for *Aleurites montana*, *Bryophyllum daigremontiana*, *Saccharum officinarum* and *Capsicum frutescens* at 9 a.m. (beginning of light period) and at 2 p.m. (middle of light period).

Organism	Greenhouse		Growth chamber normal air		Growth chamber 700 ppm CO_2	
	9 a.m.	2 p.m.	9 a.m.	2 p.m.	9 a.m.	2 p.m.
Aleurites	13.9 ± 1.7	7.6 ± 0.7	11.6 ± 0.4	13.9 ± 0.8	11.9 ± 0.6	14.6 ± 0.7
Bryophyllum	3.2 ± 0.2	6.6 ± 0.6	5.7 ± 0.7	9.3 ± 0.6	4.5 ± 0.3	7.2 ± 0.5
Saccharum	18.9 ± 1.2	14.9 ± 0.5	not determined			
Capsicum	32.5 ± 0.2	32.6 ± 1.6	32.2 ± 2.3	35.1 ± 4.8	25.6 ± 0.8	36.7 ± 4.6

Data are averages of 4–8 independent measurements (∅ 350 ppm CO_2 in air).

However, being determined in identical crude cell extracts, i.e. based on total protein contents, they should allow relative comparison of enzyme actions. Thus, the calculated ratio of the average PEP-Co to Rubisco capacity is close to one (0.9:1) in *Aleurites*. In contrast to this the ratio is 10:1 for *Saccharum* and 20:1 for *Bryophyllum* (data from 2 p.m.), whereas the ratio for the C_3-plant *Capsicum* is close to that, determined for the tung-oil tree (0.86:1).

Malic enzyme

$NADP^+$-ME is an important enzyme for the decarboxylation of malate in C_4-plants and serves as a source of CO_2 for the Calvin cycle. It is only active in the bundle sheat chloroplasts, the site where the CO_2 is refixed by Rubisco (Edwards and Walker, 1983; Hatch, 1987). The genes encoding for $NADP^+$-ME and PEP-Co are highly expressed. They are differentially expressed in mesophyll and bundle sheat cells (Shen and Bogorad, 1987; Langdale *et al.*, 1988; Nelson and Langdale, 1989 and Maurino *et al.*, 1997). In plants having CAM, the CO_2, trapped by fixation into malate, is released by the enzyme to support the reductive pentose phosphate pathway during the day (e.g. Edwards and Andreo, 1992). The capacity of ME in C_4 or CAM plants is comparably high and matches that of photosynthetic carbon assimilation (Edwards and Walker, 1983).

Another prominent role of ME is the decarboxylation of malate and the concomitant reduction of $NADP^+$, required for the biosynthesis of lipids, as has been shown for *Ricinus communis* (Smith *et al.*, 1992 and Colombo *et al.*, 1997) and also for the C_4-plant *Zea mays* (Preis *et al.*, 1994). As has been mentioned above in *Aleurites*, high amounts of lipids are accumulated in the seeds and even in the leaves (Radunz *et al.*, 1998), a fact which also might suggest the contribution of the enzyme. However, in extracts of *Aleurites* an only low capacity of $NADP^+$-Me could be detected and this independent on the light period (Table IV). A 40% higher ME activity was found if plants were grown in 700 ppm CO_2. The capacity of the enzyme in extracts of *Bryophyllum*, grown in the growth chamber, was significantly higher and almost doubled between 9 a.m. to 2 p.m. to approx. 80 nmol × min^{-1} × mg $protein^{-1}$. In plants from the greenhouse the capacity increased over the same time period from 6.6 to 32.8 nmol × min^{-1} × mg $protein^{-1}$. The activity of ME from *Saccharum* was higher than in the other pants investigated.

Table IV. Activity of malic enzyme in nmol × min^{-1} × mg $protein^{-1}$ for *Aleurites montana*, *Bryophyllum daigremontiana*, *Saccharum officinarum* and *Capsicum frutescens* at 9 a.m. (beginning of light period) and at 2 p.m. (middle of light period).

Organism	Greenhouse		Growth chamber normal air		Growth chamber 700 ppm CO_2	
	9 a.m.	2 p.m.	9 a.m.	2 p.m.	9 a.m.	2 p.m.
Aleurites	14.8 ± 1.8	14.7 ± 1.5	16.7 ± 0.7	17.4 ± 1.5	23.4 ± 1.8	21.1 ± 1.5
Bryophyllum	6.6 ± 0.5	32.8 ± 1.9	42.6 ± 4.4	80.5 ± 3.9	33.9 ± 1.2	82.7 ± 1.7
Saccharum	381.8 ± 32.4	230.7 ± 5.7	not determined			
Capsicum	18.4 ± 0.6	19.3 ± 0.6	26.4 ± 1.3	27.2 ± 1.5	28.5 ± 1.1	31.8 ± 1.9

Data are averages of 4–8 independent measurements (∅ 350 ppm CO_2 in air).

Table V. Activity of malate dehydrogenase in nmol × min^{-1} × mg $protein^{-1}$ for *Aleurites montana*, *Bryophyllum daigremontiana*, *Saccharum officinarum* and *Capsicum frutescens* at 9 a.m. (beginning of light period) and at 2 p.m. (middle of light period).

Organism	Greenhouse		Growth chamber normal air		Growth chamber 700 ppm CO_2	
	9 a.m.	2 p.m.	9 a.m.	2 p.m.	9 a.m.	2 p.m.
Aleurites	3269 ± 287	3522 ± 135	1395 ± 161	1255 ± 42	1484 ± 184	1615 ± 31
Bryophyllum	13453 ± 586	19565 ± 1151	23123 ± 2041	11702 ± 556	15621 ± 876	9142 ± 605
Saccharum	7821 ± 511	5536 ± 111	not determined			
Capsicum	6909 ± 572	6838 ± 260	5309 ± 395	5105 ± 379	4995 ± 303	5222 ± 242

Data are averages of 4–8 independent measurements (CO2-content 350 ppm in air).

This was expected from literature. Compared to *Aleurites*, the ME-capacity was 16 to 20 times higher in the monocot C_4-plant.

For *Capsicum* no influence of growth in 700 ppm CO_2, nor of the light period was found. In plants from the greenhouse, ME-rates were 35% lower, than in plants from the growth chamber.

Summerizing, the capacity of ME, in extracts of the tung-oil tree is as low as in those of the C_3-plant *Capsicum*. There is no increase in activity in the light, as one finds it for the CAM-plant *Bryophyllum* (Table IV). The comparably low ME-capacity, together with low malate contents of extracts, do not speak in favour of a malate dependent synthesis of lipids, as was supposed above. In order to get further insights concerning the fates of CO_2, affinities of the enzymes, as well as turnover rates of metabolite pools should be determined.

Malate dehydrogenase

MDH is used in the NAD^+-type of C_4-plants for the reduction of oxaloacete, produced by the PEP-Co reaction. In all plants having CAM, the enzyme generates malate, which is stored in the vacuole over night and mobilized again during the day.

In extracts of the different plants studied, MDH capacity was extremely high, compared to that of other enzymes (Table V). Highest rates between 10 and 20 μmol × min^{-1} × mg $protein^{-1}$ were found for *Bryophyllum*, followed by *Saccharum* and *Capsicum* (5 to 8 μmol × min^{-1} × mg $protein^{-1}$) (Table V). Again, the lowest capacity was found in *Aleurites*, with approx. 1.3 to 1.6 μmol × min^{-1} × mg $protein^{-1}$ in the growth chamber and with 3.5 μmol × min^{-1} × mg $protein^{-1}$ in the greenhouse. Whereas the activity of MDH from *Bryophyllum* and *Saccharum* decreased after 5 hours in the light, the enzyme activity from *Aleurites* and *Capsicum* was more or less unchanged (Table V). As has been stated for the other enzymes studied, the data for NAD^+-ME do not support the existence or presence of CAM- or C_4-type photosynthesis in the tung-oil tree.

In conclusion, the data obtained for enzyme capacities of *Aleurites montana* do not support the idea of a CO_2-fixation via a *Crassulacean* type metabolism in this plant. All capacities match more or less the enzyme rates observed with the C_3-plant *Capsicum*, which served as a control. From this, the high CO_2-uptake can not easily be explained by a high expression of specific enzymes, known to occur in CAM- and C_4-plants. This holds true even for plants, grown under an increased CO_2-content of 700 ppm in air. Correspondingly, the high content of lipids and of fatty acids in leaves and in seeds of the tree, can not be explained by enhanced capacities of malate metabolising enzymes. Since CO_2 is not stored inside the vacuole (bound in malate), a rapid turnover might be reached by high substrate affinities of the respective enzymes involved. A study of regulatory and kinetic properties might give further insights into the fate of the fixed CO_2 in this exotic tree.

Acknowledgement

The work was financially supported by the *Deutsche Forschungsgemeinschaft* (DFG) within the frame of a cooperation between the DFG and the *Bundesminister für wissenschaftliche Zusammenarbeit* (BMZ) and the National Natural Science Foundation of China (NSFC).

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