

## Photosynthetic activity of stems in two *Clusia* species

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### Abstract

**Key message** In stems of *Clusia*, CO<sub>2</sub> concentrated in the xylem sap in CAM trees can be fixed by PEPC and Rubisco, while in C<sub>3</sub> trees only Rubisco is engaged.

**Abstract** The photosynthetic characteristics of 7–8-year-old stems of two tropical trees representing the Clusiaceae family were compared: *Clusia multiflora* Kunth. described as an obligate C<sub>3</sub> and *Clusia rosea* Jacq. as an obligate CAM plant. Photosynthetic gas exchange, xylem CO<sub>2</sub> concentration, chlorophyll distribution, <sup>13</sup>C discrimination, daily malate and citrate fluctuations and the abundance of Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) and PEPC (phosphoenolpyruvate carboxylase) proteins were measured in leaves and stems. In stems of both species a low CO<sub>2</sub> efflux (in the range of 0.05–0.1 μmol m<sup>-2</sup> s<sup>-1</sup>) was observed as a result of

extremely low cork conductance for water vapor (0.15–0.2 mmol m<sup>-2</sup> s<sup>-1</sup>). This led to the CO<sub>2</sub> concentration in xylem sap reaching 5.2 (CO<sub>2</sub>\*) mmol l<sup>-1</sup>. The substantial amount of chlorophyll in the outer part of the bark and light-induced decrease of CO<sub>2</sub> concentration within the xylem can be explained by photosynthetic activity in this tissue. Moreover, Western blotting analyses proved the presence of Rubisco in the stems of both *Clusia* species; however, PEPC was only found in *C. rosea*. Additionally, daily fluctuations in the concentration of citrate and malate (higher than in leaves) and significant enrichment in <sup>13</sup>C in *C. rosea* stems were observed. These facts allow us to conclude that the examined stems of *C. rosea* and *C. multiflora* represent specific types of photosynthetic metabolism.

**Keywords** β-Carboxylation · Citrate · <sup>13</sup>C discrimination · Malate · Corticular photosynthesis · Stems

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### Introduction

Most photosynthesizing plant species are equipped with two carboxylating enzymes, Rubisco and phosphoenolpyruvate carboxylase (PEPC). Usually in C<sub>3</sub> plants PEPC is present in much lower amounts than Rubisco in many tissues and can fix (prefix) CO<sub>2</sub> (as HCO<sub>3</sub><sup>-</sup>) in the cytoplasm, which results in an increase of malate or citrate levels. These processes can be very intensive and are characteristic for some heterotrophic tissues in C<sub>4</sub> and also CAM (crassulacean acid metabolism) plants (both described as β-carboxylating plants). In photosynthetic tissues after decarboxylation of malate or citrate, CO<sub>2</sub> can be fixed again by Rubisco. PEPC expression is a major factor underpinning the genotypic capacity for CAM (Taybi et al. 2004).

Most literature concerning photosynthesis describes the process of fixing atmospheric CO<sub>2</sub> in leaf blade mesophyll tissues. We know that within leaf blades also cells associated with veins are performing photosynthesis and also cells within stems contain significant amounts of chlorophyll. In addition, the carbon present as malate in the transpiration stream may be used for photosynthesis. This was shown in *Arabidopsis* leaves, a C<sub>3</sub> model plant species (Janacek et al. 2009). As was reported by Hibberd and Quick (2002) in experiments on the C<sub>3</sub> plant *Nicotiana* sp., leaf veins are able to perform C<sub>4</sub>-similar photosynthesis using carbon fixed by PEPC in their roots. Malate (after pre-fixation of CO<sub>2</sub>) is transported within the vascular bundles to leaf mesophyll tissues. It seems that photosynthesis taking part within stems and leaf veins plays a significant role in many physiological processes. It was also possible to determine that photosynthesis in cells close to veins was quantitatively important for growth fitness (Janacek et al. 2009). According to other authors, most CO<sub>2</sub> within the stem originates from living cells in the stems and roots. In woody plants, because of the large size of xylem, CO<sub>2</sub> accumulation can occur. It has been found that the concentration of CO<sub>2</sub> in the xylem sap changes daily and seasonally, and it also depends on many environmental factors including temperature, drought and light conditions (Saveyn et al. 2007; Teskey et al. 2008; Etzold et al. 2013).

According to the type of the photosynthetic mechanism performed within leaves, most trees are classified as C<sub>3</sub> plants, but in vascular tissues of stems, especially young branches, photosynthesis occurs mostly by refixing the internal respiratory CO<sub>2</sub> (Berveiller and Damesin 2008). However, in this research the possibility of CO<sub>2</sub> production due to decarboxylation of malate and citrate was not taken into account.

In experiments on eight tree species (deciduous and evergreen: *Ginkgo biloba*, *Fagus sylvatica*, *Tilia cordata*, *Betula pendula*, *Quercus robur*, *Fraxinus excelsior*, *Alnus glutinosa*, *Picea abies*, *Pinus sylvestris*), it was also shown that the decrease in CO<sub>2</sub> efflux from stems in the light is mainly due to increased assimilation rather than to reduced respiration (Berveiller et al. 2007). Stem maximal Rubisco and PEPC activities occurred at the beginning of the season when the chlorophyll content, *chl*a/*chl*b ratio and CO<sub>2</sub> assimilation rate are also maximal. The PEPC/Rubisco activity ratio in the stems of the tested tree species was on average 3.6, while the value for leaves in C<sub>3</sub> plants is 0.1 and for C<sub>4</sub> plants 10.0 (Berveiller and Damesin 2008).

Different methods can be used to evaluate the role of both carboxylases in gaining atmospheric CO<sub>2</sub>, but their participation in the general metabolism is difficult to estimate. Analyzing the net photosynthesis, it is quite easy to

distinguish between C<sub>3</sub> and CAM plants (Taybi et al. 2004; Teskey et al. 2008). When measuring the activity of the enzymes involved in both metabolic pathways, we have to take seasonal and daily changes into account. Their activities can be subjected to many transcriptional and post-transcriptional regulations. Both carboxylating enzymes, PEPC and Rubisco, discriminate the <sup>13</sup>C isotope differently, and this phenomenon can help to evaluate their overall activity during growth.

The relative abundance of the stable carbon isotopes varies in nature (Nier and Gulbransen 1939). Such variation was reported to exist among plant taxa and among plants growing in different environments (Cernusak et al. 2013) as well as species using different photosynthetic metabolism, C<sub>3</sub>, C<sub>4</sub> and CAM (Craig 1953; Farquhar 1983; Cernusak et al. 2008). The <sup>13</sup>C discrimination values for the new biomass of CAM plants obtained solely during the dark (PEPC) and light (PEPC and Rubisco) were estimated as −8.7 and −26.9 ‰, respectively. Farquhar (1983) showed that fractionation associated with PEPC is −5.7 ‰ and that associated with Rubisco −29.0 ‰. Following this, according to Cernusak et al. (2009), non-photosynthetic or heterotrophic tissues in C<sub>3</sub> plants tend to be enriched in <sup>13</sup>C (less significant discrimination) compared with the leaves that supply them with photosynthate. This would indicate that non-photosynthetic tissues can fix CO<sub>2</sub> independent of the Calvin cycle pathway, and we may explain this by <sup>13</sup>C discrimination caused by PEPC and Rubisco. We can also expect that stems can use, at least in part, other sources of carbon than that fixed by Rubisco in leaves. Craig (1953) observed that the branch wood of several tree species was enriched in <sup>13</sup>C compared with leaves. This means that the <sup>13</sup>C discrimination factor of branch wood reaching −25.8 ‰ was significantly less negative than that of leaves at −26.8 ‰. Badeck et al. (2005) also calculated that woody stems were on average enriched in <sup>13</sup>C by 1.9 ‰ compared with leaves. Also young emerging leaves of C<sub>3</sub> plants, for which growth may be mostly heterotrophic, tend to be <sup>13</sup>C-enriched (Damesin and Lelarge 2003), and later during the development of the photosynthetic apparatus this factor is lowered, indicating an increase in Rubisco activity.

In addition to the above-mentioned literature, it is worth noting that <sup>13</sup>C-rich molecules are discriminated in many reactions. It was shown that significant discrimination of <sup>13</sup>C-rich pyruvate by pyruvate dehydrogenase causes reduced <sup>13</sup>C levels in fatty acids. Therefore, thylakoid membranes have reduced <sup>13</sup>C levels. This may affect <sup>13</sup>C levels in tissues, especially those rich in chloroplasts (DeNiro and Epstein 1977).

Malate and citrate synthesized in β-carboxylation because of the PEPC action play a crucial role, and both acids

can provide CO<sub>2</sub> during the time when it is limited. In our previous experiments (Miszalski et al. 2013) done on *Clusia hilariana* CAM tree leaves, we showed that fluctuations of malate and citrate are independent, and at low light citrate can even be accumulated opposite to malate, which is decarboxylated. In *C. alata* CAM plants, decarboxylation of citrate during the daytime is delayed in comparison to decarboxylation of malate (Kornas et al. 2009).

In many stem succulent CAM plants, accumulation of carbon takes place just in stems (Lüttge 2004). Leaves of many *Clusia* plants show very high flexibility in  $\beta$ -carboxylation, but some species are obligatory C<sub>3</sub> or CAM. We know that the main veins behave differently from the leaf blade in *C. minor* leaves exposed to photorespiratory and non-photorespiratory conditions (Lüttge 2008). Nothing is known about the activity of  $\beta$ -carboxylation in stems of *Clusia* plants that use this type of metabolism intensively and flexibly in their leaves. Are stems of the obligatory C<sub>3</sub> plants able to perform intensive  $\beta$ -carboxylation similar to obligatory CAM species? Are stems of the obligatory CAM species active in using this type of CO<sub>2</sub> gaining? Is carbon fixed within stems also transported to other tissues?

The present study aims to improve the physiological characterization of stem photosynthesis by examining the enzymatic characteristics. The two enzymes that are crucial in CO<sub>2</sub> fixation and  $\beta$ -carboxylating metabolism, respectively, were investigated in stems of obligatory C<sub>3</sub> and CAM species (*C. multiflora* and *C. rosea*, respectively) and were compared to the corresponding characteristics of leaves.

## Materials and methods

### Plant material

The experiments were carried out on 8–10-year-old *C. multiflora* and *C. rosea* trees. Plants were grown under a natural photoperiod in a greenhouse at Cracow Botanical Garden. Trees of similar height (1.2–1.5 m) and stem diameter (2.0–2.5 cm) at a height of 1 m were grown in 20-l containers. Soil composed of sand and compost (Universal Potting Soil, Pokon Naturado, Veenendaal, The Netherlands) was used in 1:1 proportion. Gas exchange measurements on leaves and bark were taken at a height of about 70–100 cm. After that, samples of leaves and bark were collected for biochemical analyses and epifluorescence microscopy. Samples for isotope analysis, chlorophyll content, acid concentration, and protein extraction were immediately immersed in liquid nitrogen and stored at –80 °C until usage.

### Carbon isotope analysis in organic samples

The frozen samples were oven dried for 24 h at 105 °C before being ground to a fine powder for isotopic analysis. Isotope ratio measurements of C were performed on a Finnigan MAT 253 Mass Spectrometer coupled with a Flash HT Elemental Analyzer in continuous flow mode. Samples were weighed in tin capsules and introduced into the combustion furnace with a temperature of 1,020 °C. A small volume of oxygen was added to the system to ensure the full combustion of organic compounds and conversion into elemental gases. CO<sub>2</sub> was then separated in a chromatographic column (heated to 45 °C) and transferred in a carrier gas (He) via a ConFlo IV Interface to the isotope ratio mass spectrometer. International isotope standards were used to calculate the results: USGS 40, USGS 41 and IAEA 600 (Coplen et al. 2006).

### Measurement of the CO<sub>2</sub> concentration in stem tissues

The CO<sub>2</sub> concentration ([CO<sub>2</sub>]; %) was measured at the height of 1 m from the base of the trees with a CO<sub>2</sub> microelectrode (model MI-414, Microelectrodes Inc., Bedford, NH) (McGuire and Teskey 2002). Four-mm-diameter holes were drilled 5 mm deep into the xylem. Teflon tubes (3.5 mm inner diameter and 30 mm length) were tightly fitted into the holes, and the tubes were sealed to the trees with flexible adhesive putty (Terostat IX, Henkel, Heidelberg, Germany).

The CO<sub>2</sub> microelectrode was inserted in the tube, and adhesive putty was used to provide a gas-tight seal. The microelectrode measured the [CO<sub>2</sub>] of the gas in the headspace of the hole, which was proportional to the concentration of all products of CO<sub>2</sub> dissolved in the xylem sap. The [CO<sub>2</sub>] of the gas (%) was converted to total dissolved carbon ([CO<sub>2</sub>\*] mmol l<sup>-1</sup>) by equations based on Henry's law (McGuire and Teskey 2002). For this conversion, the pH of the xylem sap must be known. Therefore, sap was expressed from an excised twig of the tree with a pressure chamber (PMS Instruments, Corvallis, OR) and measured with a pH electrode (model MI-720, Microelectrodes Inc., Bedford, NH). The microelectrode was calibrated with two air samples of known [CO<sub>2</sub>], and an exponential equation was developed to convert the millivolt output to [CO<sub>2</sub>]. The results were compensated for temperature by applying the equation empirically derived by McGuire and Teskey (2002).

### Gas exchange measurements

The measurements of gas exchange were conducted in the leaves and stems under greenhouse conditions at

24 °C using a Portable Photosynthesis System LI-6400 (LI-COR Inc., Lincoln, NE, USA). The rates of CO<sub>2</sub> and H<sub>2</sub>O efflux were measured using a standard chamber for leaves and a 6400-05 conifer chamber for stems. Measurements were performed under constant conditions: 24 °C, 25–30 % relative humidity (RH), CO<sub>2</sub> concentration 385–400 μmol mol<sup>-1</sup> and PPFD 400 μmol photons m<sup>-2</sup> s<sup>-1</sup>.

### Chlorophyll content

Photosynthetic pigments *chl a* and *b* were measured in 80 % acetone using the method described by Wellburn (1994).

### Protein extraction and quantification

To determine the amount and diurnal changes of PEPC and Rubisco proteins in different plant parts of *Clusia*, the plant material, i.e., roots, stems, petioles and leaves, of *C. rosea* and *C. multiflora* was harvested at 7 a.m. and 2 p.m., then powdered in N<sub>2</sub> and homogenized at 4 °C according to the method described by Taybi et al. (2004) with modification. Proteins (35 μg) of each species were used for SDS PAGE electrophoresis in 12 % polyacrylamide gels. Blotting was performed on a polyvinylidene fluoride membrane (PVPD) (Merck Millipore). Identification of PEPC and Rubisco (large subunit) bands was done with anti-PEPC polyclonal antibody (Agrisera), and detection was performed with alkaline phosphate buffer (AP) with BCIP/NBT as a substrate.

### Chlorophyll localization in stems

The distribution of chlorophyll in stems was determined by using the autofluorescence of *chl a*. Cross-section fragments of stems with a thickness of about 1 mm were soaked in a few drops of polyethylene glycol. The fluorescence images were recorded with a Bio-Rad MRC 1024 (Bio-Rad Microscience, Herts, UK) confocal microscope equipped with a Nikon Diaphot 300 microscope (Nikon, Amsterdam, The Netherlands) and a PlanFluor 10×, NA 0.3 lens. The autofluorescence in the studied plant tissues was excited by a 488-nm line emitted by a 100-mW argon laser (ILT, USA). The images of square-shaped areas with 1,040-μm sides were recorded using 512 × 512 pixels and 8-bit resolution. Fluorescence was recorded within a 515–630-nm range (cell walls) and 640–700-nm range (*chl*). In the combined confocal images, the fluorescence within the 515–630-nm range—representing the cell autofluorescence—had a green color and within the 640–700-nm range—autofluorescence of *chl*—a red color.

### Malate and citrate determination

Prior to cell sap collection, frozen bark and leaf samples were thawed for 3 min. Then cell sap was obtained by extrusion with a squeezer and subsequently centrifuged for 4 min at 12,000×*g*. Malate and citrate were determined enzymatically according to Möllering, respectively (1974) and (1985).

### Statistics

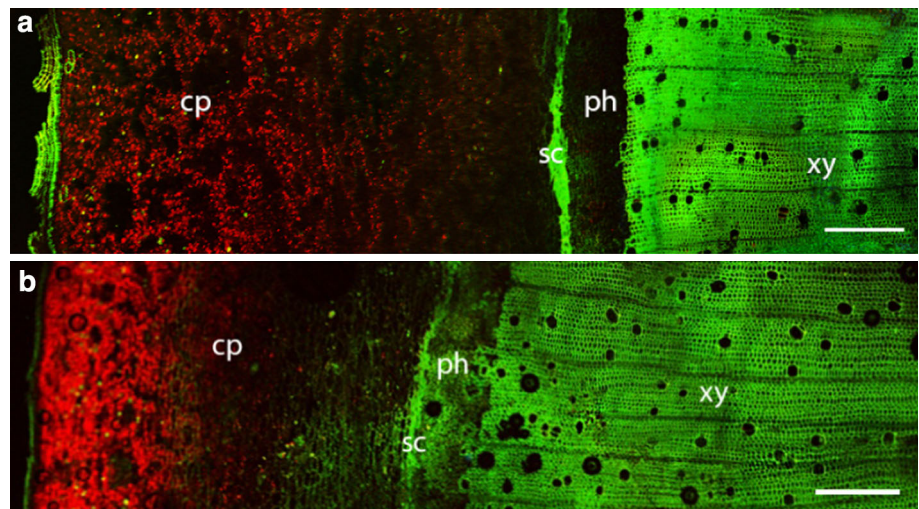
Statistical analyses of the data were made with Statistica 12.0 (Statsoft, Tulsa, OK, USA). The δ<sup>13</sup>C values, CO<sub>2</sub> efflux and chlorophyll content were evaluated by statistical analysis of variance (ANOVA). Oscillations of the daily acid concentration in leaves and bark were analyzed by Student's *t* test. Pictures from epifluorescence microscopy and electropherograms represent typical examples of five repetitions. Detailed information about statistic tests and the number of replicates is provided in the tables and figures.

### Results and discussion

Cross sections of stems of *C. multiflora* and *C. rosea* (Fig. 1) reveal a similar anatomic structure of stems where a substantial part (about 25 %) consists of bark and is covered with a thin cork lacking stomata and lenticels. In the examined 8–9-year-old stems, chlorophyll is concentrated mostly in the outer part of the bark. The presented pictures show that in these tissues the chlorophyll amount decreases in more deeply located cell layers and that the bark of *C. multiflora* is less rich in chlorophyll. The largest part of the stems in *Clusia* species is wood (approximately 65 %) and only about 10 % the pith, both without visible amounts of chlorophyll. Pigment localization differs strongly from other typical C<sub>3</sub> trees with thin cork (e.g., 6-year-old beech trunks) where chlorophyll is abundant in all living tissues including the pith and wood parenchyma (Pilarski and Tokarz 2006).

In the constitutive C<sub>3</sub> plant *C. multiflora*, PEPC protein was detected in neither the leaves nor other plant organs. The level of Rubisco remained stable independently of the time point during the day (7 a.m., 2 p.m.) in leaves and other organs in both examined species. The abundance of the Rubisco protein was higher in *C. rosea* compared to *C. multiflora*. Immunoblot analysis indicated that in the constitutive CAM plant *C. rosea*, PEPC protein is present in all parts of the plant except the roots, and its abundance increases in the order: bark of the stem–petioles–leaves (Fig. 2). An effect of daytime on enzyme amount, however, was not observed. In *C. rosea* extracts, several bands

**Fig. 1** Cross-sections of 8-year-old stems of *C. multiflora* (a) and *C. rosea* (b), observed by epifluorescence microscopy. Red and green colors correspond to the autofluorescence of chlorophyll and cell walls respectively. cp cortex parenchyma, sc sclerenchyma, ph phloem, xy xylem. Scale bar 500  $\mu$ m



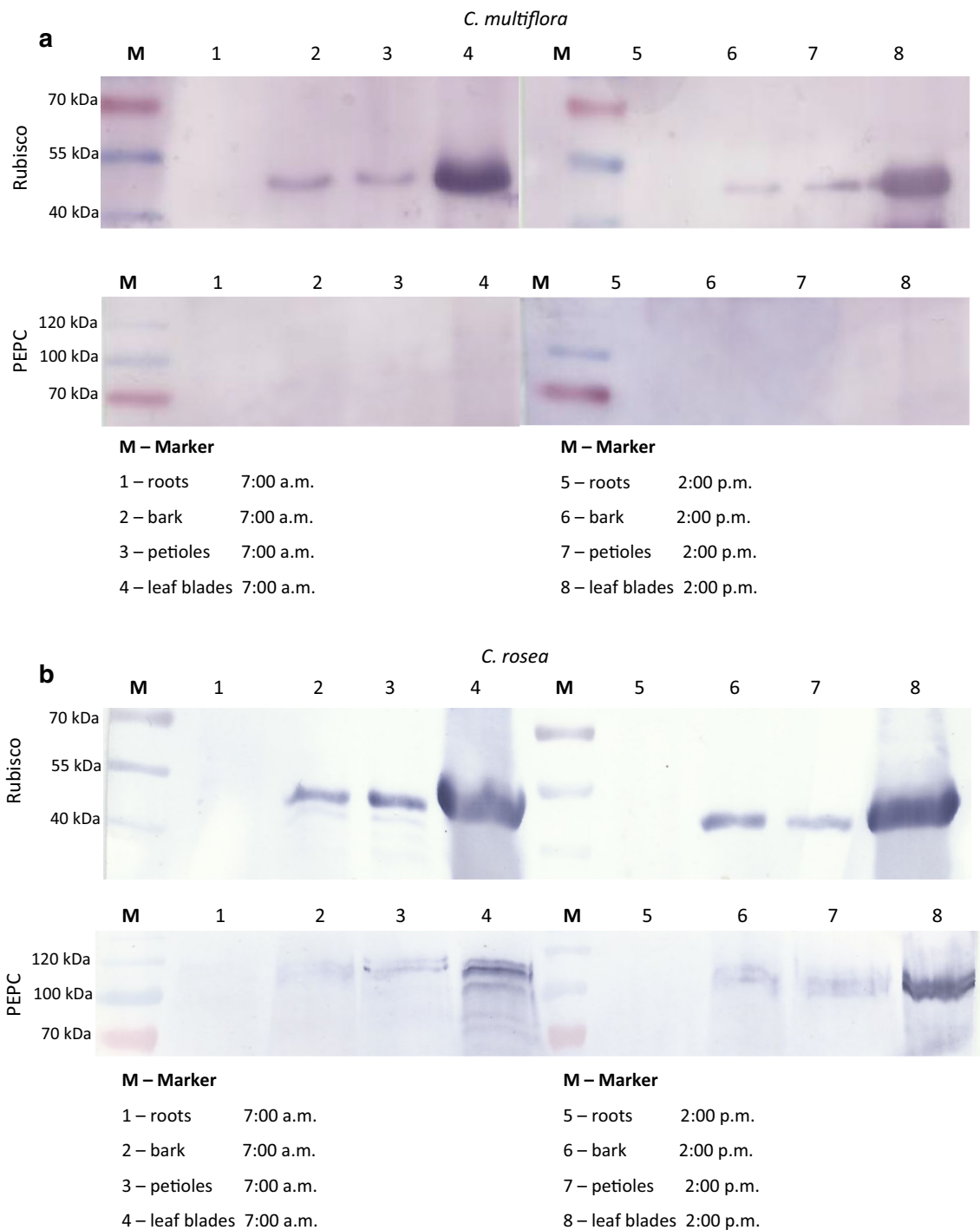
of PEPC protein were identified, most probably resulting from intensive day/night regulation of this enzyme. However, the other possible explanation could be the existence of functionally different PEPC isoforms as is the case in many CAM plants (Taybi et al. 2004).

In the experiments presented here, we were able to show significant amounts of  $\text{CO}_2$  within stems of both  $\text{C}_3$  and CAM *Clusia* species. In both plants kept under greenhouse conditions (day/night temperature 35/25  $^\circ\text{C}$ ), fluctuations of the  $\text{CO}_2$  concentration showed a clear increase during the morning hours reaching the maximal value around midday, and much a lower concentration was observed during the night (Fig. 3). These fluctuations reflect changes in temperature well, and most probably they are due to respiratory and decarboxylating processes. Some cells in the bark of stems are equipped with chloroplasts, as shown in Fig. 1, and there is the possibility to use  $\text{CO}_2$  for photosynthesis. However, they are not able to fix all available carbon dioxide. To check the effectiveness of  $\text{CO}_2$  fixation in the analyzed plant organs excluding a temperature effect, we measured its concentration at a constant temperature of 24  $^\circ\text{C}$  during the day/night cycle. In measurements with both tested *Clusia* species, a clear increase during the night period and a decrease of the  $\text{CO}_2$  concentration during the daytime were found. When comparing the shapes of both curves (Fig. 3), it is observed that in *C. multiflora* these processes are less intensive during the first hours of light as well as during the first hours of dark periods in comparison with *C. rosea*. It is tempting to speculate that changes in  $\text{CO}_2$  fixation processes in *C. rosea* are more active in comparison to *C. multiflora*. However, we can not exclude that this is due to different respiratory characteristics in *C. rosea*.

$\text{CO}_2$  gas exchange between stems and the atmosphere has been well described in the case of several trees and

shrubs (Pfanz and Aschan 2001; Teskey et al. 2008; Bloemen et al. 2013), but nothing is known about these processes in the stems of Clusiaceae. In our experiments we compared the  $\text{CO}_2$  exchange in leaves and stems of *C. multiflora* and *C. rosea*, and we found clear diel changes in the net photosynthesis characteristic for  $\text{C}_3$  and CAM plants, respectively (Fig. 4a). *C. multiflora* leaves start to fix  $\text{CO}_2$  with the onset of daytime, and a similar behavior was noted in fluctuations of  $\text{H}_2\text{O}$  conductance (Fig. 4b). *C. rosea* leaves absorb large amounts of  $\text{CO}_2$  during the night, and this process decreases sharply with the onset of daytime. The total conductance to  $\text{H}_2\text{O}$  shows a similar daily course. During the nighttime, leaves of *C. rosea* lose much more water than during the daytime; especially in the afternoon this process is very slow (Fig. 4a). In the stems of both plants these diel cycles did not occur (Fig. 4). Analyzing  $\text{CO}_2$  and  $\text{H}_2\text{O}$  exchange (influx/efflux) in stems and leaves (at 24/24  $^\circ\text{C}$ , day/night temperature) of *C. multiflora* and *C. rosea* (Table 1), we can state that stems are very tight structures, and only very small amounts of  $\text{CO}_2$  and  $\text{H}_2\text{O}$  diffuse from them to the atmosphere.

Stems of succulent CAM plants with highly reduced or lacking leaves are able to effectively fix carbon during hot and dry periods. Their stems show net  $\text{CO}_2$ -fixation rates of up to 10–20  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$  during the night (Nobel and Hartssock 1986). Also in many non-succulent  $\text{C}_3$ -perennials originating from semi-desert, desert and other seasonally dry habitats, green stems and twigs contribute an important proportion to the whole-plant carbon gain. In these plants the uptake of atmospheric  $\text{CO}_2$  reaches maximum rates of net stem photosynthesis of ca. 8–10  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$  during the day (Nilsen and Sharifi 1994). In woody stems and branches with strongly lignified tissues and bark layers, net photosynthesis was observed almost exclusively with halved twig segments or isolated

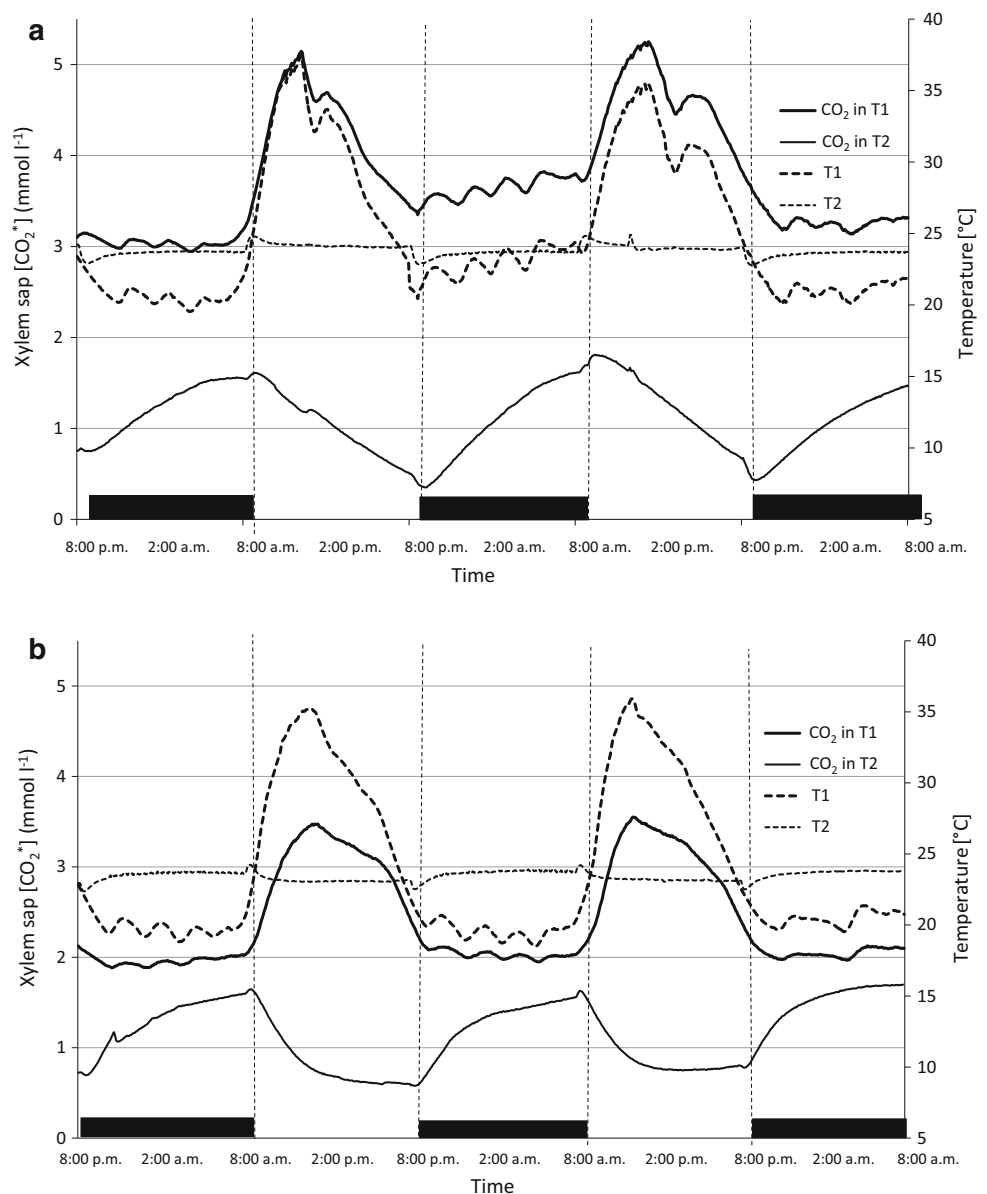


**Fig. 2** Changes in Rubisco and PEPC abundance in roots, bark, petioles and leaf blades in *C. multiflora* (a) and *C. rosea* (b) at 7 a.m. (1–4) and 2 p.m. (5–8), respectively. An amount of marker (M) (5  $\mu$ g) and protein extract (35  $\mu$ g) was loaded to each lane

chlorenchymal tissues (Pfanzen and Aschan 2001). In trees and shrubs, instead of net photosynthesis, respiratory  $\text{CO}_2$  efflux is widely observed in the range of 1–4  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (Saveyn et al. 2007; Cerasoli et al. 2009). Calculated from  $\text{CO}_2$  efflux (in light and dark conditions), efficient refixation of the respiratory  $\text{CO}_2$  is considered a

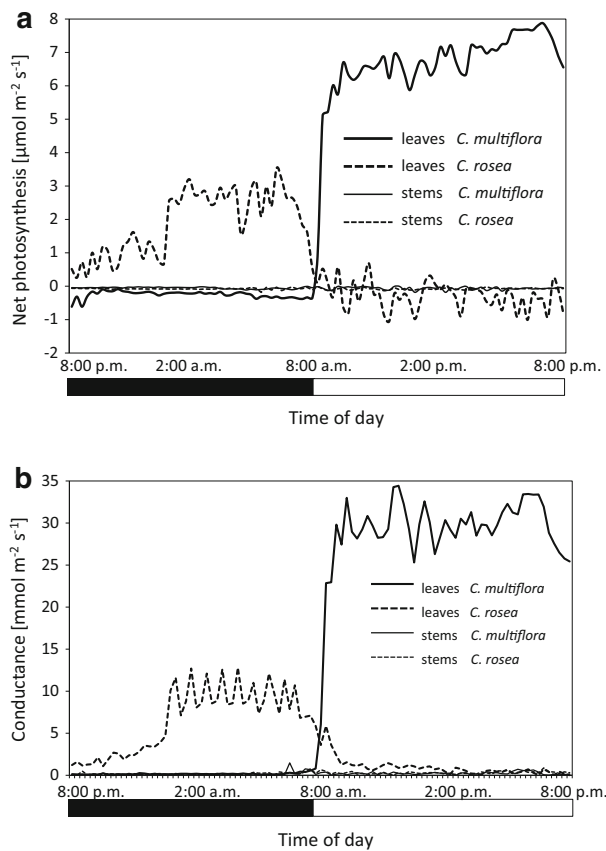
photosynthetic phenomenon of plant stems (Pfanzen and Aschan 2001; Cerasoli et al. 2009). Such refixation of internally available  $\text{CO}_2$  is also described for the chlorophyllous stems of herbaceous plants, such as, e.g., parasitic *Cuscuta* species (Hibberd et al. 1998). In the case of the tested two *Clusia* species, only low levels of  $\text{CO}_2$  efflux,

**Fig. 3** Daily temperature fluctuations (*dotted lines*) and CO<sub>2</sub> concentration changes (*solid lines*) within stems of *C. multiflora* (**a**) and *C. rosea* (**b**). *T*<sub>1</sub> greenhouse conditions: temperature day/night 35/20 °C, 20–35 % relative humidity, CO<sub>2</sub> concentration 410–480 μmol mol<sup>-1</sup>, PPFD = 250–700 μmol photons m<sup>-2</sup> s<sup>-1</sup>; *T*<sub>2</sub> growth chamber conditions: constant temperature 24 °C, 25–30 % relative humidity, CO<sub>2</sub> concentration 470–530 μmol mol<sup>-1</sup>, PPFD = 400 μmol photons m<sup>-2</sup> s<sup>-1</sup>. Each curve is for a representative stem of three replicate determinations



similar in the light and dark, were observed mostly in the range of 0.07–0.1 μmol m<sup>-2</sup> s<sup>-1</sup>, and as a consequence, in our experiments, no measurable refixation was identified (Table 1). Our calculations of CO<sub>2</sub> refixation in light in *Clusia* contrast with those reported for other species (Cernusak and Marshall 2000; Teskey et al. 2008; Cerasoli et al. 2009). The very low level of CO<sub>2</sub> efflux in our plant material could be an effect of extremely low cork conductance (0.15–0.2 mmol m<sup>-2</sup> s<sup>-1</sup>). Other trees showed higher conductance, e.g., ca. 1.0 mmol m<sup>-2</sup> s<sup>-1</sup> in 4-year-old stems of *Pinus monticola* (Cernusak and Marshall 2000) or 1.1 mmol m<sup>-2</sup> s<sup>-1</sup> in young stems of *Betula pendula* (Wittmann et al. 2006). Thus, in *Clusia* plants, the carbon dioxide present inside stems most probably comes from respiration or is transported from other plant organs.

“Saturation” of tissues with CO<sub>2</sub> in *Clusia* stems decreases with the onset of day (Fig. 3). We may expect that limited decarboxylation of citrate and malate together with the high capacity of Rubisco-catalyzed carboxylation causes some of the CO<sub>2</sub> to be used up during this part of the day. In stems of both plants, depletion of the CO<sub>2</sub> concentration during the day (Fig. 3) cannot be the effect of PEPC activity, which is present in low but measurable amounts only in *C. rosea*. It is worth noting that malate-insensitive PEPC (very crucial for CAM metabolism) is mostly active during the night. Additionally, changes in the CO<sub>2</sub> concentration during the light period are similar in both C<sub>3</sub> and CAM plants. CO<sub>2</sub> transport within some plants is strongly correlated with changes in the transpiration stream. According to Teskey and McGuire (2007), over a



**Fig. 4** Daily gas exchange fluctuations of leaves and stems of *C. multiflora* and *C. rosea*; **a** net photosynthetic rate ( $P_N$ ); **b** total conductance to water vapor ( $g_{tw}$ ). Measurements were performed under constant conditions: 24 °C, 25–30 % relative humidity,  $\text{CO}_2$  concentration  $385\text{--}400 \mu\text{mol mol}^{-1}$ , PPF =  $400 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Gas exchange curves are representative of three replicate runs with  $\text{SE} < 5 \%$ . ( $n = 3$ )

**Table 1**  $\text{CO}_2$  release of stems of *C. multiflora* and *C. rosea* in the dark and light; after 30 min under illumination with  $2000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$

| Treatment | $\text{CO}_2$ efflux ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$ ) |                     |
|-----------|--|---------------------|
|           | <i>C. multiflora</i>   | <i>C. rosea</i>     |
| Light     | $0.073 \pm 0.008^a$  | $0.100 \pm 0.009^b$ |
| Dark      | $0.070 \pm 0.007^a$  | $0.103 \pm 0.009^b$ |

Measurements were performed under constant conditions (20 °C, 40–45 % relative humidity) and at controlled  $\text{CO}_2$  supply ( $400 \mu\text{mol mol}^{-1}$ ). Data are mean  $\pm$  SD ( $n = 10$ ). Values marked with the same letters do not differ significantly according to Duncan's test;  $P \leq 0.01$

whole day, 55 % of the  $\text{CO}_2$  efflux to the atmosphere consists of  $\text{CO}_2$  that had been transported from lower parts of the tree. Moreover, stem girdling decreased the xylem  $\text{CO}_2$  concentration, indicating that belowground respiration contributes to the aboveground transport of internal  $\text{CO}_2$

**Table 2**  $^{13}\text{C}$  discrimination in leaves and bark of *C. multiflora* and *C. rosea*

| Part of plant | $\delta^{13}\text{C}$ (‰) |                     |
|---------------|---------------------------|---------------------|
|               | <i>C. multiflora</i>      | <i>C. rosea</i>     |
| Leaves        | $-30.58 \pm 0.36^a$       | $-23.50 \pm 1.29^b$ |
| Bark          | $-30.81 \pm 0.08^a$       | $-20.77 \pm 0.62^c$ |

Data are mean  $\pm$  SD ( $n = 3$ ). Values marked with the same letters do not differ significantly according to Duncan's test;  $P \leq 0.05$

(Bloemen et al. 2014). Considering the conductance of leaves and the transpiration stream, which would move  $\text{CO}_2$  upward within the whole plant, this should occur in different time periods of the day: for *C. rosea* during the night and in *C. multiflora* during the day. Thus, we cannot expect that this is the main reason explaining the presence of  $\text{CO}_2$  in the stems of both *Clusia* plants. We expect that neither the transpiration stream nor PEPC activity, but rather photosynthetic activity, is responsible for the depletion of the  $\text{CO}_2$  concentration during the day in the stems of both plants.

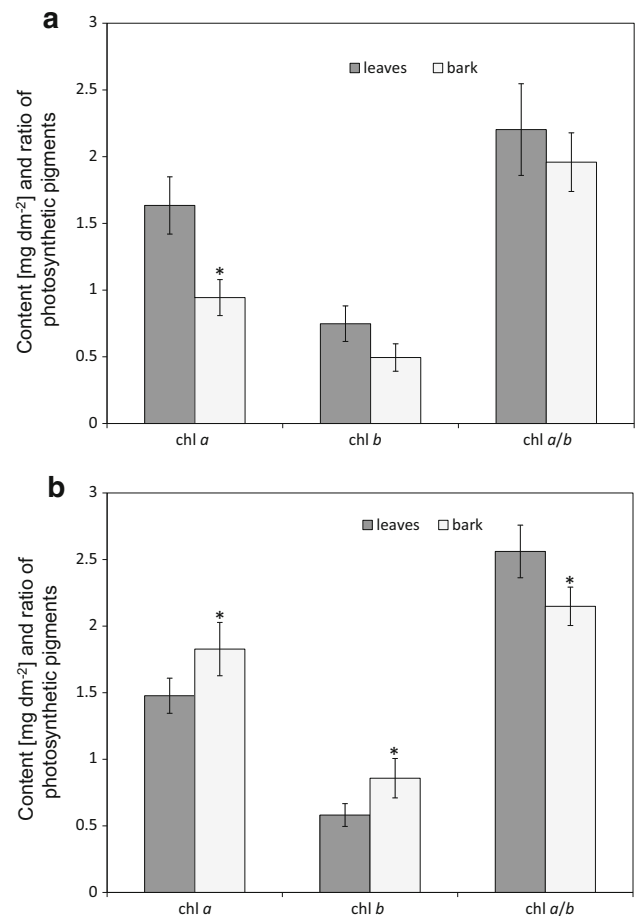
There is a high difference in the  $\delta^{13}\text{C}$  discrimination value in *C. rosea* leaves (around  $-23.5$  ‰) compared to *C. multiflora* (around  $-30.6$  ‰) (Table 2). In stems this difference is even higher, ranging from almost  $-21$  to  $-31$  ‰, respectively. Enrichment in  $^{13}\text{C}$  in bulky organs in comparison to leaves is well known, but the mechanism of this phenomenon is not well understood (Badeck et al. 2005; Cernusak et al. 2009). According to Farquhar (1983), the main physiological factor responsible for shaping  $^{13}\text{C}$  is the discrimination of the  $^{13}\text{C}$  isotope by Rubisco and to a much lesser extent by PEPC. In fact, PEPC activity in stems of  $\text{C}_3$  plants (in 1-year *Fagus sylvatica* and the herbaceous plant *Reynoutria japonica*) can be even higher than in leaves (Berveiller et al. 2007; Kocurek and Pilarski 2011). The results presented here on *C. multiflora* and *C. rosea* do not support these observations, and we show some amount of PEPC in *C. rosea* and no measurable amounts in *C. multiflora* (Fig. 2). In *C. rosea* this protein is much better expressed in leaves than in stems, which is characteristic for CAM plants. If so, most carbon fixed in the *C. multiflora* body is due to Rubisco activity, without pre-fixation (with PEPC).

In *C. multiflora* the  $\delta^{13}\text{C}$  value reaches less than  $-30$  ‰ and is similar in leaves and bark. It seems interesting that when comparing the  $\delta^{13}\text{C}$  for *C. rosea*, bark tissue shows less discrimination than leaves (Table 2). Similar results concerning strong and weak CAM *Clusia* were obtained by Cernusak et al. (2008). Thus, a substantial part of carbon incorporated in stems possibly was fixed in a pre-fixation process. Continuing this line of interpretation, we can expect that the origin of carbon within stems of *C. rosea* is



more due to PEPC activity than in leaves. We can speculate that if this carbon originated from the  $\text{CO}_2$  produced in respiratory processes, this would show similar discrimination as in other plant tissues. Thus, the less negative  $\delta^{13}\text{C}$  value in the bark tissue in *C. rosea* (and the difference between leaves and bark) would mean that at least some part of  $\text{CO}_2$  in the bark is fixed because of PEPC activity. It can be expected that  $\text{CO}_2$  is fixed in roots and transported via vascular tissues to stems, as shown by Hibberd and Quick (2002), and in this way participates in building carbon skeletons of the cell walls in the bark. However, this transpiration stream does not seem very intensive in the case of *Clusia* species (Herrera et al. 2008). If we assume that the final  $^{13}\text{C}$  discrimination level depends mostly on Rubisco and PEPC activities, we may also expect that more carbon incorporated in bark tissue comes from pre-fixation in comparison to leaves. We may suspect that bark tissue is growing more intensively during time periods when the PEPC/Rubisco activity ratio is increased. Moreover, in *C. rosea* the low value of  $\delta^{13}\text{C}$  in stems (which is lower than in leaves) would indicate that photosynthesizing cells in bark tissue intensively use carbon dioxide pre-fixed in the reaction catalyzed by PEPC, and this may also be transported from the other tissues. In addition to this, we know that tissues rich in chloroplasts (lipid-rich organelles) show higher discrimination (more negative  $\delta^{13}\text{C}$  values). We have not observed such differences in *C. multiflora* (Table 2), but in *C. rosea* this difference was higher than observed for *Mimosa nagi* (Fernandes et al. 1998). Thus, in our experiments about a 3 ‰ difference between the bark and leaves in *C. rosea* is probably the effect of both: the depletion in chloroplasts and PEPC activity as shown in Fig. 2b.

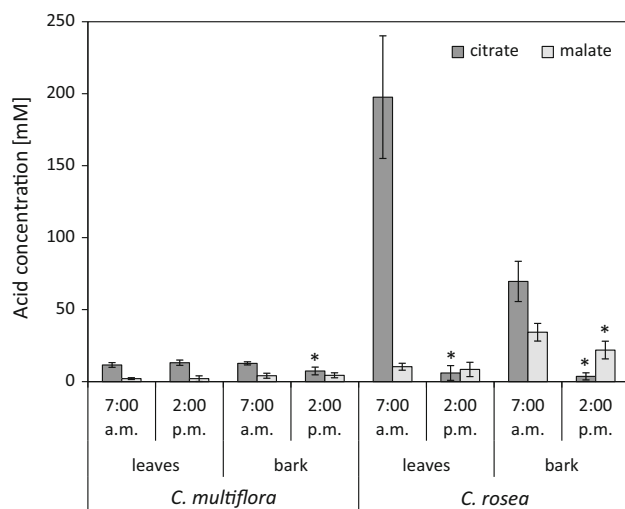
The chlorophyll amount calculated on the area of the plant organ in leaves of *C. multiflora* is higher than in bark (Fig. 5). Opposite to this in *C. rosea*, the total amount of both chlorophylls is lower in leaves in comparison to bark. A high amount of chlorophyll in bark (comparable to *C. multiflora* leaves) could be an additional factor enabling efficient photosynthesis in this plant organ. The chl $a$ /chl $b$  ratio is somehow lower or significantly lower in bark in comparison to their leaves in both tested *Clusia* species, and probably most of these differences are because of lower light intensity and different wavelengths of light reaching photosynthesizing cells localized in the bark. A lower chl $a$ /chl $b$  ratio indicates that the PSI/PSII activity ratio is lower (Walters 2005). Thus, we can expect that in the bark oxygen production in the water splitting system within PSII is important. The relationship between the  $\text{CO}_2$  and  $\text{O}_2$  concentration in photosynthesizing organs was studied also in detail in the case of fruits, organs with a limited possibility to exchange  $\text{CO}_2$  and  $\text{O}_2$  with the atmosphere (Borisjuk and Rollenschek 2009). In these bulky organs, covered with a



**Fig. 5** Chlorophyll content and chlorophyll  $a/b$  ratios of leaves and bark of *C. multiflora* (a) and *C. rosea* (b). Data are mean  $\pm$  SD ( $n = 5$ ). Asterisks indicate significant differences between leaves and stems according to Duncan's test;  $P \leq 0.05$

low-permeable epidermis, photosynthesizing cells deliver  $\text{O}_2$  for deeper heterotrophic layers including seeds (Lytovchenko et al. 2011). As mentioned for *Mesembryanthemum crystallinum* leaves in the CAM state (Miszalski et al. 2001), an excess of  $\text{O}_2$  in *Clusia* probably cannot diffuse out of stems and can be removed during respiration in mitochondria or photorespiration in chloroplasts. This can help in  $\text{CO}_2$  production and its fixation by Rubisco in chloroplasts. Sap flux is considered the major source of  $\text{O}_2$  in the xylem (Gansert 2003), but the importance of  $\text{O}_2$  generated by stem photosynthesis may increase when sap flux is diminished. Gansert (2003) reported that when sap flow was reduced, the  $\text{O}_2$  deficit in *Betula pubescens* stems increased dramatically; under these conditions, any  $\text{O}_2$  provided by stem photosynthesis would help in maintaining adequate rates of respiration.

When comparing day/night fluctuations of malate and citrate, the two most important acids responsible for the accumulation of  $\text{CO}_2$  in CAM plants, we have shown that there are differences between both tested *Clusia* species



**Fig. 6** Malate and citrate concentrations in cell sap in the leaves and bark of *C. multiflora* and *C. rosea* collected at 7 a.m. and 2 p.m.; data are mean  $\pm$  SD ( $n = 5$ ). *T* tests were performed for values of corresponding pairs (7 a.m. and 2 p.m.) for leaves and stems separately. Asterisks indicate significant differences compared to 7 a.m. at  $P \leq 0.05$

(Fig. 6). As expected, in leaves of  $C_3$  *C. multiflora*, no day/night differences were identified in either the level of citrate or malate. In bark tissue only very small differences were observed in the citrate level, indicating that some small amounts of citrate (5 mM) are accumulated during the night hours and are decarboxylated during the morning hours. In leaves of CAM *C. rosea* no significant malate fluctuations were observed, and the very high differences in citrate levels show that mostly citrate acid participates in active CAM-type metabolism. Very clear nocturnal accumulation in citrate levels was also noted in bark tissue. Accumulation of citrate during night hours was not as high as in leaves, and its decarboxylation was very efficient. Thus, the citrate level during the afternoon hours was very low.

If we assume that photosynthesizing cells in bark tissue obtain much less light energy than leaf cells, we can compare these measurements with those we presented in experiments made on another CAM plant. *C. hilariana* (Miszalski et al. 2013). Also in these experiments leaves kept at low light accumulated much less malate than leaves growing in high light, and the citrate level even increased during the day. Our experiments on *C. hilariana* allowed us to speculate that in plants growing at high light, the TCA cycle is less active during the daytime because photosynthesis delivers most of the necessary energy and helps to use citrate. As suggested by the results in Fig. 3, the function of mitochondria during the day has to be reduced because of limited access to oxygen from the air. Mitochondria can use only the oxygen that is produced in photosynthesis. However, this citrate level and mitochondrial

activity are reduced, and the  $CO_2$  produced in the decarboxylation process is fixed in photosynthesis. However, the  $O_2$  concentration in phase III of CAM behind closed stomata can be very high so that even photorespiration occurs (Lüttge 2011). It can be also suggested that an important role of chloroplasts in the stems of *Clusia* is to deliver oxygen for respiration during the daytime. This explanation would be also helpful to understand why many *Clusia* plants occupy very dry niches and may grow with relatively low photosynthetic gas exchange, and they often show parallel malate and citrate fluctuations during the day/night cycle. This can also explain the high plasticity of the Clusiaceae family to changing environments (Lüttge 2006).

## Conclusions

1. Stems of both *Clusia* species representing  $C_3$  and CAM are closed structures, characterized by low gas exchange with the atmosphere.
2. Available  $CO_2$  within stems is limited, and possibly also efflux of  $O_2$  produced because of photochemical activity of PSII is slow. Thus, internally produced  $O_2$  can be used for photorespiration and dark respiration. This seems to be more important for trees from arid environments like *Clusia* in comparison to trees in temperate forests.
3. Not only in leaves but also in stems, the obligatory CAM plant *C. rosea* performs  $\beta$ -carboxylation.

**Author contribution statement** ZM, AK, and JP conceived and designed experiments; MK and ZM wrote the manuscript; AK and UL prepared the figures and redaction of the manuscript; MK performed most experimental analyses; KT performed Western blot analysis; ZM, AK, and UL carried out data analysis and JP pigment analysis.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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