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Impact of anatomical traits of maize (*Zea mays* L.) leaf as affected by nitrogen supply and leaf age on bundle sheath conductance

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ABSTRACT

The mechanism of photosynthesis in C_4 crops depends on the archetypal Kranz-anatomy. To examine how the leaf anatomy, as altered by nitrogen supply and leaf age, affects the bundle sheath conductance (g_{bs}), maize (*Zea mays* L.) plants were grown under three contrasting nitrogen levels. Combined gas exchange and chlorophyll fluorescence measurements were done on fully grown leaves at two leaf ages. The measured data were analysed using a biochemical model of C_4 photosynthesis to estimate g_{bs}. The leaf microstructure and ultrastructure were quantified using images obtained from micro-computed tomography and microscopy. There was a strong positive correlation between g_{bs} and leaf nitrogen content (LNC) while old leaves had lower g_{bs} than young leaves. Leaf thickness, bundle sheath cell wall thickness and surface area of bundle sheath cells per unit leaf area (S_b) correlated well with g_{bs} although they were not significantly affected by LNC. As a result, the increase of g_{bs} with LNC was little explained by the alteration of leaf anatomy. In contrast, the combined effect of LNC and leaf age on S_b was responsible for differences in g_{bs} between young leaves and old leaves. Future investigations should consider changes at the level of plasmodesmata and membranes along the CO_2 leakage pathway to unravel LNC and age effects further.

1. Introduction

Improving the efficiency of photosynthesis could contribute to better food security under an unprecedented rise in global population and climate-change [1,2]. The photosynthesis pathway in C_4 plants enables them to be more efficient in solar-use, nitrogen-use and water-use than C_3 plants [3,4]. In C_4 plants, CO_2 is initially fixed by phospho*enol*pyruvate carboxylase (PEPc) in mesophyll cells, and the resulting metabolites move into the bundle sheath cells where they are decarboxylated into CO_2 and re-fixed by Rubisco. The association of the two cell types, combined with highly regulated enzyme activities, creates a biochemical carbon concentration mechanism (CCM) resulting in an elevated CO_2 concentration nearby the fixation sites of Rubisco [5]. This mechanism effectively suppresses photorespiration, thereby yielding high photosynthetic resource-use efficiencies.

The efficiency of the CCM relies on the concerted action of anatomical, biochemical and biophysical mechanisms [5–8]. It has been well known from C_3 photosynthesis studies that leaf anatomy impact photosynthesis as it influences the physical obstruction to CO_2 diffusion. The leaf boundary layer and stomatal conductance affect diffusion of CO_2 towards the stomatal cavity. The mesophyll conductance (g_m) constrains the diffusion from sub-stomatal cavities into CO_2-fixation sites in mesophyll. The distribution of stomata and the connectivity of intercellular airspaces affect the diffusion of CO_2 in the gaseous phase, while the properties of the cell wall such as thickness and porosity, the plasma membrane and presence of carbonic anhydrase affect the diffusion in the liquid phase [9–11]. While these phenomena occur in C_4 photosynthesis as well, C_4 photosynthesis is also affected by CO_2 retro-diffusion from bundle sheath cells back into mesophyll cells. This retro-diffusion, also called 'CO_2 leakage', partially increases the CO_2 levels of the mesophyll cells [12] and is constrained by resistance of the mesophyll-bundle sheath interface [13]. The inverse of this resistance is known as the bundle sheath conductance (g_{bs}). The lower g_{bs}, the lower is CO_2 retro-diffusion from bundle sheath cells, and

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thus the higher is the efficiency of the CCM [5,8,14,15]. Leakiness, a physiological variable often used to characterize retro-diffusion of CO_2 from bundle sheath cells back to mesophyll cells relative to the rate of PEP carboxylation, depends greatly on g_{bs}.

C_4 photosynthetic efficiency has been proposed to depend on a number of anatomical properties of the leaves. For instance, a low permeability of bundle sheath cell wall to CO_2, a high surface of mesophyll cells to volume ratio and features such as close proximity of mesophyll and bundle sheath cells, among others, are essential to the effectiveness of the CCM [5,9,16,17]. Moreover, the shorter vein spacing in C_4 plants than in C_3 plants has been shown to be beneficial for high quantum yield [18]. CO_2 retro-diffusion has also been found to be influenced by the diffusive properties of the stroma and the chloroplast envelope [19]. Thus, the significance of leaf anatomy and ultrastructure of C_4 plants to the efficiency of C_4 photosynthesis continues to be extensively studied [6,16,17,20–23].

CO_2 conductances in C_4 plant leaves were recently estimated using combined gas exchange and chlorophyll fluorescence measurements [24,25] or with carbon isotope discrimination measurements [8,15,25,26] in analogy to the methods used to estimate g_m in C_3 leaves [27–29]. Gas exchange and chlorophyll fluorescence measurements result in CO_2 and irradiance responses of net photosynthesis and quantum efficiency of PSII electron transport, which are then used to parameterize biochemical model of von Caemmerer & Furbank [30] and estimate g_m and/or g_{bs}. The procedures to estimate these conductances using various software tools are readily accessible [24,32]. In addition, the benefits of chlorophyll fluorescence measurements in C_4 plants have been substantiated [24,25,33,34]. Using these methods, g_{bs} was found to vary with nitrogen supply [24], growth light [7,25,26], leaf age [24,35,36], and temperature [34].

Very few studies measured leaf anatomical properties and estimated g_{bs} or g_m in C_4 plants to examine their relationship [7,26]. These properties include the exposed surface area of mesophyll cells per unit of leaf area (S_{mes}), surface area of bundle sheath cells per unit of leaf area (S_b), leaf thickness and diameter of mesophyll and bundle sheath cells. When maize and *Flaveria bidentis* were grown under contrasting light environment, differences in S_{mes}, S_b [7], leaf thickness and cell diameter [26] contributed to the variations in g_{bs} or g_m. A negative correlation of bundle sheath resistance with leaf nitrogen content was reported for maize in a recent study [24]. At that time, it was only presumed to be due to S_b and cell wall thickness being altered by nitrogen treatment. In addition, an increase in g_{bs} was suggested when C_4 plants were grown at elevated CO_2 [37] or temperature [38] due to a decrease in wall thickness of the bundle sheath.

The relationships between photosynthesis and leaf anatomical properties have commonly been investigated using chemically fixed leaf tissue samples [6,7,17,39,40]. X-ray micro-computed tomography (X-ray micro-CT) also gives high-quality images that render the airspace between cells at sufficient contrast to allow quantification of anatomical features with the additional advantage of no requirement of intensive sample preparation and thus measurement artefacts are minimized [41–43]. In addition, X-ray micro-CT allows measurements over the intricate three-dimensional leaf geometry of any thickness but has a limitation in resolving leaf ultrastructural components [44,45].

In C_3 plants, it is well known that the cell wall strongly influences CO_2 diffusion and hence CO_2 fixation rate [46]. Whether and how the cell wall of the bundle sheath contributes to the variations in g_{bs} for C_4 plants with leaf nitrogen content and age were not investigated. The objectives of this research were (i) to study how bundle sheath conductance is affected by leaf nitrogen content and leaf age, (ii) to quantify leaf anatomical properties as altered by leaf nitrogen and age using combined microscopy and micro-tomography measurements, (iii) to relate these properties to CO_2 conductances of a maize (*Zea mays* L.) leaf. This will be achieved by using gas exchange and chlorophyll fluorescence measurements with biochemical models of C_4 photosynthesis [30] to estimate g_{bs}, and X-ray micro-CT, light and electron microscopy images to obtain microstructure and ultrastructure details of the leaf anatomy.

2. Materials and methods

2.1. Plants, treatments and photosynthetic measurements

Part of the data of our experiment was used to validate predictions of a C_4 photosynthesis model we presented in a recent publication [12]; therefore, the growth conditions and gas exchange measurements were described therein. In brief, maize (*Zea mays* L.) plants, hybrid 2-02R10074, were grown in a controlled glasshouse in four blocks. In each block, the three nitrogen treatment levels were 0.15 (N1), 0.50 (N2) and 1.25 (N3) g N per pot. There were two leaf ages: 19 d (young leaves) or 32 d (old leaves) counted after their first appearance. For the old leaves, the frequency of applying nutrients was increased to twice weekly after the fourth week (since nutrition application started) to minimize the decline of leaf nitrogen content with leaf age.

Combined gas exchange and chlorophyll fluorescence measurements were done in four replicates on the mid-portion of the 6th leaf, using a LI-6400XT open gas exchange system with an integrated fluorescence chamber head, enclosing a 2 cm^2 leaf area (LI-COR, Lincoln, NE, USA). The CO_2 responses of photosynthesis were measured at an incident light intensity of 1500 $\mu mol\ m^{-2}\ s^{-1}$ in steps of 380, 200, 100, 90, 80, 70, 60, 50, 380, 380, 500, 1000 and 1500 $\mu mol\ mol^{-1}$ allowing three minutes per step for photosynthesis to reach a steady-state. The light response curve was measured in leaves that were first dark-adapted for 25 min, in steps of 20, 40, 60, 80, 100, 200, 500, 1000, 1500 and 2000 $\mu mol\ m^{-2}\ s^{-1}$ allowing six minutes per step. The response curves were measured both at 2% and 21% O_2, and the IRGA calibration was adjusted for O_2 composition of the gas mixture according to the manufacturer's instructions. The ambient CO_2 was 250 $\mu mol\ mol^{-1}$ at 21% O_2 and 1000 $\mu mol\ mol^{-1}$ at 2% O_2. All measurements were made at leaf temperature of 25 °C and a leaf-to-air vapor difference within 1.0–1.6 kPa, with measurement flow rate of 400 $\mu mol\ s^{-1}$. In addition, using thermally killed leaves, the gas exchange data were corrected for CO_2 diffusion into and out of the leaf cuvette [47]. Simultaneously with the gas exchange measurements, the steady-state fluorescence (F_s) and maximum relative fluorescence (F'_m) were also measured. F_s was measured after photosynthesis reached a steady-state after each of the CO_2 or light steps. F'_m was measured after a saturating light-pulse of intensity greater than 8500 $\mu mol\ m^{-2}\ s^{-1}$ for a duration of 0.8 s. The quantum efficiency of PSII electron transport was calculated as $\Delta F/F'_m = (F'_m - F_s)/F'_m$ [48]. Following the photosynthetic measurements, leaf nitrogen content (Micro-Dumas combustion method, Thermo Scientific, elemental C/N analyzer, type: Flash 2000) and dry mass were determined from three leaf samples (per plant) of having an average area of 2.15 cm^2 that were dried to constant weight in an oven at 70 °C for 48 h.

2.2. X-ray micro-CT imaging

Maize plants of the same cultivar were grown in three replicates simultaneously with those used in the gas exchange measurement to study the leaf anatomy using microscopy and the tomography experiments. The effect of nitrogen on these plants was assessed from readings of a portable chlorophyll meter (SPAD-502, Minolta, Japan) [49]. Maize leaf tissue samples (5 mm × 5 mm), both for