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Leaf development, gas exchange characteristics, and photorespiratory activity in maize seedlings

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Abstract

Five decades ago, a novel mode of CO_2 assimilation that was later described as C_4-photosynthesis was discovered on mature leaves of maize (*Zea mays* L.) plants. Here we show that 3- to 5-day-old developing maize leaves recapitulate the evolutionary advance from the ancient, inefficient C_3 mode of photosynthesis to the C_4 pathway, a mechanism for overcoming the wasteful process of photorespiration. Chlorophyll fluorescence measurements documented that photorespiration was high in 3-day-old juvenile primary leaves with non-specialized C_3-like leaf anatomy and low in 5-day-old organs with the typical "Kranz-anatomy" of C_4 leaves. Photosynthetic gas (CO_2)-exchange measurements on 5-day-old leaves revealed the characteristic features of C_4 photosynthesis, with a CO_2 compensation point close to zero and little inhibition of photosynthesis by the normal oxygen concentration in the air. This indicates a very low photorespiratory activity in contrast to control experiments conducted with mature C_3 sunflower (*Helianthus annuus* L.) leaves, which display a high rate of photorespiration.

Additional key words: leaf development, maize seedlings, photorespiration, photosynthesis.

Introduction

Fifty years ago, shortly after Melvin Calvin and coworkers had elucidated the "path of carbon in photosynthesis" (Bassham and Calvin 1957), the Russian scientist Y. S. Karpilow published a remarkable paper in the *Annual Report of the Kazan Agricultural Institute*. Using mature leaves of the tropical grass maize (*Zea mays* L.) as experimental material, Karpilow (1960) discovered that this plant is characterized by a unique mode of CO_2-fixation that did not fit into the "universal scheme" known at that time. Based on extensive studies with suspension cultures of unicellular green algae (*Chlorella pyrenoidosa*), the "Calvin-team" had unequivocally shown that the first product of CO_2 assimilation is the C_3-compound 3-phosphoglycerate and concluded that this photosynthetic pathway may be established in all green (drifting and sessile) organisms on Earth (*i.e.*, planktonic algae, water- and land plants) (Bassham and Calvin 1957). Contrary to his biased expectation, Karpilow (1960) found that in maize leaves the fixed radioactivity ($^{14}CO_2$) first appeared in C_4-compounds such as malate and aspartate, with only *ca*. 10 % of the labelled carbon in "Calvin's C_3-acid" 3-phosphoglycerate. Karpilow (1960) concluded that his results were "not characteristic of other plant species". However, in subsequent publications, Karpilow retracted this interpretation and attributed his unexpected maize data to experimental artefacts (*see* Hatch 1992, 2002, El-Sharkawy 2009).

The definitive discovery and detailed reconstruction of the mechanism of C_4-photosynthesis occurred in the early 1960s in the laboratory of the *Colonial Sugar Refining Company* in Brisbane, Australia. As described in excellent reviews (Hatch 1992, 2002), experiments were designed to trace the exact pathway of CO_2 assimilation in sugercane (*Saccharum officinarum*) leaves, using the ^{14}C-labelling protocols originally developed by Calvin and coworkers (Bassham and Calvin 1957, Calvin 1989). In accordance with Karpilow (1960), it was documented that most of the radioactivity incorporated after brief periods of $^{14}CO_2$-application was localized in C_4-acids

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Abbreviations: Chl – chlorophyll; CO_2-CP – carbon dioxide compensation point; $\Delta F/F_m'$ – effective quantum yield of PSII of light-adapted leaves; PSII – photosystem II.
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(malate, aspartate), which were transformed into the C_3-compound 3-phosphoglycerate, later into hexose phosphates, and finally into sucrose and starch (Hatch and Slack 1966). At about the same time, Forrester *et al*. (1966) discovered that mature maize leaves lacked photorespiration, a process that leads to oxygen inhibition of photosynthesis in most other plants.

Despite of decades of research on C_4-photosynthesis in maize and related taxa one question related to Karpilow's original finding is still a matter of debate (Dai *et al*. 1995, Ghannoum *et al*. 1998, Leakey *et al*. 2006): Is the mechanism of carbon assimilation in young maize leaves the same as that of C_3 plants (and the green algae *Chlorella*), which represents the phylogenetically "ancient", less efficient mode of photosynthesis (Sage 2004, Kutschera and Niklas 2006), or do maize seedlings develop as C_4-photosynthesizers (*see* Ghannoum *et al*. 1998)?

To address this question, we analysed the development of the primary leaf in maize seedlings and measured light-dependent chlorophyll (Chl) fluorescence- and gas (CO_2)-exchange characteristics of this developing organ. Based on our anatomical and physiological data we document that the first leaf of the maize seedling exhibits C_3-like features, but some days later, the unfolding lamina exhibits Chl fluorescence and resistance to O_2 inhibition of photosynthesis typical of the C_4-mode of CO_2-assimilation.

Materials and methods

The experiments were carried out with a commercial variety of maize (*Zea mays* L. var. Hybridmais Liberal), obtained from *Schmitz and Laux*, Hilden, Germany. Caryopses were pre-soaked for 4 h in water and thereafter grown in moist general purpose soil (closed, transparent plastic boxes, 14 × 12 × 9 cm; depth of the soil *ca*. 5 cm) in a 16 h dark/8 h white light cycle [*ca*. 150 µmol (photon) m^{-2} s^{-1} at plant level; temperature: 25 ± 1°C]. For determination of the length of the primary leaf and fresh mass of the organ, seedlings of average size were harvested 3, 4, and 5 days after sowing (Fig. 1).

Anatomical studies and quantification of leaf thickness: Sections, 1–2 mm in length, were excised from 3-, 4-, and 5-day-old organs and preserved in 70% formalin, acetic acid and alcohol. The samples were dehydrated in a graded alcohol series and thereafter embedded in Spurr's resin. Leaf cross sections (thickness *ca*. 2–4 µm) were cut by a microtome *1512* (*Leitz*, Wetzlar, Germany), mounted on coated slides and stained with 0.03% toluidine blue O, pH 7.0. Sections were observed under bright-field microscopy using a *Leitz* light microscope, equipped with a digital camera as described by Scherp *et al*. (2001).

Determination of leaf pigments: 10 sections, 10 mm in length, were cut from the sub-apical region (*ca*. 2 mm below the tip) of 3-, 4-, and 5-day-old leaves and weighed. The samples were homogenized (mortar and pestle, quartz sand) in the presence of 1 ml of ethanol *p. a*., transferred into Eppendorf cups and centrifuged. The supernatant (pigment extract) was transferred into a cuvette and Chl (*a*+*b*) and the total amount of carotenoids determined, using a spectrophotometric assay. Pigment contents were calculated as described (Jucknischke and Kutschera 1998).

Photosynthesis and photorespiratory activity were measured with a *LI-6400 Portable Photosynthesis System* (*LI-COR, Inc*., Lincoln, Nebraska, USA) as described by Jucknischke and Kutschera (1998). The expanded leaf blade of an intact 5-day-old maize seedling (Fig. 1*C*) was clamped into the leaf chamber (3 × 2 cm) of the system. Net photosynthesis (uptake of CO_2 per m^{-2} s^{-1} at within-leaf concentrations, C_i , that varied between 0 and 1,200 µmol(CO_2) mol^{-1}(air) at a photon fluence of 1,000 µmol(photon) m^{-2} s^{-1}; 25°C; 70% rel. humidity) was measured in air (at 21 vol. % O_2). The leaf area within the cuvette was determined with a mm-scale and the values were recalculated accordingly. Stomatal conductance of the leaves varied between 0.1 and 0.5 mol m^{-2} s^{-1}.

To evaluate photorespiratory activity, each series of measurements was repeated on the same leaf at O_2-concentration of the air of *ca*. 1%. This was achieved by mixing nitrogen gas, N_2, with normal air in a mixing ratio that resulted in about 1% O_2 which was monitored with an oxygen electrode. A series of control measurements were performed on the expanded primary leaves of 28-day-old sunflower seedlings (*Helianthus annuus* L., cv. Sunspot) that were raised as described above. All measurements were repeated at least 4 times with different batches of seedlings (10 per experiment) and representative results are depicted in the figures.

Chlorophyll fluorescence was measured with a *Portable Chlorophyll Fluorometer PAM-2100* (*Heinz Walz GmbH*, Effeltrich, Germany) on the tip of a maize leaf enclosed in the chamber of the *LI-6400* with a mounting for the fibre optics of the fluorometer. Prior to the experiment, the seedlings were kept in darkness for at least 30 min, and quantum yield of dark-adapted leaves (F_v/F_m) was measured, calculated as $F_v/F_m = (F_m - F)/F_m$, with F as the steady-state and F_m as the maximum fluorescence. Then, the actinic light was turned on [200 µmol(photon) m^{-2} s^{-1}] and a saturating pulse [duration: 0.8 s, intensity *ca*. 5,000 µmol(photon) m^{-2} s^{-1}] was applied every 60 s. The effective quantum yield of photosystem (PS) II of light- adapted leaves ($\Delta F/F_m'$) was calculated as follows: $\Delta F/F_m' = (F_m' - F')/F_m'$, with F′ as the steady-state

fluorescence prior to the flash, and F_m' as maximum fluorescence, measured while saturated light flashes were applied. The temperature was kept constant at 25°C and CO_2 concentration at 400 μmol mol^{-1}. The experiment started with ambient oxygen concentration (21 vol. %) and, after 10 min, the oxygen content was changed to *ca*. 1 vol. % O_2 by substituting air with nitrogen gas. Experiments with 3- to 10-day-old seedlings were repeated 7 times.

Statistical analysis was performed with the average values of the $\Delta F/F_m'$-measurements obtained under ambient- and low oxygen concentrations by applying the *t*-test with the null hypothesis that there is no significant difference between the treatments and significant differences for $P<0.05$. The test was performed with the software *SigmaPlot V10.0* (*Systat Software Inc.*, Chicago, USA).

Results

Representative individuals selected from populations of 3-, 4-, and 5-day-old maize seedlings that were grown in a 16 h white light/8 h dark-cycle are shown in Fig. 1. Under these conditions, the pale-green, enrolled primary leaf emerged on day 3 (Fig. 1*A*); 24 h later, this organ had entirely pierced and destroyed the tip of the coleoptile (Fig. 1*B*). On day 5 after sowing, the green leaf blade unfolded and the next, tube-shaped leaf of the seedling emerged (Fig. 1*C*).

The changes in fresh mass, length, and average thickness of the primary leaf, measured between days 3 and 5 after sowing, are summarized in Table 1, together with the contents of the photosynthetic pigments Chl *a*, Chl *b*, and carotenoids. It is apparent that the concentration of leaf pigments is very low at day 3 and displays a large increase during the unfolding of the organ.

The changes in leaf anatomy during development of the maize seedling are shown in Fig. 2. In the lamina of the unfolded primary leaf of 5-day-old plants, the features of atriplicoid Kranz-anatomy are apparent (Fig. 2*C*). The lignified vascular tissues are entirely surrounded by large, round bundle sheath (Bs) cells that are characterized by centripetally arranged chloroplasts. The Kranz cells are surrounded by a tissue composed of mesophyll cells (Mc) with chloroplasts equally distributed in the peripheral cytoplasm of these non-specialized leaf cells. This specific internal arrangement of cells surrounding the vascular bundles was not detectable in the enrolled primary leaf of 3-day-old seedlings (Fig. 2*A*). In 4-day-old leaves that were not yet unfolded an intermediate stage in organ development was apparent (Fig. 2*B*). Some juvenile Bs cells can be identified that are round and morphologically distinguishable from the outer mesophyll tissue, but no complete wreath surrounding each bundle was detectable. Hence, our anatomical studies document that in juvenile maize leaves no specialized ring of cells, which encircles the vascular bundle, has yet developed. The unique C_4-type "Kranz-anatomy" is only established after the unfolding of the lamina of the primary leaf (Fig. 2*C*).

Fluorescence measurements documented that quantum yield of PSII in dark-adapted leaves (*i.e.*, quantum efficiency) increased (F_v/F_m-values of 0.41 ± 0.04 in 3-, 0.62 ± 0.03 in 4-, 0.69 ± 0.02 in 5-, and 0.76 ± 0.01 in 10-day-old seedlings, respectively, were measured; means ± SE, n = 7). In light, steady-state values of $\Delta F/F_m'$ were obtained after 4 to 5 min of illumination at 400 μmol mol^{-1} and after switching to oxygen-reduced

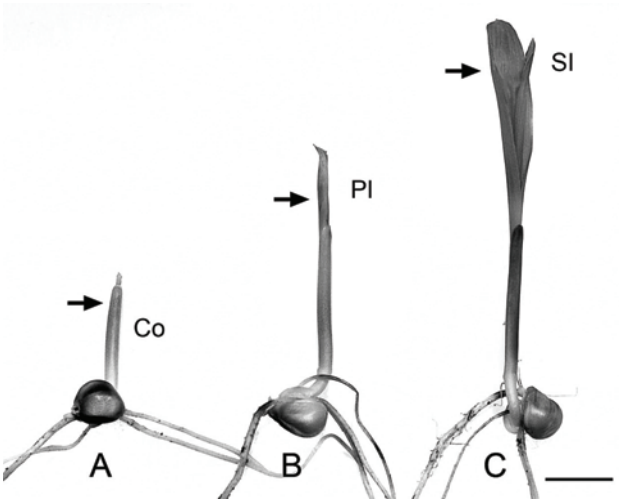

Fig. 1. Stages in the development of maize (*Zea mays*) seedlings that were raised in a light/dark regime. Photographs of juvenile plants of average size were taken at day 3 (*A*), 4 (*B*) and 5 (*C*) after sowing. The *arrows* point to the sub-apical region of the primary leaf from which 1-mm sections were excised for anatomical analyses. Co – coleoptile, Pl – primary leaf, Sl – secondary leaf. *Bar* = 1 cm.

Table 1: Changes in fresh mass (FM), leaf length (L), thickness of the organ in the subapical region (Th) and pigment contents (Chl *a/b*, carotenoids) in the primary leaf of each maize (*Zea mays*) seedlings. Data represent means (± SE) of 12 measurements.

Time [d]	FM [mg]	L [mm]	Th [μm]	Chl *a* [mg g^{-1}(FM)]	Chl *b* [mg g^{-1}(FM)]	Carotenoids [mg g^{-1}(FM)]
3	26.6 ± 1.3	25.4 ± 0.6	55 ± 2	0.24 ± 0.02	0.16 ± 0.01	0.11 ± 0.01
4	80.9 ± 4.0	45.3 ± 0.9	89 ± 2	0.89 ± 0.02	0.33 ± 0.01	0.23 ± 0.01
5	130.0 ± 5.2	86.0 ± 1.7	133 ± 3	1.91 ± 0.02	0.57 ± 0.02	0.47 ± 0.02

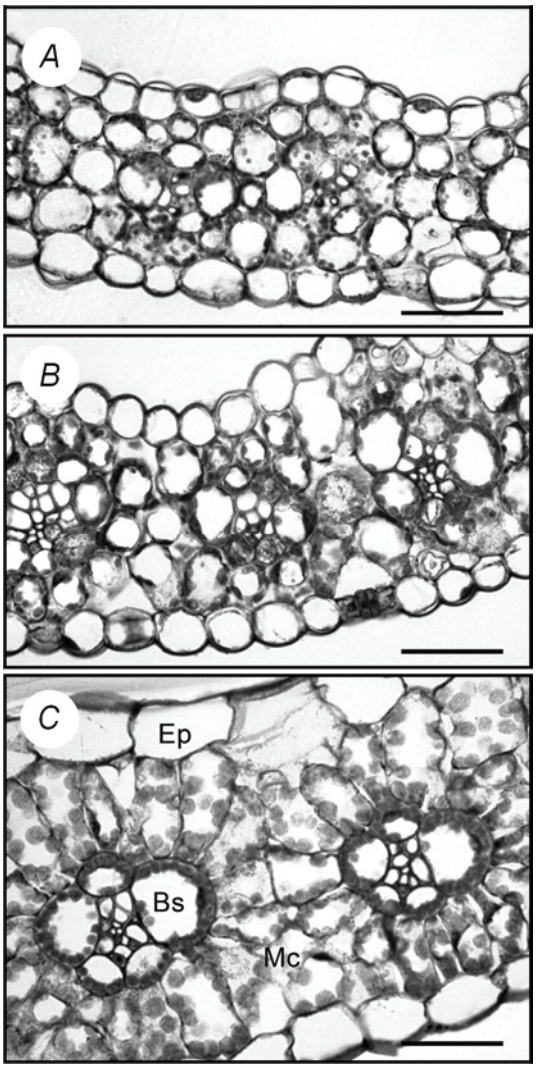

Fig. 2. Light micrographs of cross sections through the subapical region of the primary leaf of developing maize seedlings. The plants were 3 (*A*), 4 (*B*), and 5 (*C*) days old, respectively (*see* Fig. 1). Note that the leaf of the 5-day-old seedling (*C*) had a fully developed Kranz-anatomy, which was not apparent in 3- and 4-day-old samples (*A*, *B*). Bs – bundle sheath tissue, Ep – epidermis, Mc – mesophyll cell. *Bars* = 25 µm.

environmental conditions. In the almost complete absence of O_2 (*ca*.1 vol. %), the effective quantum yield ($\Delta F/F_m'$) was significantly lower in the 3- and 4-day-old seedlings, while this treatment exerted no detectable effect in 5- and 10-day-old plants (Fig. 3). The considerable decrease in $\Delta F/F_m'$ documents that oxygen is a sink for electrons in 3-day-old, but not in 10-day-old seedlings. This indicates that photorespiration is large in 3-day-old organs, and very low or zero in more mature maize leaves.

A representative photosynthetic gas (CO_2)-exchange analysis of the unfolded lamina of a 5-day-old primary leaf of average size (Fig. 1*C*) is shown on Fig. 4. Internal CO_2 concentration was enhanced and reached

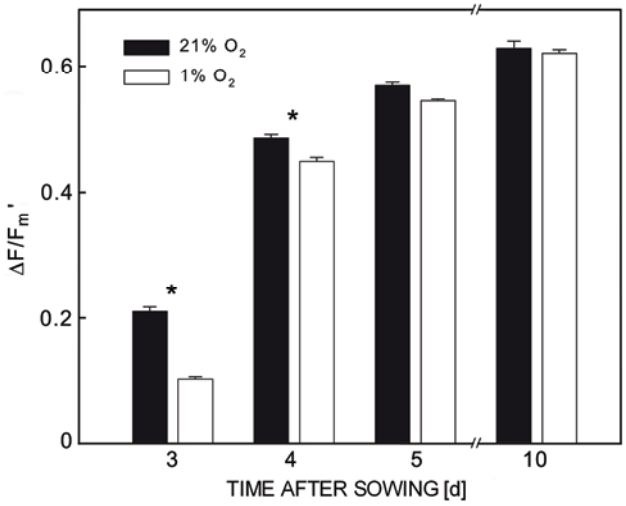

Fig. 3. The dependence of the effective quantum yield of PSII in light-adapted primary leaves ($\Delta F/F_m'$) of maize seedlings on the changes from ambient (*black bars*) to an oxygen-reduced atmosphere (*white bars*) (21 and *ca*. 1 vol. % O_2, respectively). Statistically significant differences between the treatments were recorded on days 3 and 4 after sowing ($P<0.05$, indicated by the symbol *). On days 5 and 10, no significant differences were measured (means ± SE, $n = 7$).

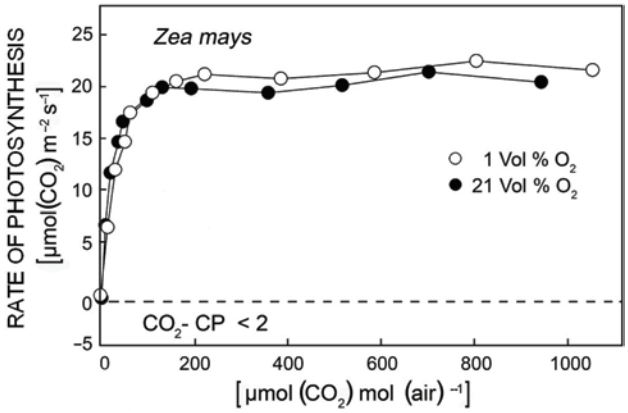

Fig. 4. Representative gas-exchange curves of an intact primary leaf from a 5-day-old maize seedling measured in normal air (21 vol. % O_2) and under anaerobic conditions (*ca*. 1 vol. % O_2), respectively. The CO_2-compensation point (CP) was lower than 2 µmol(CO_2) mol^{-1}(air). During the measurements at 12 different external CO_2 concentrations the photosynthetic photon flux density was 500 µmol m^{-2} s^{-1} at plant level.

a constant value of about 21 µmol CO_2 m^{-2} s^{-1} at saturating gas concentrations between 200 and 1,000 µmol(CO_2) mol^{-1}(air) (average value of 4 independent measurements: 21.8 ± 0.8 µmol(CO_2) m^{-2} s^{-1}, mean ± SE). Under anaerobic conditions (O_2 concentrations *ca*. 1 vol. %), similar assimilation/CO_2-curves were obtained, but the rate of photosynthesis at saturating CO_2-concentrations was slightly (2 to 5%) lower than in the control (21 vol. % O_2). In addition, the photosynthetic CO_2 assimilation rates, measured at 21 vol. % of

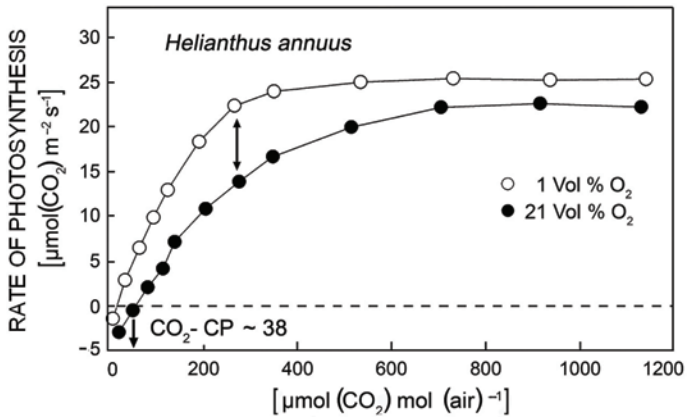

Fig. 5. Representative gas-exchange curves of an intact primary leaf from a 4-week-old sunflower seedling measured in normal air (21 vol. % O_2) and under anaerobic conditions (*ca*. 1 vol. % O_2), respectively. The CO_2-compensation point (CP) was about 38 µmol(CO_2) mol^{-1}(air) (21 vol. % O_2) and considerably lower under anaerobic conditions (*ca*. 1 vol. % O_2). During the measurements at 12 different external CO_2 concentrations the photosynthetic photon flux density was 500 µmol m^{-2} s^{-1} at plant level. *The arrow* indicates the extent of photorespiration at normal external CO_2 concentration [~ 350 µmol(CO_2) mol^{-1}].

atmospheric oxygen (*i. e*., in normal air), rapidly increased as the compensation point (CP) was estimated; very low values of less than 2 µmol (CO_2) mol^{-1}(air) were obtained. Similar assimilation/O_2-curves were measured when leaves of 14-day-old and mature maize plants were clamped into the *LI-COR* gas-exchange analyzer, but the maximum values were higher [25 to 28 µmol(CO_2) m^{-2} s^{-1}] (data not shown).

As a control, additional gas-exchange measurements were performed on the unfolded primary leaves of 4-week-old sunflower plants (Fig. 5). Assimilation rates, measured at 21% O_2, were similar as in maize leaves, but a large (*ca*. 25 to 30%) enhancement in photosynthesis was recorded when the oxygen content of the air was lowered to *ca*. 1 vol. % (anaerobic conditions). At 21 vol. % O_2, the CO_2-CP was *ca*. 38 µmol(CO_2) mol^{-1}(air).

Much lower CP values of *ca*. 2 to 4 µmol (CO_2) mol^{-1}(air) were obtained after the atmospheric oxygen concentration was reduced. Hence, the primary leaf of 5-day-old maize seedlings (Fig. 4) shows CO_2 gas-exchange characteristics of a mature C_4 plant, whereas sunflower (Fig. 5) behaves like a typical C_3 photosynthesizer (Tregunna *et al.* 1966).

Discussion

Numerous studies have unequivocally shown that in C_3 leaves the production of oxygen-dependent photorespiratory CO_2 decreases net carbon dioxide assimilation by approximately 25% at 25°C and that this futile process increases greatly at higher temperatures (Zelitch *et al*. 2009). The mechanism of this loss of carbon dioxide in irradiated C_3 leaves is likewise no longer a matter of debate. It has been documented that the dual function of the enzyme ribulose-1,5-bisphosphate carboxylase/ oxygenase at high O_2 (21 vol. % in normal air) leads to the toxic by-product glycolate, which is converted into other compounds *via* the photorespiratory C_2 cycle (Berry *et al*. 1978, De Veau and Burris 1989, Dai *et al*. 1995, Sage 2004, Zeltich *et al*. 2009). Moreover, it is well established that in mature leaves of typical C_4 plants, such as maize, photorespiratory CO_2 loss is greatly reduced, due to the cooperation between the mesophyll and bundle sheath-cells, which act as a "CO_2-pump" (Berry *et al*. 1970, 1978; Sage 2004, Majeran and van Wijk 2009, El-Sharkawy 2009; *see* Voznesenskaya *et al.* 2001 for the description of C_4 photosynthesizers without Kranz-anatomy).

Despite of these insights, a controversy exists in the literature whether or not young leaves of the C_4 plant *Zea mays* are characterized by a C_3-like pathway or develop directly as C_4-type organs (Dai *et al*. 1995, Ghannoum *et al*. 1998). This open question is related to the expected rise in atmospheric CO_2 concentration over the next decades with respect to a possible stimulation of photosynthesis in C_3 plants, while maize and other C_4 plants may be unaffected (Leakey *et al*. 2006).

Our results document that the juvenile, enrolled primary leaf lacks "Kranz-anatomy" and hence has no "CO_2-pump." Concomitant with the unfolding of the lamina of the primary leaf, the "CO_2-pump" (Kranz-anatomy) develops (Figs. 1, 2) and the juvenile maize seedling starts to behave more and more like a mature C_4-photosynthesizer (Figs. 3, 4). The decreasing sensitivity of the effective quantum yield ($\Delta F/F_m'$) to atmospheric oxygen indicates that the rate of photorespiration decreases rapidly during early development of the maize seedling. By day 10 after sowing, no effect of oxygen-free air on $\Delta F/F_m'$ could be detected (Fig. 3), indicating that photorespiration is negligible at this stage of organ development. Hence, the juvenile maize leaf "recapitulates" the unspecialized anatomy of its evolutionary C_3-like ancestor according to Ernst Haeckel's classical "ontogeny *vs.* phylogeny"-hypothesis (Stebbins 1950; Kutschera and Briggs 2009; Kutschera and Niklas 2009; Niklas and Kutschera 2009, 2010). It is obvious that the rate of net photosynthesis of the pale-green, enrolled 3-day-old primary leaf that is surrounded by the coleoptile and characterized by low chlorophyll content (Table 1) must be low. This conclusion is supported by

a low quantum yield of PSII Chl fluorescence in dark- and light-adapted leaves which are very sensitive to lowered oxygen concentrations, documenting a high rate of photorespiration under ambient environmental conditions (21 vol. % O_2) (Fig. 3).

Finally, it should be mentioned that our data show that the rate of photosynthesis in the primary leaf of maize seedlings reaches a constant value at a low carbon dioxide concentration of *ca*. 200 μmol(CO_2) mol^{-1}(air) (Fig. 4). In contrast, in the leaf of the representative C_3 plant studied here (Fig. 5), at 21 vol. %, a "CO_2-enhancement effect" of net carbon assimilation was measured that reached a plateau at *ca*. 800 μmol(CO_2) mol^{-1}(air). We conclude that the first unfolded leaf of the juvenile *Zea mays* plant rapidly develops an efficient biochemical "CO_2-pump" that is largely insensitive to rising concentrations of carbon dioxide in the surrounding air.

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