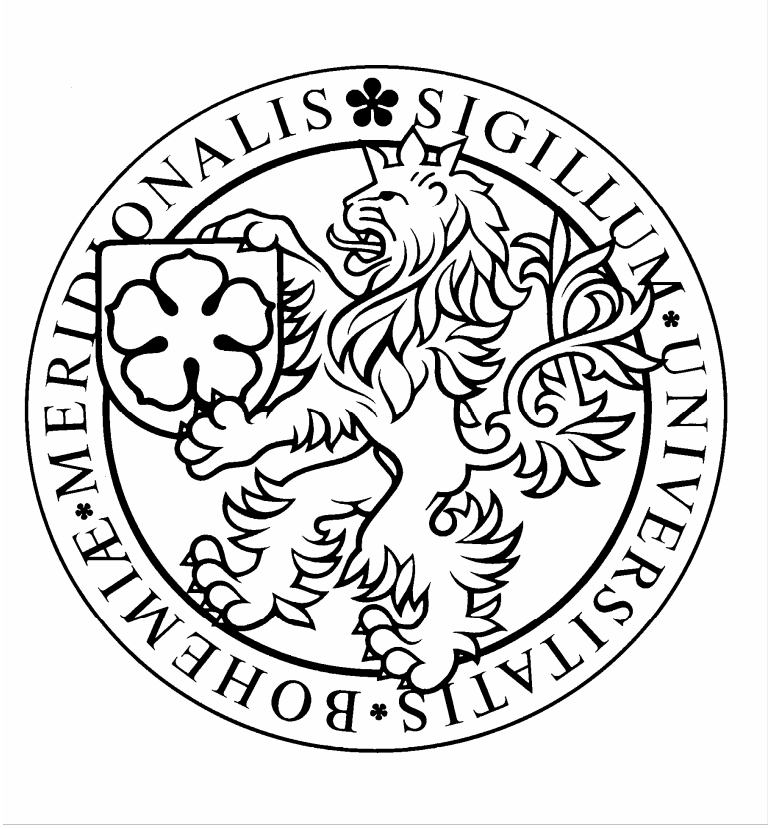
UNIVERSITY OF SOUTH BOHEMIA
FACULTY OF SCIENCE



Master Thesis

Effect of abscisic acid on mesophyll conductance at different CO_2 concentrations

Daniel Hisem, Bc.

Supervisor: Daniel Vrábl, Mgr.
Faculty of Science, University of South Bohemia

Supervisor specialist: Jiří Šantrůček, Doc., Ing., CSc.
Faculty of Science, University of South Bohemia

České Budějovice 2011

HISEM, D. 2011: Effect of abscisic acid on mesophyll conductance at different CO_2 concentrations. Mgr. Thesis, in English. – 53 p., *Faculty of Science, University of South Bohemia, České budějovice, Czech Republic.*

Annotation:

Mesophyll conductance to CO_2 transport is one of crucial components of diffusionall limitations of photosynthesis and is characterized by CO_2 flux from sub-stomatal cavity to chloroplast stroma. Using *variable J* method, mesophyll conductance was estimated over the range of CO_2 concentrations in absence and presence of low concentration of abscisic acid in hydroponically grown sunflower and poplar plants. Presented study proved that mesophyll conductance is sensitive to varying CO_2 concentration and is increased in presence of low concentration of abscisic acid. Results were discussed with respect to possible regulation of mesophyll conductance.

Prohlašuji, že svoji diplomovou práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své diplomové práce, a to v nezkrácené podobě elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

V Českých Budějovicích dne 3. ledna 2011 .....

Daniel Hisem

Poděkování

Rád bych poděkoval všem, kteří přispěli k úspěšnému dokončení mé diplomové práce, čili v první řadě školiteli Danovi a konzultantovi Jirkovi. Obou si velmi vážím nejen jako odborníků, ale hlavně jako lidí a děkuji jim. Martině děkuji za pozitivně kritické připomínky k textu i za odborné rady a věcnou diskuzi. Petře děkuji za pomoc s pěstováním rostlin. Celé Katedře fyziologie rostlin, včetně všech psích mazlíčků, chci poděkovat za příjemné a důstojné zázemí, kterého si také vážím a myslím, že není běžné.

Velký dík patří také mým rodičům, dále Tomášovi a Věře a ostatním blízkým a přátelům za jejich vytrvalou podporu. Ze všeho nejvíce chci poděkovat své Markétce za její obětavou podporu, trpělivost a vynikající zázemí. Velmi to oceňuji a děkuji ji za to.

Content

1. Introduction

1.1 Carbon dioxide diffusion and mesophyll conductance

Carbon dioxide (CO_2), which is necessary for photosynthesis, comes from the atmosphere surrounding a leaf of a plant (C_a) and it has to reach the sites of carboxylation in the chloroplast. Within its pathway, several barriers occur that finally reduce CO_2 concentration in the site of carboxylation. First diffusional barrier to CO_2 transport is thin, non-moving layer of air at the leaf surface called boundary layer. The second one is stomatal resistance, then resistance of intercellular airspaces and finally mesophyll resistance – on a boundary between gaseous environment outside the mesophyll cell and liquid environment inside the cell (**Fig. 1.1**).

Although calculating with resistance is more convenient for a pathway with a series of limitations (Evans and von Caemmerer, 1996), components of CO_2 diffusion pathway are often described as conductance rather than resistance, especially when considering fluxes. Mathematically, conductance is reciprocal to resistance ($g = 1/r$). Presented study is focused on CO_2 fluxes; hence it will consider conductance rather than resistance.

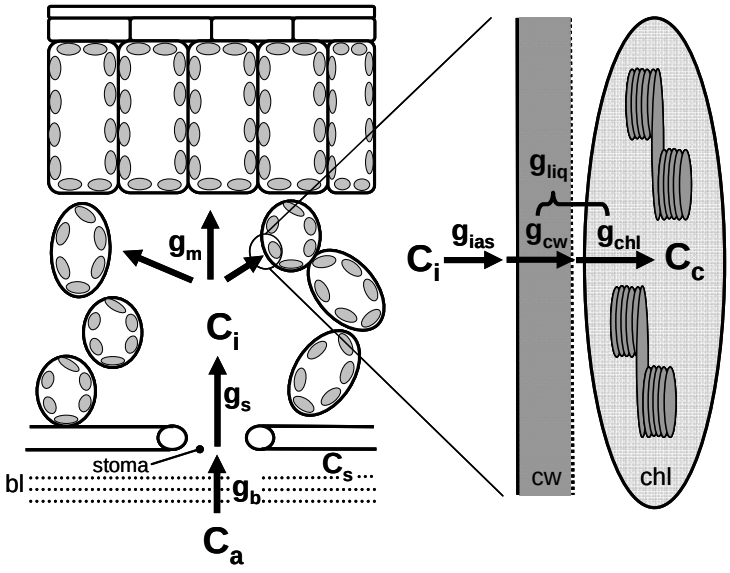


Figure 1.1. Schematic illustration of diffusion pathway of CO_2 from the ambient (C_a) through leaf surface (C_s) and intercellular air spaces (C_i) to the chloroplast (C_c). Boundary layer conductance (g_b), stomatal conductance (g_s) and mesophyll conductance (g_m) are figured. The partition of mesophyll conductance into three components - intercellular air space conductance (g_{ias}), cell wall conductance (g_{cw}) and chloroplast conductance (g_{chl}) - is figured on the right side of the figure where the cell wall (cw) and the chloroplast (chl) with granum is featured in detail. g_{cw} and g_{chl} are often being joined and called as liquid phase conductance (g_{liq}) since CO_2 has to diffuse through liquid phase inside the cell.

Two of above mentioned components of diffusional conductances dominate the CO_2 transport to chloroplast – stomatal and mesophyll conductance. Stomatal conductance is given by the aperture of stomatal pore that actually enables CO_2 uptake accompanied by loss of water. Stomatal conductance is large but flexible, and it responds to environmental variables such as photosynthetic active photon flux density (*PPFD*), CO_2 concentration and atmospheric moisture (Evans and Loreto, 2000).

Mesophyll conductance involves CO_2 diffusion from the substomatal cavity to the sites of carboxylation inside the cell (Warren, 2008a). There, the CO_2 diffusion occurs in both gas and liquid phases. Subsequently, several components of mesophyll conductance can be distinguished. The first component includes diffusion of CO_2 in gas phase (g_{ias}) before it enters the cell. Formerly, g_{ias} was regarded to be significant limitation (Parkhurst and Mott, 1990). However, this component does not influence the CO_2 flux notably, and therefore can be neglected (Parkhurst, 1994). When entering the cell, CO_2 has to cross the cell wall (g_{cw}) and dissolve in the solution. Finally, it diffuses in liquid phase (g_{chl}) in the chloroplast stroma where it reaches the carboxylation enzyme (**Fig. 1.1**).

Formerly it was impossible to partition g_m into its individual components. However, Gillon and Yakir (2000) presented possibility to provide an estimate of conductance from the intercellular air spaces to the chloroplast surface (g_{cw}) and from the chloroplast surface to the sites of carboxylation (g_{chl}). So nowadays, it is possible to distinguish what part of mesophyll conductance posses the greatest diffusional limitation for photosynthesis.

1.2 Importance of mesophyll conductance

Mesophyll conductance was previously assumed to be infinite and constant, that means $C_i = C_c$, in other words, CO_2 concentration in the chloroplast is the same like CO_2 concentration in sub-stomatal cavity (Farquhar *et al*., 1980). It was also considered that g_m is determined only by the structure of mesophyll and movements of chloroplasts (Sharkey *et al*., 1991; Tholen *et al*., 2007). Therefore, g_m was not regarded significant when modeling CO_2 transport through leaf mesophyll. But later studies confirmed that C_c is significantly lower than C_i suggesting the finite (i.e. limiting) mesophyll conductance. Evans *et al*. (1986), Evans and Terashima (1988)

and also Bongi and Loreto (1989) came to this conclusion by using several different methods, respectively.

Moreover, mesophyll conductance was confirmed not to be constant but highly variable among species and responding rapidly to environmental factors as temperature, CO_2 concentration or water stress (Bernacchi *et al*., 2002; Flexas *et al*., 2007a; Galmés *et al*., 2007a). Moreover, g_m was found to respond to environmental factors as rapidly as or even faster than stomatal conductance (g_s), i.e. within seconds to minutes (Flexas *et al*., 2008).

The process of CO_2 diffusion in leaf mesophyll is in agreement with first Fick's law of diffusion:

$$A_N = g_m(C_i - C_c), \qquad \text{(Eq. 1.1)}$$

where A_N is the net photosynthetic rate at steady state, g_m is the mesophyll conductance to CO_2 diffusion and C_i, C_c are the concentrations in the sub-stomatal cavity and in the chloroplast stroma, respectively. As mentioned above, mesophyll conductance significantly reduces C_c relative to C_i and so it is responsible for creating the CO_2 gradient between sub-stomatal cavity and the site of carboxylation. Therefore, g_m determines the difference between C_i and C_c as a function of photosynthetic rate (Flexas *et al*., 2008, Harley *et al*., 1992, Loreto *et al*., 1992). As a result, concentration of CO_2 decreases to approximately half of atmospheric concentration, from ~400 μmol mol^{-1} to ~200 μmol mol^{-1}. Approximately 20-50% of this drawdown is attributed to g_m (**Fig. 1.2**) (Warren, 2008a and references therein; Warren, 2008b).

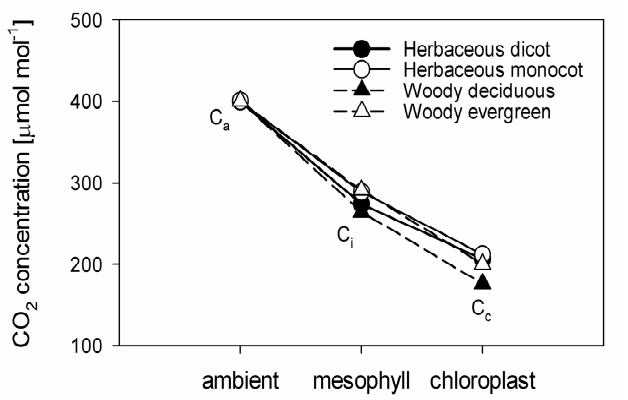


Figure 1.2. Decrease of CO_2 concentration during its diffusion from ambient through leaf mesophyll. Mesophyll conductance accounts for 20 to 50% decrease of CO_2 concentration from sub-stomatal cavity (C_i) to chloroplast stroma (C_c). Data have been taken and modified according to Warren (2008a).

1.3 Range of variation in mesophyll conductance

Mesophyll conductance is of similar magnitude to and quite closely correlated with stomatal CO_2 conductance across C_3 species from different functional types (Evans *et al*., 2004; Loreto *et al*., 1992). However, it was proven that g_m differs between different plant functional groups or even within the one functional group (Warren, 2008a).

A comprehensive review published recently by Flexas *et al*. (2008) includes 122 different species, subspecies, hybrids, forms and varieties (13 evergreen gymnosperms, 32 evergreen angiosperms, 3 semi-deciduous angiosperms, 37 deciduous angiosperms, 23 herbaceous annuals, 13 perennial annuals, and 1 CAM plant). Comparison of these functional groups showed that herbaceous annuals and biannuals present the largest values of g_m around 0.4 mol CO_2 $m^{-2}s^{-1}bar^{-1}$. Perennial herbs and deciduous angiosperms present lower values around 0.2 mol CO_2 $m^{-2}s^{-1}bar^{-1}$ while semi-deciduous angiosperms show intermediate values. The lowest values under 0.1 mol CO_2 $m^{-2}s^{-1}bar^{-1}$ were found in evergreen gymnosperms and CAM plants; however, only one CAM plant was included. Evergreen angiosperms present values slightly above 0.1 mol CO_2 $m^{-2}s^{-1}bar^{-1}$.

Flexas *et al*. (2008) confirmed what was previously suggested by Evans *et al*. (2004), that differences in g_m are associated with leaf forms or anatomy and plant functional groups. As shown in Warren (2008a), g_m is positively correlated with the rate of photosynthesis (A_{max}) (**Fig. 1.3**).

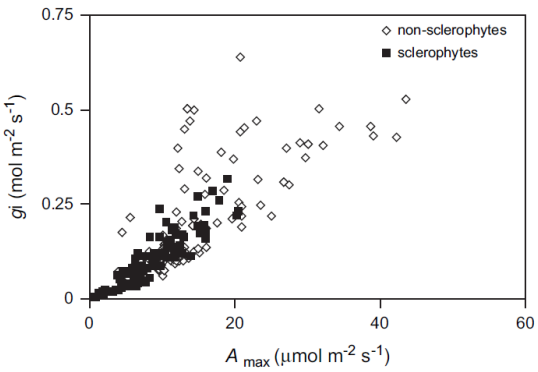


Figure 1.3. The relationship between light-saturated rate of net photosynthesis (A_{max}) and mesophyll conductance (g_i) in non-sclerophytes (open symbols) and sclerophytes (closed symbols). Taken from Warren, 2008a.

That is in agreement with fast growing strategies of annual and biannual herbs (with high rate of photosynthesis) having the highest values of g_m. Sclerophytic plants

have generally low values of g_m and low rate of photosynthesis, but the relationship between A and g_m is the same (Loreto *et al*., 1992; Flexas *et al*., 2008; Warren, 2008a) (**Fig. 1.3**).

High variability can be observed not only between the plant functional groups but also within a single group, genus or even species. For instance, in annual herbs, g_m varied from 0.08 mol CO_2 $m^{-2}s^{-1}bar^{-1}$ in wild-extinct Mediterranean species *Lysimachia minoricensis* (Galmés *et al*., 2007b) to >1 mol CO_2 $m^{-2}s^{-1}bar^{-1}$ in fast growing crops such as cotton (Brugnoli *et al*., 1998) and sunflower (Laisk and Loreto, 1996). This variability can be even higher in woody deciduous angiosperms (Flexas *et al*., 2008).

Although functional plant groups differ in g_m to some extent, high variability suggests that g_m is "a rapidly adapting trait" (Flexas *et al*., 2008). Chapter 1.4 is therefore dealing with adaptation or response of g_m to environmental conditions, especially to CO_2 concentration.

1.4 Response of g_m to environmental factors

As mentioned above, g_m is finite and therefore limiting diffusional factor for photosynthesis. Plants live in changing conditions so one of the major questions on this field is if g_m can respond to environmental variables. Quite wide range of external factors was tested for effect on mesophyll conductance. Most studies are focused on CO_2 concentration, temperature, less on irradiance (Flexas *et al*. 2007a; Bernacchi *et al*. 2002; Tazoe *et al*., 2009, respectively), although the results are inconsistent (**Fig. 1.4**). Besides this, water stress, high altitude, high O_3 content, light availability, low N availability, salinity or even virus infection have been studied as well as application of exogenous ABA (abscisic acid), $HgCl_2$ or PEG. (polyethylene glycol) (for review, see Flexas *et al*., 2008). Recently, Warren (2008a) and Flexas *et al.* (2008) has published a very fine and comprehensive review on literature dealing with external factors affecting g_m.

It was revealed that mesophyll conductance surely responds to environmental variables. Warren (2008a) and also Flexas *et al*. (2008) divided types of responses into two groups: short-term responses that occur within minutes and long-term responses that occur within days or growing season.

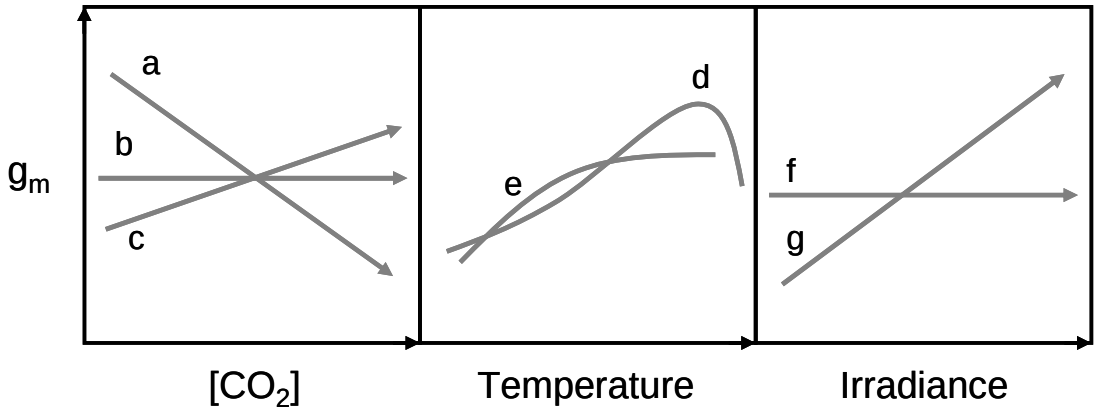


Figure 1.4. Schematic illustration of different g_m responses to three main environmental factors – CO_2 concentration, temperature and irradiance – as published in the literature. Letters at curves state for responses found in different studies: **a)** Düring, 2003; Flexas *et al*., 2007a; Vrábl *et al*., 2009; Hassiatou *et al*., 2009; Yin *et al*., 2009; **b)** Loreto *et al*., 1992; Tazoe *et al*., 2009; **c)** Harley *et al*., 1992; **d)** Bernacchi *et al*., 2002; **e)** Warren and Dreyer, 2006; Yamori *et al*., 2006; **f)** Tazoe *et al*., 2009; **g)** Flexas *et al*., 2007a; Gorton *et al*., 2003.

In presented study, I am going to focus on CO_2 response of g_m since published data have brought the most discrepancies and so the contribution to this issue would be valuable. Moreover I want to shed more light on the link between stomatal and mesophyll conductance in the conditions when CO_2 concentration around leaves is decreased and so the rate of photosynthesis can be restricted due to diffusional limitations.

1.4.1 CO_2 concentration

To investigate the effect of CO_2 concentration on g_m, one need the values of C_c for calculation according to Eq. 1.1 as $C_c = C_i - A/g_m$. This is possible by using the technique based on carbon isotope discrimination (Evans *et al*., 1986). An alternative method that allows estimating g_m over a large range of CO_2 concentrations is variable J method proposed by Di Marco *et al*. (1990) and further evolved by Harley *et al*. (1992). The method is based on the gas exchange measurements coupled with chlorophyll fluorescence assessment and is substantially easier than the isotopic method. Therefore, it is suitable to use it for more measurements on different plant species. Although high endeavor is made to understand the plant responses to climate change as well as to correctly interpret A_N-C_i curves, response of g_m to varying CO_2 has received only little attention and only several studies were published, despite the simplicity and advantages of variable J

method.

Early studies have shown that g_m do not vary with different CO_2 concentration. For example, g_m did not alter in the *Raphanus sativus* when C_a was changed from 350 to 220 μmol CO_2 mol^{-1} air (von Caemmerer and Evans, 1991). However, developing variable J method, Harley *et al*. (1992) has discovered that g_m decreased from 0.2 to about 0.1 μmol m^{-2} s^{-1} bar^{-1} when C_i was increased from 100 to 450 μmol CO_2 mol^{-1} air in *Quercus rubra*, but it was rather unaffected or slightly increased in *Eucalyptus globulus* (**Fig. 1.5a, b**). Using method of isotope discrimination, Loreto *et al*. (1992) have shown that g_m was reduced after increasing C_i from ambient to 750 μbar in *Ouercus rubra* and even more in *Xanthium strumarium* (**Fig. 1.5c**). However, neither Harley *et al*. (1992) nor Loreto *et al*. (1992) discussed the issue.

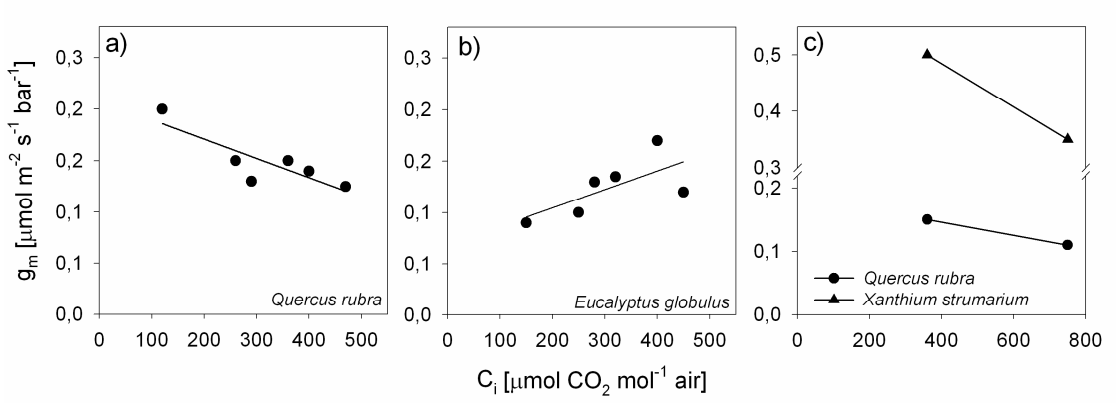


Figure 1.5. Mesophyll conductance as a function of CO_2 concentration in sub-stomatal cavity (C_i). Re-drawn according to Harley *et al*., 1992 for *Q. rubra* (a) and *E. globulus* (b), and according to Loreto *et al*., 1992 for *Q. rubra* and *X. strumarium* (c).

Later, Düring (2003), using variable J method, clearly showed significant change in g_m over the wide range of C_a (from 50 to 2000 μmol CO_2 mol^{-1} air) in grapevine. It decreased six-fold as C_a was increased from 300 to 1000 μmol CO_2 mol^{-1} air (**Fig. 1.6**). This demonstrates very fast respond (i.e. within minutes) of g_m to varying CO_2 concentration as the values were obtained during typical A_N-C_i curve. g_m was proved to control the transport of CO_2 into the chloroplast stroma. Author stated that C_c remained constant at Ci >340 μmol CO_2 mol^{-1} air due to distinct decline of mesophyll conductance (**Fig. 1.6**). Moreover, it was the first hint that showed that the stomatal and mesophyll conductance are somehow linked together and cooperate in regulation of photosynthesis.

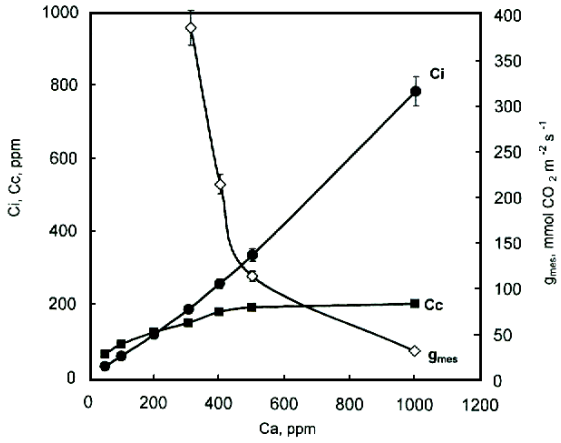


Figure 1.6. The sub-stomatal CO_2 concentration (C_i), the chloroplastic CO_2 concentration (C_c) and the mesophyll conductance (g_{mes}) as a function of ambient CO_2 concentration (C_a). Taken from Düring (2003).

Another step in the knowledge about the effect of CO_2 on g_m was made by Centrito *et al*. (2003) using salt stressed olives. The leaves were exposed to very low CO_2 concentration (50 μmol mol^{-1} air) to force stomatal opening. After an hour, stressed plants recovered the same A_N values and similar A_N-C_i curves as controls. This would be not possible only due to increased stomatal conductance, but due to its cooperation with mesophyll conductance that was increased either. Authors have found out that biochemical apparatus was not damaged, and therefore, the sum of diffusional resistances (or conductances) set the limit to photosynthesis. Moreover, from linear positive relationship ($r^2 = 0.68$) between g_m and g_s at 350 μmol CO_2 mol^{-1} air, they have concluded that changes in g_m can be as fast as those in g_s (Centrito *et al*., 2003). Similar results were published by Flexas *et al*. (2004) on drought-stressed sunflower.

More recently, Flexas *et al*. (2007a) has studied rapid variations of mesophyll conductance in response to changes in CO_2 concentration around leaves of six different C_3 species (*Arabidopsis thaliana*, *Limonium gibertii*, *Nicotiana tabacum*, *Vitis berlandieri* x *Vitis rupestris*, *Cucumis sativus* and *Olea europaea* var. *europaea*). In all of them, g_m responded rapidly to varying CO_2 concentration and varied as much as five to nine-fold along the range of CO_2 concentrations from 50 to 1500 μmol mol^{-1} air, which is in agreement with Düring (2003) (**Fig. 1.6**). The pattern of g_m response to CO_2 was not strictly uniform but was rather species dependent. However, g_m decreased rapidly at high C_i when photosynthesis is no longer limited by CO_2 availability in all species (Flexas *et al*., 2007a). The same was observed for *Nicotiana tabacum* mutants – aquaporin anti-sense and over-expressed – as well as for their

controls with normal aquaporin expression. All measurements were made during the performance of classic A_N-C_i curve. These results suggest that mesophyll conductance responds to varying CO_2 concentration even faster than stomatal conductance. The conclusions were supported by using three different methods to evaluate g_m – isotopic (Evans *et al*., 1986), variable J (Harley *et al*. 1992) and curve-fitting (Ethier and Livingston, 2004) method. According to personal communication, authors noticed that similar results were obtained by Ethier and Pepin, and also by Warren. Therefore, the clear picture about the issue started to rise up.

Most recently, two other studies contributed to the knowledge about the issue to make the picture be clear. Hassiatou *et al*. (2009) observed a six-fold decline of g_m in respose to increasing CO_2 at high irradiance in *Banksia* species using variable J method of Harley *et al*. (1992). By using two independent methods (isotopic and variable J method) (**Fig. 1.7**), Vrábl *et al*. (2009) compared sensitivity of g_m to CO_2 between control and plants treated with abscisic acid (ABA). Although ABA did not exhibit any effect to g_m, there was the same significant sensitivity of g_m to variable CO_2 as in controls.

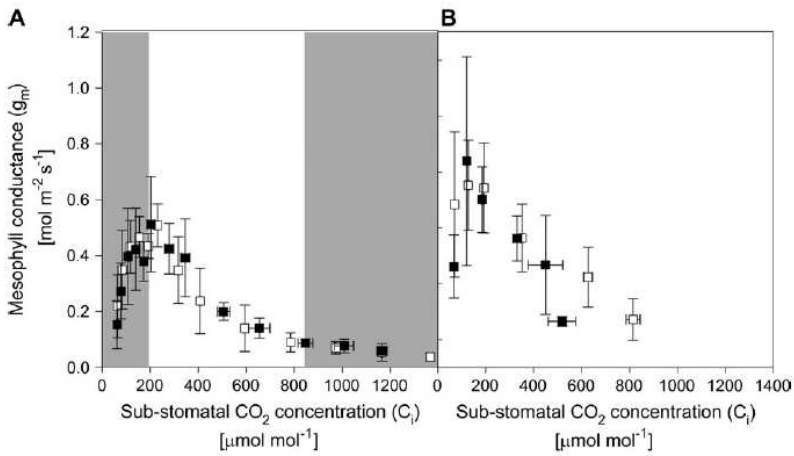


Figure 1.7. Response of mesophyll conductance to substomatal CO_2 concentration in controls (open symbols) and ABA-treated plants (full symbols) estimated by two independent methods: (A) Variable J method of Harley *et al*. (1992) and (B) isotopic method according to Evans *et al*. (1986).

The CO_2 response pattern was similar to that of *Nicotiana tabacum* and *Vitis* hybrid in Flexas *et al*. (2007a). At first, g_m increased at low sub-stomatal CO_2 concentrations (C_i), then peaked at 200 μmol mol^{-1} air and subsequently decreased at higher C_i (**Fig. 1.7**). Eventually, also Yin *et al*. (2009) observed the same dependency of g_m to CO_2. Together, these studies proved high sensitivity of g_m to CO_2 concentration and rapidness of its response.

However, there are still some discrepancies in the literature since several studies brought contrary results. Although Loreto *et al*. (1992) found decrease in g_m when CO_2 was increased in *Q. rubra* and *X. strumarium* (using isotopic method) as mentioned above, they did not observe CO_2 dependency in other two species (using variable J method) over a range of 100 to 500 μmol CO_2 mol^{-1} air C_i. Recently, Tazoe *et al*. (2009) has presented results from *PPFD* and CO_2 dependency measurements using isotopic method. They did not find any sensitivity of g_m to CO_2 over the same range of C_i as Loreto *et al*. (1992). This range is, however, three-times smaller than that used in previous studies that proved CO_2 dependency of g_m and thus, possible effect on g_m could be hidden. Nevertheless, measuring over a bit larger range of CO_2 (100 – 700 μmol CO_2 mol^{-1} air), Tazoe *et al*. (2010) did not find any g_m-CO_2 dependency again in all three species studied – *Nicotiana tabacum, Arabidopsis thaliana* and *Triticum aesativum*. Moreover, Ethier and Pepin (2010) showed that g_m responded to CO_2 in *Nicotiana tabacum* but not in *Helianthus annuus* and *Populus* x *jackii* using isotopic method. On the other hand, g_m responded in all species using variable J method. This could suggest that g_m response to CO_2 is species dependent and/or that some methodological artifacts are present.

Finally, responses of g_m to long-term acclimations to high CO_2 are rather questionable. g_m was observed to be unaffected in some studies (Eichelmenn *et al*., 2004; Bernacchi *et al*., 2005) or to be rather species-dependent in other (Singsaas *et al*., 2004).

So far, we can declare that range of g_m is species dependent when measured at ambient [CO_2] on non-stressed plants. But we are not as sure about its response to varying CO_2. Hence, more measurements should be made to get a higher number of observations to make a final generalization about the issue. Is it also species dependent or not?

1.5 Regulation of mesophyll conductance

Studies, mentioned above, proved that g_m is crucial component of CO_2 diffusion because it reduces its concentration to quite great extent and therefore, g_m became recognized as considerable limitation for photosynthesis. Moreover, g_m is sensitive to many environmental factors and varies between plant functional groups and/or even within group or species. Hence, it is crucial for plant to regulate the mesophyll

conductance very delicately, and more attention is paid to the principle of its regulation nowadays. However, the puzzle is far from being completed.

1.5.1 Leaf structure

In earlier studies, leaf structure was considered the only determinant of g_m. Although this statement was displaced (see bellow), leaf structure is still considered having an important role in determining g_m. As indicator of leaf structure, leaf dry mass per area unit (LMA) is used. As summed up in Flexas *et al*. (2008), relationship between g_m and LMA is asymptotic (**Fig. 1.8 A**). Thus, species with low LMA present a wide range of g_m values. As LMA is increasing, range of g_m values decreases indicating that leaf structure strongly limits g_m. g_m was extrapolated to zero at the value of 240 g m^{-2} (Flexas *et al*., 2008). However, LMA in sclerophyllous plants can be much higher than this value. Therefore, Hassiotou *et al*. (2009) examined *Banksia* species that ranges in LMA from 134 to 478 g m^{-2} to see what would be the g_m/LMA relationship behind the value 240 g m^{-2}. By adding measured data to that previously summarized by Flexas *et al*. (2008), the notional upper bound was redrawn as concave curve with an unknown asymptotic value at LMA grater than 500 g m^{-2} (**Fig. 1.8 B**). This relationship represents clear evidence that leaf structure is somehow limiting for g_m or set the limit for maximum g_m rather than its actual value. Let's say it is kind of "passive" regulation in terms of fast responses of g_m on environmental variables. LMA can be considered and included during the long term experiments because leaf structure can change over that time.

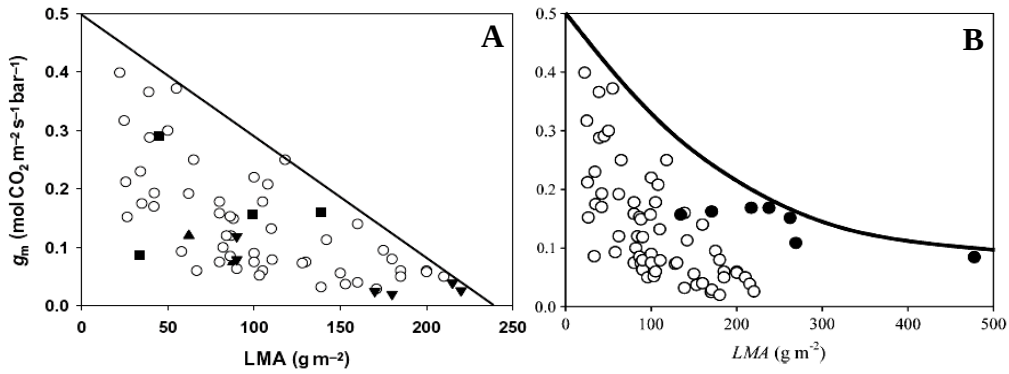


Figure 1.8. The relationship between mesophyll conductance and leaf mass per area (LMA) in the absence of stress in different species. (**A**) Data have been taken from 17 studies and compiled by Flexas *et al.* (2007a). (**B**) Data from Hassiotou *et al*. (2009) on *Banksia* species (filled symbols) were added to 17 previous studies (empty symbols) and the asymptotic line was changed.

1.5.2 Metabolic processes

Studying the temperature response of any diffusional limitation is a useful tool to uncover the mechanism of diffusion. By calculation of temperature coefficient (Q_{10}), it can be estimated what is the portion of pure or facilitated diffusion. Experimentally ascertained Q_{10} for diffusion of CO_2 in pure water is close to the predicted one, 1.25 (Tamimi, 1994). Q_{10} of 2 or higher is typical for metabolic process. When studying the temperature response of g_m, Bernacchi *et al.* (2002) found out that Q_{10} was approximately 2.2. Thereby, this indicates that g_m is more associated with facilitated diffusion than pure diffusion. Recently, it has been suggested that g_m could be controlled by protein-facilitated process regarding aquaporins and/or carbonic anhydrase as two hot candidates for this function.

1.5.2.1 Aquaporins

As the first candidate for regulation of CO_2 diffusion, aquaporins (AQP) are taken into account. Aquaporins are small pore-forming transmembrane protein channels first discovered in mammalian erythrocytes and renal tubules (Denker *et al*., 1988). Recently, they have been found in nearly all living organisms (Maurel and Chrespeels, 2001) including plants (Maurel *et al*., 1993) where they express high number of homologues (Johanson *et al*., 2001; Chaumont *et a*l., 2001). Name of aquaporins indicates their primary role as water channels in both animals and plants. The amount of water in a leaf has to be balanced for proper function of physiological processes in leaf such as photosynthesis, especially during CO_2 uptake when water is unavoidably lost. The amount of water transported by aquaporins may reach 90% of all transported water (Evans *et al*., 2004). These reasons clearly show how important aquaporins are for plants. However, it was revealed that animal aquaporin 1 transports CO_2 as well as water (Cooper and Boron, 1998; Nakhoul *et al*., 1998; Prasad *et al*., 1998).

1.5.2.2 Plant aquaporins and CO_2 transport

First evidence for AQP contribution in CO_2 transport in plants was provided by Terashima and Ono (2002) who found out that hydraulic permeability of plasma

membrane was decreased by 70-80% when leaf discs of *Vicia faba* and *Phaseolus vulgaris* were treated by $HgCl_2$, non-specific inhibitor of aquaporins. In consequence, mesophyll conductance decreased to 40 and 30% of the control value, respectively. This decline was attributed to aquaporins that are mercury-sensitive. However, mercurial compounds often inhibit metabolism by non-specific way and exert a broad range of other secondary effects (Hanba *et al*., 2004). Later, Uehlein *et al*. (2003) shown that transmembrane CO_2 transport was mediated by tobacco aquaporin NtAQP1 expressed in *Xenopus* oocytes by injection of NtAQP cRNA. The decrease of intracellular pH indicated the transport of CO_2 into the oocytes. For NtAQP1 it was found out that CO_2 uptake was 45% higher compared to the control.

More direct evidence was proposed by Hanba *et al*. (2004) using transgenic rice with over-expression of barley aquaporin HvPIP2;1. The leaves of transgenic plants with higher content of aquaporins expressed 40% increase in g_m, and leaves with lower content of aquaporins showed decrease in g_m values as compared to wild-eype. However these evidences might be misleading due to increased transpiration of water caused by huge over-expression of aquaporins that led to water stress. Therefore, it was suggested that also anatomical or physiological (like Rubisco concentration) properties of leaf could be included and affect the g_m in the study (Hanba *et al*., 2004).

More recently, Flexas *et al*. (2006a) has provided strong evidence supporting the hypothesis that tobacco aquaporin NtAQP1 is involved in g_m *in vivo*. Mesophyll conductance was estimated to be 30% lower in anti-sense and 20% higher in over-expressed plants as compared to their wild types.

Possible pathway for CO_2 through aquaporins has been most recently suggested by Wang *et al*. (2007) who has provided evidence that CO_2 can permeate the membrane through the central pore created by tetramer of aquaporins (**Fig. 1.9 A**). Evidence for this was obtained from calculation of free energy profiles (PMF) that suggests that the central pore is the most favorable location for CO_2 permeation (**Fig. 1.9 B**). Additionally, also side pore in the gap between the two monomers (**Fig. 1.9 A**) is considered as possible way for CO_2 diffusion through AQP.

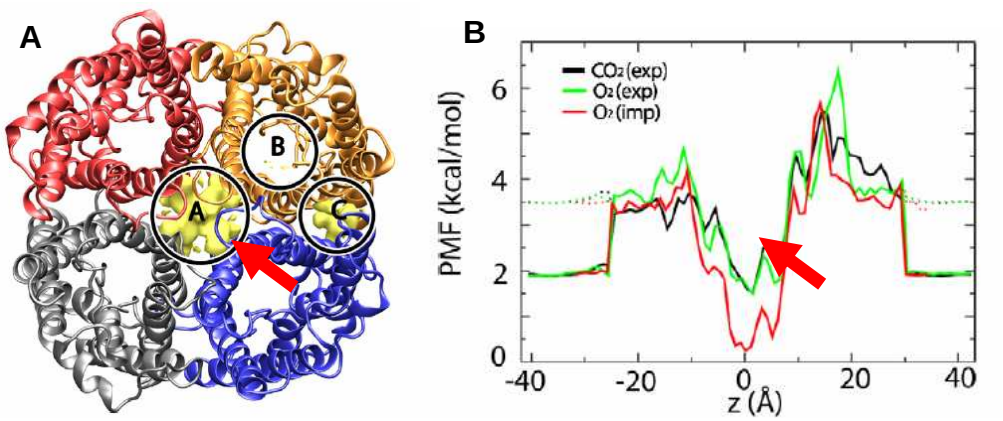


Figure 1.9. (A) Top view of four AQPs in tetramer composition. There is one pore in each monomer (B), and tetramer creates one extra pore in center (A) that is marked by arrow. Last, there is one small pore between the each two monomers (C). **(B)** Free energy profiles (PMF) associated with gas permeation through the AQP1 central pore, calculated from the explicit gas diffusion simulations (exp) and implicit ligand sampling (imp). The dotted lines correspond to the PMFs of gas molecules in water of the same area as the central pore. z = 0 is defined as the center of aquaporin tetramer. All PMFs were calculated assuming a density of 1 AQP1 per 50 nm2 of bilayer. Taken and modified according to Wang *et al*. (2007).

However, although the central pore of AQP1 was found to be permeable for CO_2, gas conduction through AQP1 may only be physiologically relevant either in membranes of low gas permeability or in cells where a major fraction of the cellular membrane is occupied by AQPs since lipid bilayer provides a much larger cross-section area.

Since aquaporins play a crucial role in plant-water relations and maybe also in CO_2 transport, many studies have tried to reveal the mechanism of regulation of aquaporin activity. So far, several ways of regulation have been discovered including direct phosphorylation (Johansson *et al*., 2000), molecular trafficking (see Chaumont *et al*., 2005 for review), heteroizomerization (Fetter *et al*., 2004), pH-dependent gating of AQP (Chaumont *et al*., 2005; Gerbeau *et al*., 2002; Tournaire-Roux *et al*., 2003), divalent cations (like Ca^{2+}) (Gerbeau *et al*., 2002), cohesion/tension forces in the presence of high concentrations of osmotic solutes (Ye *et al*., 2004) and transcriptional regulation and protein stability (Eckert *et al*., 1999). Yet, this task is not completely resolved.

Missner *et al*. (2008) has recently shown that CO_2 transport through membrane is not limited by lipid bilayer but near-membrane unstirred layers and that facilitation of CO_2 transport by AQP or any other protein is highly unlikely. They suggested that aquaporins could regulate CO_2 transport through membrane indirectly by reducing the thickness of unstirred layers.

As shown above, there are many ways to regulate aquaporin activity, and hence, mesophyll conductance. Wan *et al*. (2004) proposed another interesting possibility of its regulation. Studying gating of aquaporins in cortical cells of young corn roots by mechanical stimuli, they found out that abscisic acid (ABA) can be involved. They applied a pressure pulses into a single cell using a cell pressure probe (Steudle, 1993). When larger pulses were employed, changes in turgor were not reversible within 1-3 hours, but were able be reverse within 30 minutes in the presence of ABA (**Fig 1.10**). Therefore, authors speculated that ABA binds to aquaporins thus reducing the activation energy for a change from closed states to the opened (Wan *et al*., 2004). If so, ABA could be one of key players in AQP regulation.

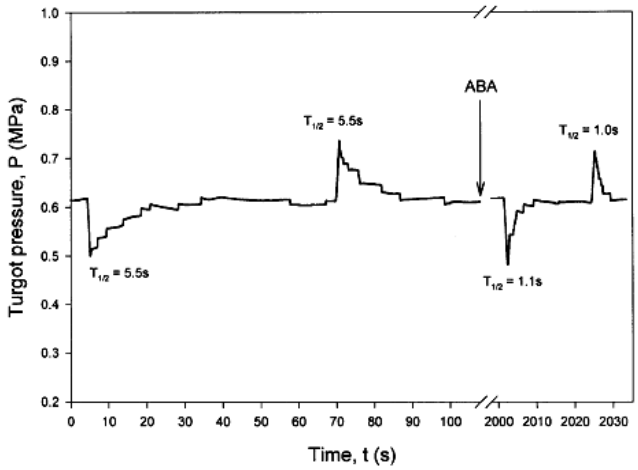


Figure 1.10. Treatment with ABA (500 nM) changed $T_{1/2}$. Applying the pulse, the turgor pressure was either decreased or increased and recovery half-time was recorded. Doing that, it was possible to determine how long it takes the cell to exclude or absorb the water through the membrane to restore the original turgor pressure. These pressure-relaxation curves conducted on a typical cell show that ABA restored long $T_{1/2}$ induces by large pulses to short. After adding ABA, reduction of $T_{1/2}$ started after 5 min and was complete after about 30 min.

1.5.2.3 Carbonic anhydrase

As the second candidate that is considered to be connected with regulation of g_m in C_3 plants, carbonic anhydrase is taking into account (Makino *et al*., 1992; Sasaki *et al*., 1996). Carbonic anhydrase (CA) is enzyme abundantly present in chloroplast stroma. However, only little or no change in photosynthetic rate have been revealed by using CA transgenic plants (Price *et al*., 1994; Williams *et al*., 1996), and only modest correlation was found out between CA activity and photosynthesis (Tiwari *et al*., 2006). It was proposed that the relative contribution of CA to the g_m is species

dependent and not always clearly apparent. CA may play important role in species with low g_m values (e.g. sclerophytes, Waren, 2008a) due to anatomical properties of the leaves. Yet, as implied in Flexas *et al*. (2008), influence of CA activity to g_m is rather questionable. On the other hand, Jannaud *et al*. (2010) presented results from study with transgenic *Arabidopsis* plants that were depleted in some of several CA or AQP isoforms which resulted in suggestion that mesophyll conductance is facilitated by CA rather than AQP as plants depleted in some of CA isoforms showed decreased mesophyll conductance whereas AQP depleted did not. So the field is open for investigation and discussion now.

There are surprisingly many confirmed or putative mechanisms of aquaporin regulation. Gating of aquaporins through different ways could represent a rapid pathway of response to environmental constraints. Therefore, tight regulation of AQP or CA activity is apparently essential for plants.

1.6 Motivation

ABA is a stress hormone which is known to help the plant to avoid water losses. To do that, ABA is synthesized in roots and transported to the leaves where it evokes closure of stomata and so minimizing the loose of water (Raghavendra *et al*., 2010). However, the flux of CO_2 into the sub-stomatal cavity through stomata is restricted and diffusional limitation for photosynthesis increases. We may speculate that if ABA enhances the activity of AQP for water permeability, it can also enhance permeability to CO_2 flux. If so, ABA can react as universal hormone to keep the photosynthesis rate unaffected even when the stomata are clo sed. Using ABA-treated plants, we can measure gas exchange with chlorophyll fluorescence (or isotope discrimination) to obtain the values of A_N, g_s, g_m, C_i and C_c to compare them with controls. Such measurements can bring us a hint about link between stomatal and mesophyll conductance and regulation of CO_2 diffusion from sub-stomatal cavity to the chloroplast.

Such measurements have been made by Vrábl *et al*. (2009). After addition of 20 μM ABA, stomatal conductance decreased significantly in comparison to controls. However, photosynthetic rate was unaffected and even slightly increased in saturation phase of the A_N-C_i curve. As proposed by Centrito *et al*. (2003), g_s somehow cooperates with g_m. In the case of Vrábl *et al*. (2009) it means that when g_s decreases

(by ABA), g_m should increase to enhance flux of CO_2 into the chloroplast. However, it increased slightly in comparison with controls and this increase was not significant (**Fig. 1.7**). Nevertheless, this hypothesis should be further evolved and tested by more measurements on ABA treated plans.

1.7 Objectives of the presented study

Since some discrepancies occurred in the literature dealing with response of mesophyll conductance to varying CO_2 concentration around leaves, the first objective of the presented study is to measure g_m over the wide range of CO_2 concentrations to make a contribution to that issue. The second aim is to measure CO_2 dependency of g_m in ABA-treated plants to analyze the link between g_s and g_m and to see how they may cooperate to keep the photosynthetic rate unaffected when CO_2 diffusion through stomata is restricted. Finally, we aimed to make a basis for speculations about regulation of CO_2 diffusion from sub-stomatal cavities into the chloroplast stroma by regulation of AQP by ABA.

2. Materials and methods

2. 1 Plant material and growth conditions

Three different plant species were used for experiments. Plants of *Helianthus annuus* were individually hydroponically cultivated from seeds in 0.5 L pots in the growth chamber with 16/8 h and 22/18°C day/night cycle, whereas relative humidity was 70%. Thereafter, plants were transferred to 3-liter pots to be grown hydroponically. Hybrid plants of *Populus nigra* L. × *Populus maximowiczii* Henry 'Maxvier' – purchased as 15 cm-long cuttings – were directly placed into hydroponical pots mentioned above and grown in the glasshouse with ambient humidity (i.e. 70%). Photosynthetic photon flux density (*PPFD*) was approximately 300 μmol m^{-2} s^{-1} and mean day temperature in the glasshouse was 23°C. All plants were watered every 2–3 days by commercial nutrient solution (Kristalon Start, NU3 BV Vlaardingen, Netherlands).

Approximately three weeks after sowing or after several fully expanded leaves appeared in case of *Populus*, one-half of plants were exogenously treated with abscisic acid (ABA; Sigma Aldrich, Seelze, Germany) dissolved in 1 mL of methanol. Three different concentration of ABA has been supplied – 20 μM and 10 μM within the experiment with sunflower and 5 μM within the experiments with poplar plants.

2. 2 Gas exchange and chlorophyll fluorescence measurements

Measurements were made on young fully expanded leaves, 3 days after ABA addition. Day respiration (R_d) and the apparent CO_2 photocompensation point (C_c^*) were determined simultaneously using the method of the Laisk (1977) as described in von Caemmerer (2000). Set of A_N-C_i curves was measured under different *PPFDs*, whereas CO_2 concentration were in the range from 30 to 250 μmol mol^{-1} air to record only linear part of the A_N-C_i curve. Different *PPFDs* were chosen following preliminary trials to ensure a large difference between the slopes of individual A_N-C_i curves (**Fig. 2.1**). Therefore, CO_2 and *PPFD* values differ between sunflower and poplar (**Tab. 2.1**).

Table 2.1. Values of CO_2 ambient concentration and *PPFD* at which A_N has been measured to get the linear response to CO_2. C_c^* an R_d were then determined.

H. annuus	CO_2	μmol mol^{-1} air	250	200	150	100	75	50	30
	PPFD	μmol m^{-2} s^{-1}	500	300	150	100	50		
Populus	CO_2	μmol mol^{-1} air	150	100	75	50			
	PPFD	μmol m^{-2} s^{-1}	550	160	100	60			

The intersection point of A_N-C_i curves at different *PPFDs* represent C_c^* (*x*-axis) and R_d (*y*-axis) (see **Fig. 2.1**). Then, C_c^* was used as a proxy for chloroplastic photocompensation point (Γ^*) according to Warren and Dreyer (2006). All these measurements were performed by an open gas-exchange system Li-6400 (Li-Cor Inc., Lincoln, NE, USA), equipped with a 6 cm^2 broadleaf chamber and an integrated light source (Li-6400-02B; Li-Cor, Inc.).

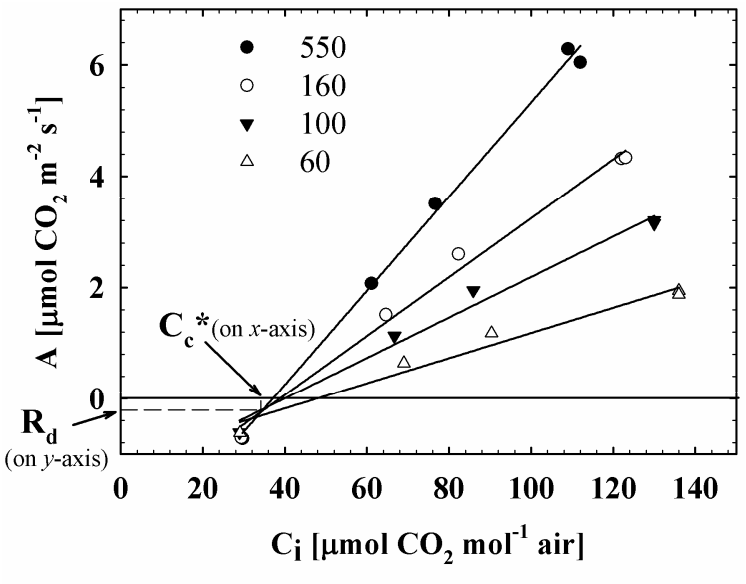


Figure 2.1. Example of estimation of apparent CO_2 compensation point (C_i^*) and day respiration (R_d) according to Laisk's method. A_N-C_i curves, of the poplar hybrid leaf were carry out at four different *PPFD*s (550, 160, 100, 60 μmol m^{-2} s^{-1}). Data were fitted by linear regression and the point of intersection represents the value of R_d (on *y*-axis) and C_i* (on *x*-axis).

The photochemical efficiency of photosystem II (Φ_{PSII}) was estimated from steady state fluorescence (Fs) and maximal fluorescence (Fm´) during light-saturating pulse according to Genty et al. (1989) as:

$$\Phi_{PSII} = (Fm´ - Fs)/ Fm´ \qquad \text{(Eq. 2.1)}$$

The rate of linear electron transport (J_f) is then related to Φ_{PSII}:

$$J_f = \Phi_{PSII}\ PPFD \times \alpha \times \beta, \quad \text{(Eq. 2.2)}$$

where *PPFD* is the photosynthetically active photon flux density, α is the total leaf absorptance (nominally 0.84), and β represents the partitioning of absorbed quanta between photosystems II and I, which is assumed to be 0.5 for C3 plants (Örgen and Evans, 1993).

The relationship between the J_f and the rate of electron transport obtained from gas exchange measurements (J_{CO2}) includes some uncertainties. Some of them can be eliminated by measuring of α and β (although β is rarely measured, Warren, 2006). However, there is still uncertainty in J_f/J_{CO2} relationship due to two other factors. Firstly, alternative electron sinks (photorespiration, Mehler reaction) can be present. Secondly, fluorescence signal is primarily emanated from upper mesophyll layer and therefore may not be a representative of the whole leaf as it is in case of gas exchange signal (Warren, 2006). These uncertainties can be eliminated by using calibration curve of the J_f/J_{CO2} relationship under non-photorespiratory conditions, when photorespiratory electron transport pathway is suppressed.

Therefore, fluorescence was calibrated by relating photochemical efficiency of photosystem II obtained from chlorophyll fluorescence (Φ_{PSII}) and gas exchange measurements (Φ_{CO2}) [(A_N+R_d)/PPFD] obtained by varying CO_2 concentration under non-photorespiratory conditions in an atmosphere containing less than 1% O_2 (Valentini et al., 1995). An example of such relationships is shown on **Figure 2.2**. Subsequently all fluorescence data measured at 21 % O_2 were corrected in accordance to the obtained calibration equation. Moreover, the linear fit of the Φ_{PSII}/Φ_{CO2} relationship shows constant uniform electron transport rate within different treatment (CO_2 concentration, ABA addition).

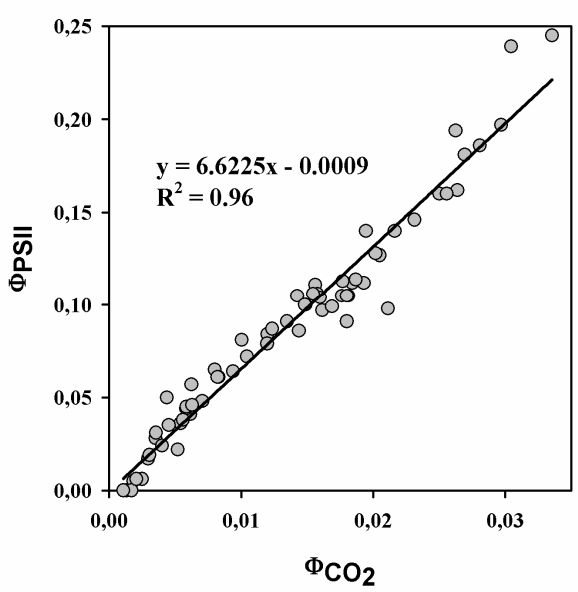


Figure 2.2. Example of the relationship between photochemical efficiency of photosystem II within A_N-C_i curve obtained from chlorophyll fluorescence (Φ_{PSII}) and from gas exchange measurements (Φ_{CO2}) in *Populus nigra* L. × *Populus maximowiczii* Henry 'Maxvier' hybrid leaves. Line was fitted with linear regression.

Mesophyll conductance (g_m) was determined at different CO_2 concentrations from simultaneous measurements of A_N-C_i and J_f-C_i curves. CO_2 response curves were performed on three light-adapted leaves of both ABA-treated and control plants. Photosynthesis was induced with a CO_2 surrounding the leaf (C_a) of 400 μmol mol^{-1}air and *PPFD* of 1500 μmol m^{-2} s^{-1} or 800 μmol m^{-2} s^{-1} (in case of *Populus*), based on previous measurements of light curves that proved these values to be saturating. The amount of blue light was set to 10% to maximize the stomatal aperture. Leaf temperature was kept close to 23°C, and leaf-to-air vapor pressure deficit was kept between 0.7 and 1.3 kPa during all measurements. CO_2 response experiment was performed right after the steady state was reached, i.e. 20-30 minutes after clamping the leaf into leaf chamber of Li-6400. Gas exchange and chlorophyll fluorescence was first measured at 400 μmol mol^{-1} air ambient CO_2 (C_a), then C_a was decreased stepwise to 150 or to 50 μmol mol^{-1} air (in case of *Populus*), and after that returned back to 400 μmol mol^{-1} air to restore the original A_N value. Thereafter, C_a was increased stepwise to 600 or 1500 μmol mol^{-1} air for *H. annuus* and *Populus* hybrid, respectively. The time lag between consecutive measurements at different C_a was 5 – 10 min.

Possible leakages into and out of the cuvette for the range of CO_2 concentrations were determined by using scrap of paper enclosed in the leaf chamber.

A_N-C_i curve was performed and measured values of assimilation rate were then corrected to actual values according to Flexas *et al*. (2007b).

2.3 Estimation of g_m by gas exchange and chlorophyll fluorescence measurements

Method of estimation of mesophyll conductance used in the presented study was originally described by DiMarco *et al*. (1990) and further evolved by Harley *et al*. (1992). It is based on simultaneous measurements of gas exchange and chlorophyll *a* fluorescence. The method provide estimation of g_m from the rate of photosynthetic electron transport (J_a), CO_2 concentration at the site of Rubisco (C_c) and the net CO_2 assimilation (A_N). The relationship between them can be expressed as (Harley *et al.*, 1992):

$$J_a = (A_N + R_d)\frac{4(C_c + 2\Gamma^*)}{C_c - \Gamma^*} \qquad \text{(Eq. 2.3)}$$

where J_a is the rate of linear electron transport, R_d is the day respiration, Γ^* is the CO_2 compensation point in the absence of R_d, and the factor 4 expresses the minimum electron requirement for assimilation of one molecule of CO_2. Substituting C_c in Eq. 2.3 with Eq. 1.1, Eq. 2.3 then becomes:

$$J_a = 4(A_N + R_d)\frac{[(C_i - A_N / g_m) + 2\Gamma^*]}{(C_i - A_N / g_m) - \Gamma^*} \qquad \text{(Eq. 2.4)}$$

where C_i is the CO_2 concentration in the intercellular air spaces and g_m is the mesophyll conductance. Rearranging Eq. 2.4 allows g_m to be calculated directly:

$$g_m = \frac{A_N}{C_i - \frac{\Gamma^*[J_a + 8(A + R_d)]}{J_a - 4(A + R_d)}} \qquad \text{(Eq. 2.5)}$$

where A_N and C_i are taken from gas-exchange measurements of CO_2-response curves and Γ^* and R_d were estimated using method of Laisk (1977) (see above).

3. Results

3.1 Correction of electron transport rate in respect to rate of photosynthesis

Measurements in air containing less than 1% O_2 revealed a strong positive relationship ($R^2 > 0.90$) between Φ_{PSII} and Φ_{CO2} at different CO_2 concentrations in controls and ABA-treated plants of both the sunflower and poplar plants (**Fig. 3.1**). This indicates a constant non-assimilatory electron flow within different treatments (CO_2 concentration and ABA). The slope of the relationship between Φ_{PSII} and Φ_{CO2} ranged from 6.4 to 7.1 in all measurements and treatments, which is slightly lower than range described previously (Vrábl *et al*., 2009; Flexas *et al*. 2007a). No significant difference was found between treatments.

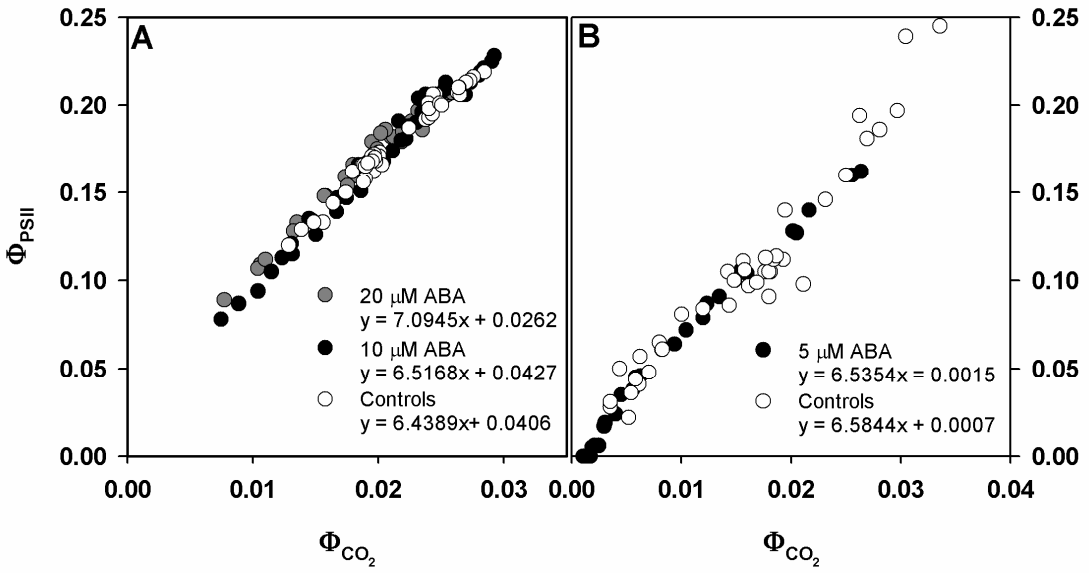


Figure 3.1. Relationship between photochemical efficiency of photosystem II estimated by chlorophyll fluorescence measurement (Φ_{PSII}) and calculated from gas exchange measurement ($\Phi_{CO2} = A_N+R_d/PPFD$) in sunflower (**A**) and poplar (**B**) obtained by varying CO_2 concentration under non-photorespiratory conditions in atmosphere containing less than 1% O_2. A strong positive relationship was observed in both sunflower ($R^2 = 0.98$ for all controls and plants treated by 20 and 10 μM ABA) and poplar ($R^2 = 0.93$ for controls and 0.99 for ABA-treated plants) when fitted by linear regression that yielded equations showed in legend. Lines of fits are not shown.

The equations of Φ_{PSII}/Φ_{CO2} linear regression fit were used for correction of electron transport rate in respect to rate of photosynthesis.

By using the method of Laisk (1977) and Warren (2006), difference between the two species were found in Γ^* and R_d. In average, values of Γ^* were lower and values of R_d two times higher in controls of sunflower than in poplar. However,

neither Γ^* nor R_d differed significantly between the controls and ABA-treated plants of both species (**Tab. 3.1**).

Table 3.1 Mean values of CO_2 compensation concentration in the absence of mitochondrial respiration Γ^* (μmol CO_2 mol^{-1} air); and day respiration, R_d (μmol m^{-2} s^{-1}). Valueas are averages ± SE of 3 - 6 replicates. T-test was used for statistical analysis to compare controls with ABA-treated plants. ns - nonsignificant difference at p = 0.05

	Helianthus annuus		***Populus* hybrid**	
	Γ^*	R_d	Γ^*	R_d
Controls	33.00 ± 0.91^{ns}	0.48 ± 0.02^{ns}	36.00 ± 1.26^{ns}	0.28 ± 0.06^{ns}
ABA-treated	33.88 ± 1.30^{ns}	0.44 ± 0.07^{ns}	38.33 ± 0.88^{ns}	0.28 ± 0.02^{ns}

3.2 Effect of abscisic acid and CO_2 concentration on the rate of photosynthesis

A_N-C_i curves were conducted within 1.5 h using different ranges of CO_2 concentrations for sunflower and poplar, respectively, and they showed the typical non-rectangular hyperbolic relationship (**Fig. 3.2 A, B**). The initial part of all A_N-C_i curves showed almost linear dependence of A_N on C_i, denoting the limitation by carboxylation (Long and Bernacchi, 2003). A curvilinearity of the second part of curves indicates limitation by regeneration of ribulose-1,5-bisphosphate. No decline of A_N was found in poplar at high C_i, indicating that photosynthesis was not limited by triose phosphate utilization. The range of CO_2 concentrations was smaller in measurements with sunflower; hence this type of limitation was probably not detectable.

When 20 μM abscisic acid was added to sunflower plants, lower photosynthetic rates over the range of C_i were observed in comparison to controls (**Fig. 3.2**). This result was, however, contrary of what we expected according to the previous study (Vrábl *et al*., 2009). Since ABA is a stress hormone with wide range of physiological effects (Raghavendra *et al*., 2010), it is probable that some secondary physiological effects that affected photosynthesis in an unexpected way due to high ABA concentration treatment were present. Therefore, concentration of added ABA was lowered to 10 μM. This concentration of ABA induced reduction of stomatal conductance (**Fig. 3.4A**), but the rate of photosynthesis was higher in contrast to plants with 20 μM ABA treatment. On the other hand the rate of photosynthesis of 10 μM ABA treated plants was lower with respect to control plants (**Fig. 3.2A**).

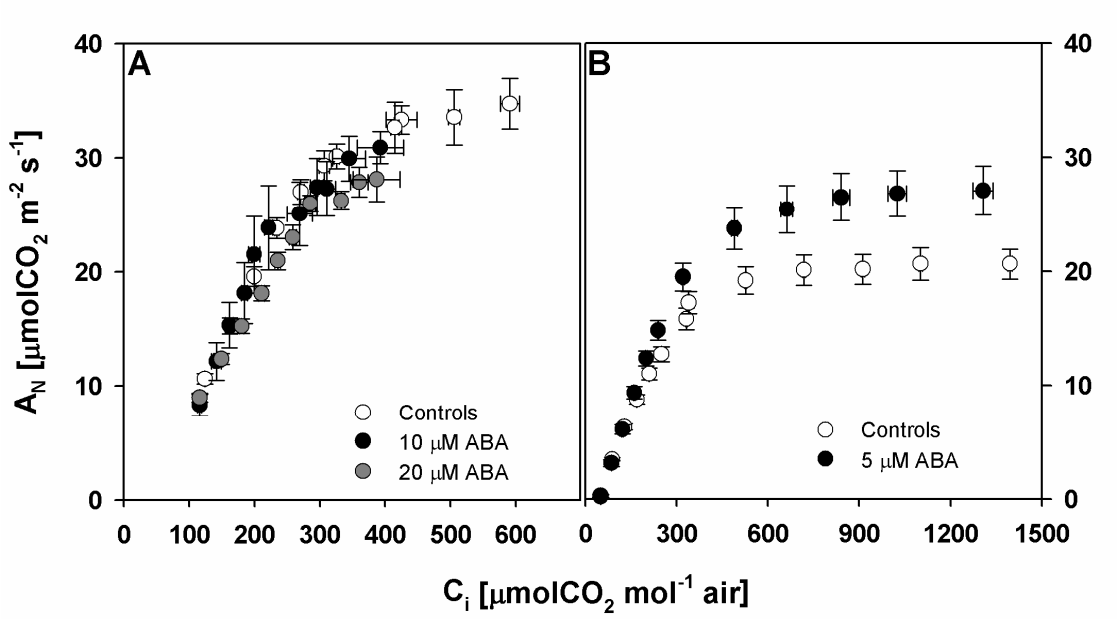


Figure 3.2. Response of net photosynthesis (A_N) to sub-stomatal CO_2 concentrations (C_i) of control and ABA-treated plants (10 and 20 μM) in sunflower (**A**), and of control and ABA-treated plants (5 μM) in poplar (**B**). Values are means ± SE of 9 (A, controls), 8 (A, 20 μM ABA), 3 (10, μM ABA) and 6 (B, both controls and 5 μM ABA) replicates.

In poplar, ABA-treated plants showed similar photosynthetic rates as controls at initial linear portion of the A_N-C_i curve but were higher in curvilinear and saturation part (**Fig. 3.2B**). Besides this, ABA-treated plants showed the general shift of data towards lower C_i. This shift was greater in sunflower since higher ABA concentration was used than in poplar and hence, stomatal conductance and finally C_i were more reduced (**Fig. 3.4 A, B**).

3.3 Effect of abscisic acid and CO_2 concentration on electron transport rate

Electron transport rate (J_f) responded in a biphasic mode in both species. Initially, J_f increased with C_i, then peaked at 300 to 500 μmol CO_2 mol^{-1} air and finally decreased (**Fig. 3.3A, B**) possibly due to feedback limitation by the utilization of triose phosphate (Sharkey *et al.*, 1988). In sunflower, this effect was observed only for controls since data set from ABA-treated plants was shifted towards lower C_i and range of CO_2 concentration was not sufficiently wide to record this type of limitation.

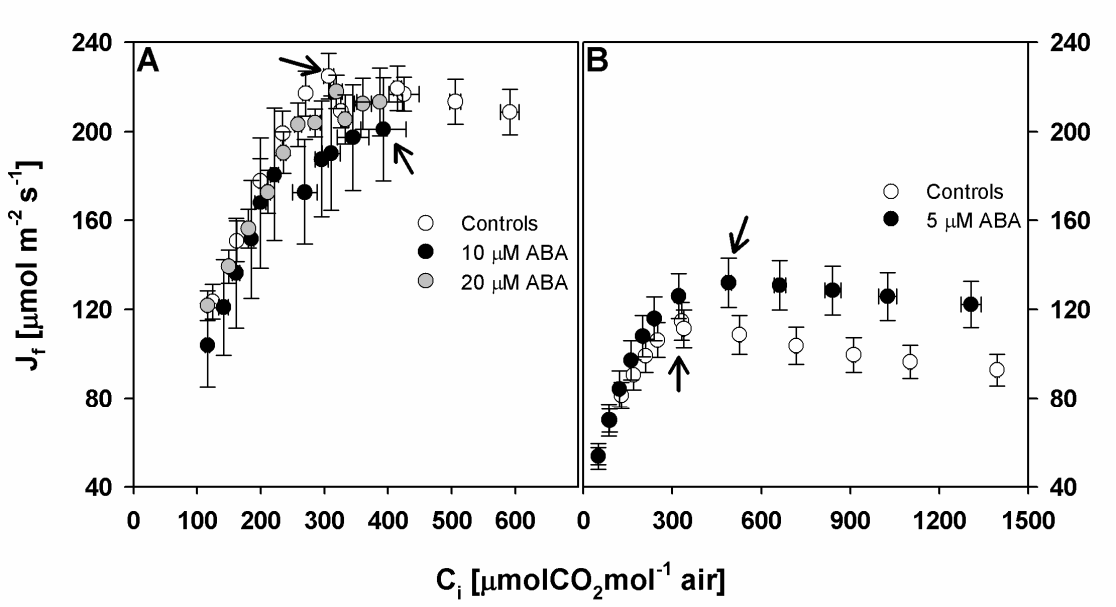


Figure 3.3. Response of electron transport rate to sub-stomatal CO_2 concentration of control and ABA-treated plants (10 and 20 μM) in sunflower (**A**), and of control and ABA-treated plants in poplar (**B**). Arrows in the graph indicate maximal value within the curve. Values are means ± SE of 9 (A, controls), 8 (A, 20 μM ABA), 3 (10, μM ABA) and 6 (B, both controls and 5 μM ABA) replicates.

In sunflower, rate of linear electron transport was affected non-significantly by addition of ABA in the initial linear portion of J_f-C_i curve. However, at C_i of 306.7 ± 8.1 μmol CO_2 mol^{-1} air where the J_f peak of 224.3 ± 10.2 μmol m^{-2} s^{-1} was observed in controls, J_f was slightly lower in plants treated with both 20 and 10 μM ABA (**Fig. 3.3 A**). After controls peaked, J_f of ABA-treated plants had still increased suggesting a peak to be shifted to the higher C_i.

In poplar, ABA-treated plants showed higher electron transport rates over the entire range of CO_2 concentrations with J_f peak of 132 ± 11.1 μmol m^{-2} s^{-1} at C_i 489.5 ± 10.1 μmol CO_2 mol^{-1} air. In control plants, peak (114 μmol m^{-2} s^{-1}) appeared already at C_i 338.7 ± 3.6 μmol CO_2 mol^{-1} air. Differences were apparently highest at the second mode of J_f-C_i curve from C_i 500 to 1500 μmol CO_2 mol^{-1} air.

In general, J_f of ABA-treated plants seemed to peak at higher CO_2 concentration in both sunflower and poplar (arrows on **Fig. 3.4** indicating the peaks).

3.4 Effect of abscisic acid and CO_2 concentration on stomatal conductance

Stomatal conductance (g_s) responded to changing CO_2 concentration by decline at higher C_i (**Fig. 3.4 A, B**) Although absolute values of g_s were higher in sunflower (maximal g_s = 0.94 mol m^{-2} s^{-1}) than in poplar (0.61 mol m^{-2} s^{-1}), the degree

of response was the same in sunflower as in poplar since both of them showed approximately 25% decrease with increasing C_i.

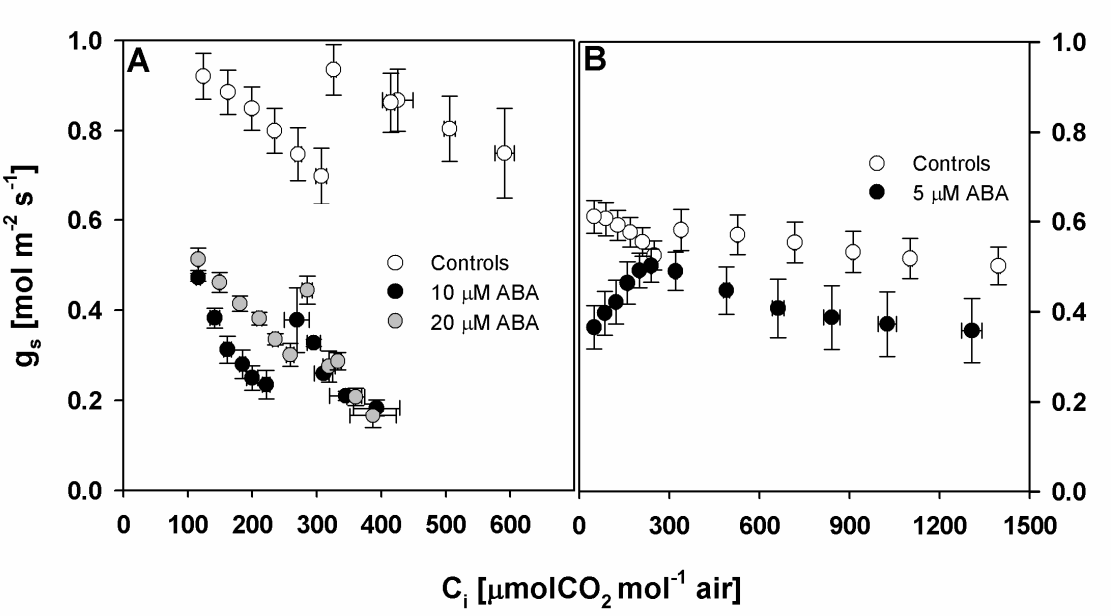


Figure 3.4. Response of stomatal conductance to sub-stomatal CO2 concentration of control and ABA-treated plants (10 and 20 μM) in sunflower (**A**), and of control and ABA-treated (5 μM) plants in poplar (**B**). Values are means ± SE of 9 (A, controls), 8 (A, 20 μM ABA), 3 (10, μM ABA) and 6 (B, both controls and 5 μM ABA) replicates.

Stomatal conductance was reduced in ABA treated plants over the entire range of CO_2 concentrations in both sunflower and poplar with greater reduction in higher CO_2 concentrations (**Fig. 3.4 A, B**). In sunflower, a reduction in g_s was as much as three-fold, from 0.86 to 0.26 (mol m^{-2} s^{-1}) at around 400 (μmol CO_2 mol^{-1} air). In poplar, reduction of g_s was not as distinct, probably due to lower ABA concentration used. Nevertheless, the reduction was observed over the entire range of C_i. Hence, it was possible to estimate the diffusional limitations of photosynthesis since the CO_2 transport from ambient to intercellular airspaces was restricted.

When measuring on ABA-treated plants, some errors can be introduced by patchy stomatal closure (Terashima, 1992). However, although reduction in g_s was substantial, g_s values in ABA-treated plants were still high (min. around 0.2 mol CO_2 m^{-2} s^{-1} in sunflower). Moreover, Flexas *et al*. (2006b) showed that exogenous ABA did not induced patchy stomatal closure even when g_s dropped to much lower values (0.03 mol CO_2 m^{-2} s^{-1}) in other herbaceous species. In addition, the close similarity in the curvature of A_N-C_i curves measured in high and low water pressure deficit (**Fig. 3.5**) has been taken as an indication for the absence of patchy stomatal closure in ABA-treated plants (Grassi and Magnani, 2005).

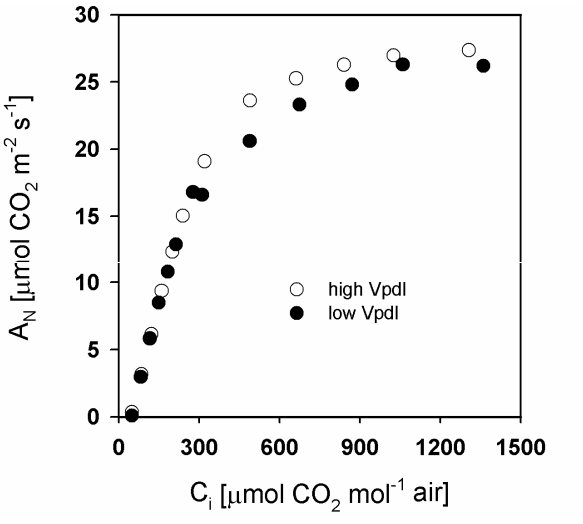


Figure 3.5. Relationship between photosynthetic rate (A_N) and sub-stomatal CO_2 concentration (C_i) under low (0.47 to 0.55 kPa) and high (0.86 to 0.99 kPa) water pressure deficit.

3.5 Response of mesophyll conductance to varying CO_2 concentration

Simultaneous measurements of gas exchange and chlorophyll fluorescence allowed g_m to be measured for all C_i concentrations used, except the very low one in poplar, where A_N was close to zero, resulting in not reliable g_m value.

Variable J method is based on the relationship between C_c and $A + R_d$, assuming Γ^* to be fixed. If so, there is a family of curves representing different values of *J*. Harley *et al*. (1992) found that if the slope of the curve was too great, the sensitivity of g_m to small errors was too great. On the other hand, if the slope was too low, the data were often unbelievable. Such data did not fit the expectable values. Therefore, Harley *et al*. (1992) introduced the criterion suggesting that estimates of g_m could be questionable when dC_c/dA_N is lower than 10 or higher than 50. So, the threshold values are set and only g_m estimates that fulfill that criterion are generally accepted as reliable (**Fig. 3.6 A, B** – shaded regions).

Applied Harley's criterion is more empirical then theoretical approach for examination of reliability of the g_m estimates. Thereby it disclaims not only negative or unlikely high g_m values but also values which are presumable. In spite of obscurities in Harley's criterion our data were tested by it to demonstrate the significance of the effect of CO_2 and ABA on g_m.

For sunflower, there are some data for controls that should not be accepted according to the criterion. However, they were regarded as acceptable in the present

study since they all were very close to the threshold value of the criterion. Moreover, all data for 10 μM ABA-treated plants fulfill the criterion.

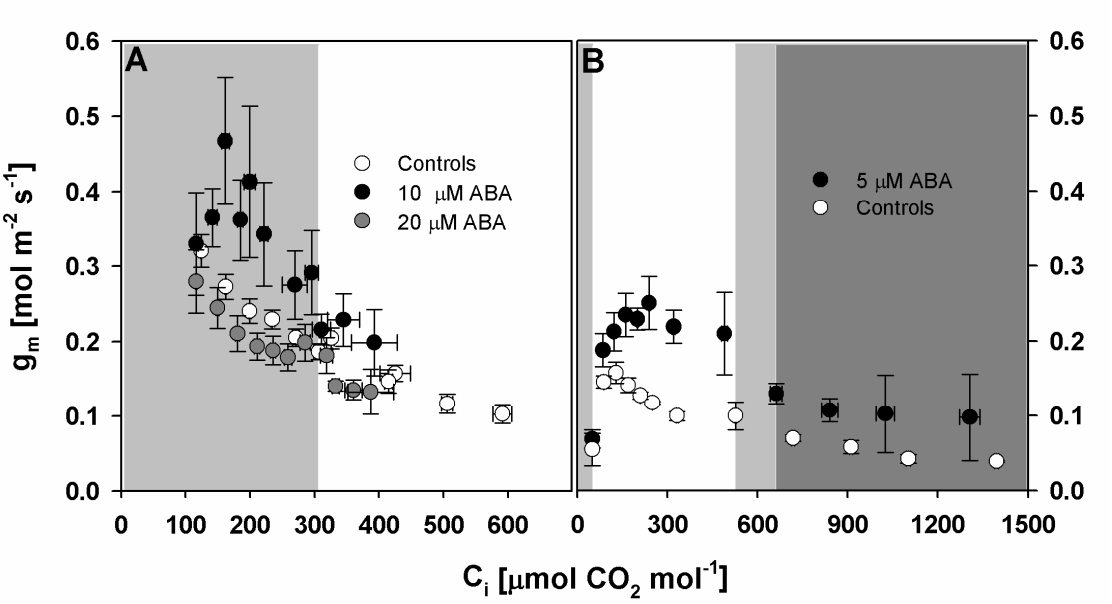


Figure 3.6. Response of mesophyll conductance to varying CO_2 concentration of control and ABA-treated plants (10 and 20 μM) in sunflower (**A**), and of control and ABA-treated (5 μM) plants in poplar (**B**). Values are means ± SE of 9 (A, controls), 8 (A, 20 μM ABA), 3 (10, μM ABA) and 6 (B, both controls and 5 μM ABA) replicates. The unshaded region indicates g_m data with a dC_c/dA_N between 10 and 50, which are reliable according to Harley *et al*. (1992). Light-grey region indicate data out of that range in controls; dark-grey region indicate data out of that range in ABA treated plants. For simplicity, only light-grey region for controls is shown in sunflower. All g_m estimates in 20 μM ABA-treated plants were slightly out of the range. Contrary, all g_m estimates in 10 μM ABA-treated plants fulfill Harley's criterion.

At ambient CO_2 concentration, g_m varied from 0.1 mol m^{-2} s^{-1} in poplar to 0.2 mol m^{-2} s^{-1} in sunflower. Mesophyll conductance was observed not to be constant along the range of CO_2 concentrations but to respond to varying CO_2 as previously described by Flexas *et al*. (2007a) and Vrábl *et al*. (2009). In both species, non-linear proportionality between g_m and C_i was found (**Fig. 3.6 A, B**). After initial growth in low C_i, g_m-C_i relationship continued with the exponential decay at C_i higher than 150 μmol mol^{-1} air with decline being steepest up to C_i of 500 μmol CO_2 mol^{-1} air. Thereafter g_m almost stabilized. In general, g_m decreases as C_i increases. At high C_i, g_m values were as low as 26 to 32% of those at lower C_i.

For ABA-treated plants, the same non-linear relationship between g_m/C_i was observed with initial growth, subsequent exponential decrease and stable values at high C_i eventually. However, peak of g_m was shifted and appeared at higher C_i in comparison to controls.

3.6 Effect of abscisic acid on mesophyll conductance

Addition of abscisic acid allowed us to introduce a diffusional limitation to photosynthesis by closing the stomata and therefore decrease stomatal conductance (g_s) markedly (see chapter 3.4). Contrary to g_s, mesophyll conductance was increased after ABA addition in both sunflower and poplar, except for the highest ABA concentration used (20 μM) in sunflower where g_m slightly decreased (**Fig. 3.6 A, B**). Mesophyll conductance in ABA-treated plants increased over the entire range of CO_2 concentrations. The increase ranged from 3 to 101% in sunflower and from 25 to 119% in poplar. Differences in g_m between controls and ABA-treated plants were most remarkable at low C_i from 100 to 300 μmol CO_2 mol^{-1} air.

Generally, values of g_m were higher in sunflower than in poplar, which is in accordance to the previously observed estimates for herbs and woody species (Warren, 2008a; Flexas *et al*., 2008).

3.7 Effect of enhanced mesophyll conductance on chloroplastic CO_2 concentration

Sub-stomatal CO_2 concentration (C_i) increased with increasing C_a in linear relationship in all treatments and species. When ABA was applied, stomatal conductance was lowered since CO_2 flux through stomata was restricted. Therefore, in comparison to controls with more opened stomata and high g_s, sub-stomatal CO_2 concentration of ABA-treated plants was lower over the entire range of C_a in both species, especially at higher C_a (**Fig. 3.7 A, B**). In sunflower, decrease ranged from 6 to 35% of C_i of controls in both the 20 and 10 μM ABA-treated plants. Since lower ABA concentration was used in poplar, decrease in C_i was less extensive (from 0 for very lowest C_a to 8% at higher C_a) but significant especially between 300 and 1500 μmol CO_2 mol^{-1} air.

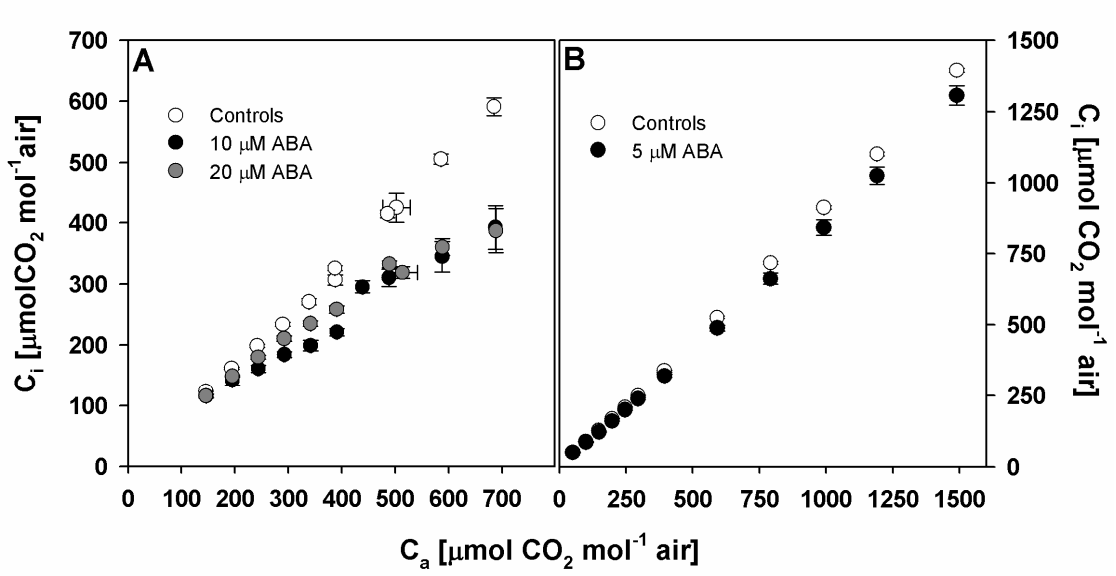


Figure 3.7. Relationship between the sub-stomatal (C_i) and ambient (C_a) CO_2 concentration of control and ABA-treated plants (10 and 20 μM) in sunflower (**A**), and of control and ABA-treated (5 μM) plants in poplar (**B**). Values are means ± SE of 9 (A, controls), 8 (A, 20 μM ABA), 3 (10, μM ABA) and 6 (B, both controls and 5 μM ABA) replicates.

Mesophyll conductance with its sub-components, as described in the Introduction, represents a great diffusional limitation to photosynthesis since it decreases chloroplastic CO_2 concentration and therefore amount of CO_2 available for carboxylation by Rubisco. In present work, mesophyll conductance increased after addition of ABA (at concentration lower than 20 μM) in both species studied. The enhancement in the g_m should affect the CO_2 concentration in the chloroplast (C_c). We found that C_c was the same (in sunflower) of slightly higher (in poplar) in ABA-reated plants than in controls (**Fig. 3.8 A, B**). Therefore, the increase of g_m corresponds to increase in C_c with respect to C_i.

Overall, the C_i/C_a ratio was higher in controls than in ABA-treated plants of both sunflower and poplar. C_i/C_a ratio ranged between 0.79 and 0.86 in sunflower controls; and between 0.84 and 0.99 in poplar controls. In ABA-treated plants, Ci/Ca ranged between 0.56 – 0.79 in both 20 and 10 μM ABA treatments of sunflower; and between 0.81–0.87 in poplar.

Contrary, C_c/C_a ratio was higher in all ABA-treated plants (0.55–0.77 in sunflower and 0.47–0.68 in poplar) in comparison to controls (0.41–0.72 in sunflower and 0.23 –0.64), except for sunflowers treated by 20 μM ABA.

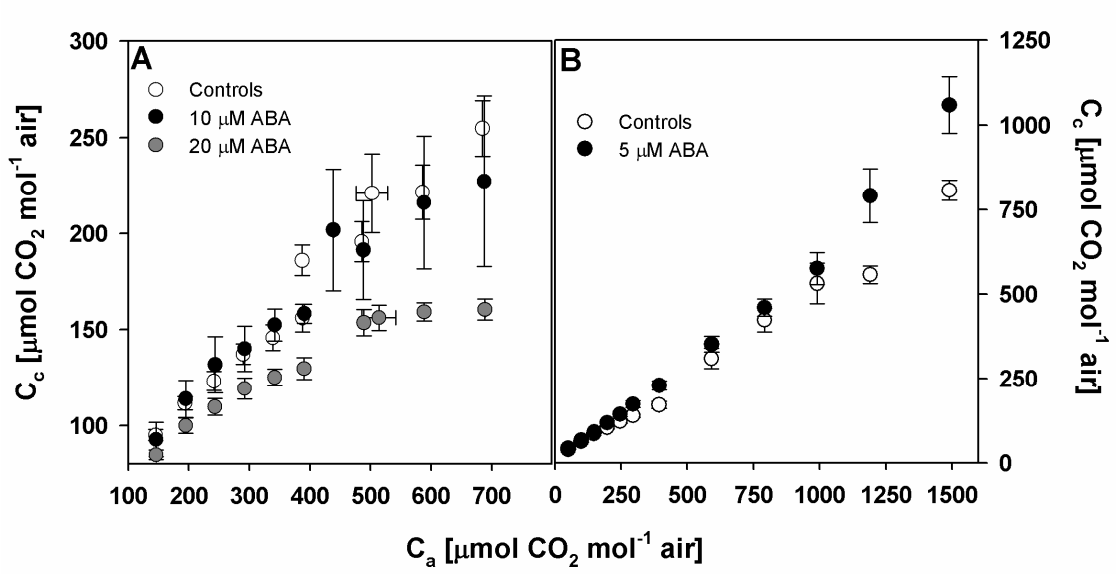


Figure 3.8. Relationship between the chloroplastic (C_c) and ambient (C_a) CO_2 concentration of control and ABA-treated plants (10 and 20 μM) in sunflower (**A**), and of control and ABA-treated (5 μM) plants in poplar (**B**). Values are means ± SE of 9 (A, controls), 8 (A, 20 μM ABA), 3 (10, μM ABA) and 6 (B, both controls and 5 μM ABA) replicates.

4. Discussion

4.1 CO_2 response of mesophyll conductance

Although CO_2 concentration is crucial for physiology of photosynthesis and g_m represents its great diffusional limitation, only several studies have been dealing with CO_2 response of g_m. In the presented study, g_m responded rapidly to varying CO_2 in two species that belong to different functional groups, herbaceous (sunflower) and woody (poplar) species. Absolute values of g_m differed between these two studied species. In sunflower, g_m was twice higher than in poplar over the whole range of CO_2 concentration with respect to actual C_i. This is in accordance with previously published data that show that annual and biannual herbs present the largest values of g_m and, in woody deciduous angiosperms being two times lower (Flexas *et al*., 2008).

However, g_m response to varying CO_2 concentration was of the same magnitude and relationship in both the sunflower and poplar. At low C_i, g_m increased, peaked from 150 to 250 μmol CO_2 mol^{-1} air and decreased exponentially until steady state was reached at C_i higher than 500 μmol CO_2 mol^{-1} air. g_m decreased as much as three to four-fold (**Fig. 3.6 A, B**).

Such type of response was initially suggested by Düring (2003) who observed six-fold decrease of g_m when increasing C_i from 300 to 1000 μmol mol^{-1} air in grapevine. Later, Flexas *et al*. (2007a) provided a more detailed analysis of g_m response to CO_2 in six different species supporting presented findings. Although magnitude of g_m response was higher (6 to 9-fold decrease over range of C_i), the relationship between g_m-C_i was similar to that presented here. Moreover, our data are consistent with most recently published results of Vrábl *et al*. (2009), Hassiatou *et al*. (2009) and Yin *et al*. (2009) from measurements on sunflower, *Banksia* and wheat, respectively. In summary, here presented and previously published data support hypothesis that g_m could be affected by CO_2 concentration. So far, two types of g_m-CO_2 relationship have been published (if study of Tazoe *et al*. (2009) is omitted). Firstly, g_m increases at low CO_2 concentrations, peaks, and declines exponentially thereafter (**Fig. 4.1, line a**). Secondly, only exponential decay without initial growth and peak was observed (**Fig. 4.1, line b**).

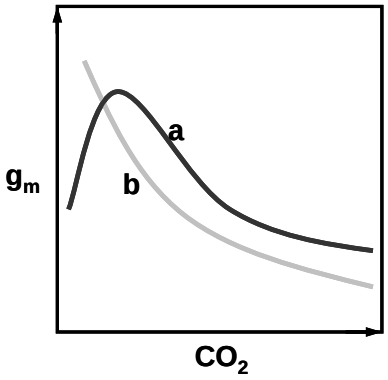


Figure 4.1. Schematic illustration of two possible types of g_m responses to varying CO_2 concentration as described mostly in the literature.

Although Loreto *et al*. (1992) and later Tazoe *et al*. (2009) found no sensitivity of g_m to varying CO_2 concentration, their data could be insufficient since they measured g_m over the three times smaller range of CO_2 concentrations (from 100 to 500 μmol CO2 mol^{-1} air) than in studies proving CO_2 dependency of g_m.

Recently, results of Ethier and Pepin (2010) has suggested that g_m response to CO_2 could be species dependent and/or that some methodological obscurities are presented, since g_m responded to CO_2 in *Nicotiana tabacum* but not in *Helianthus annuus* and *Populus* x *jackii* using isotopic method. On the other hand, g_m responded to CO_2 in all species using variable J method.

In addition, on canopy-scale, no changes in g_m were found in response to varying CO_2 concentration in sunflower grown in growth cabinet prior to ABA treatment (Schäufele *et al*., 2011). However, measurements on mesocosm-scale and leaf-scale can be different due to several factors discussed in the next chapter.

4.2 Effect of abscisic acid

Abscisic acid (ABA) is a stress hormone with wide range of effects on plant physiology. Primarily, it triggers closure of stomata to prevent water losses (Zhang and Davies, 1990). Therefore by application of ABA to plants, we can introduce a great diffusional limitation for photosynthesis since stomatal conductance is suppressed.

The ABA treatment applied in the present study was sufficient to decrease g_s over the entire range of C_i (**Fig. 3.4 A, B**). However, A_N was slightly decreased in sunflower and no decrease of A_N was recorded in poplar during the ABA treatment (10 μM ABA in sunflower and 5 μM ABA in poplar). Moreover, A_N was enhanced between 400 and 1500 μmol CO_2 mol^{-1} air in poplar (**Fig. 3.2 A, B**). Similar effect

of ABA was observed in case of electron transport rate (J_f) (**Fig. 3.3 A, B**).

These findings are in approximate accordance with those of Vrábl *et al*. (2009). Using 20 μM ABA, they found no significant decrease in A_N and J_f, although the ABA addition caused 38 to 60% reduction of stomatal conductance over the entire range of C_i. Additionally, neither g_m nor photosynthetic capacity of leaves at ambient CO_2 was significantly changed after ABA addition. The only difference observed between controls and ABA-treated plants consisted of general shift of ABA-plants towards the lower C_i (Vrábl *et al*., 2009). This displacement was caused by closure of stomata and reduced C_i in ABA-plants and was recorded also in the presented study (**Fig. 3.2 A, B**). On the other hand, supplying sunflower plants by 20 μM ABA, Schäufele *et al*. (2011) observed decrease in A_N over the entire range of CO_2 concentrations used. This was, however, on mesocosm scale in contrast to leaf-scale used in the presented study and study of Vrábl *et al*. (2009).

In presence of higher concentration of ABA (20 μM) in sunflower, g_m was observed not to differ from control plants which is the same result as previously published by Vrábl *et al*. (2009) but opposite of that published by Schäufele *et al*. (2011). However, using halved (10 μM) and even lower (5 μM) ABA concentration in case of sunflower and poplar, respectively, enhancement of g_m in comparison to controls was observed over the entire range of CO_2 concentrations (**Fig. 3.6 A, B**). ABA was previously proved to decrease g_m (Flexas *et al*., 2006b), but in much higher concentrations of 100 μM. To my best knowledge, this is the first time, when experimental evidence was provided that ABA led to increased g_m.

Canopy-scale measurements of Schäufele *et al*. (2011) brought contrary results. However, canopy-scale and leaf-scale measurements could differ due to several factors. On leaf-scale, different growing and measuring light was used and measured leaf was exposed to different CO_2 concentration than rest of the plant, in contrast to whole canopy-scale measurements where all leaves were exposed to the same CO_2 concentration and growing and measuring light was the same (Schäufele *et al*., 2011). Moreover, only young leaves were used in the leaf-scale measurements of Vrábl *et al*. (2009) and in presented study, whereas all leaf-age categories of plants contributed to the whole canopy-scale measurements.

4.3 Link between stomatal and mesophyll conductance

Simultaneous decrease of g_s and g_m was often described in drought and salt stressed plants (Bongi and Loreto, 1989; Flexas *et al*., 2002, 2006b; Centrito *et al*., 2003; Peeva and Cornic, 2009; Warren *et al*., 2004) indicating some association with ABA. Studying salt stressed olives, Centrito *et al*. (2003) found linear relationship ($r^2 = 0.68$) between g_m and g_s suggesting that response of g_m to environmental factor can be as fast as that of g_s. Moreover, besides simultaneity of g_s and g_m response to stress connected with drought, g_m was found to respond to light and CO_2 in a similar way as g_s does (Flexas *et al*., 2007a; Yin *et al*., 2009). This led to suggestion that g_s and g_m could be intrinsically co-regulated (Peeva and Cornic, 2009). However, it would be disadvantageous to decrease g_m when g_s is decreased since diffusional limitation for photosynthesis would be much higher. Therefore, Vrábl *et al*. (2009) speculated that there might be an advantage in terms of carbon gain in enhancing g_m at low g_s, and hence, keep the photosynthetic assimilation rate unchanged.

This hypothesis was proved by results presented here. When applying ABA to induce stomatal closure, g_s decreased (**Tab. 4.1**) which led to significant decrease of C_i in comparison to controls (**Fig. 4.2**). In some cases, however, differences were statistically insignificant due to high variability of data and small number of replicates. For instance, mean value of g_s in ABA-treated plants of poplar was lower than in control plants, but not significantly (**Tab. 4.1**).

Table 4.1. Mean values ± SE of rate of photosynthesis, A_N (μmol m^{-2} s^{-1}), ambient, C_a, sub-stomatal, C_i, and chloroplastic, C_c CO_2 concentration (μmol mol^{-1} air), stomatal ,g_s, and mesophyll g_m, conductance (mol m^{-2} s^{-1}); at CO_2 400 μmol mol^{-1} air for control and ABA-treated plants of sunflower (*Helianthus*) and poplar (*Populus*). T-test was used for statistical analysis to compare controls with ABA-treated plants.

Plant	Treatment	A_N	C_a	C_i	C_c	g_s	g_m
Helianthus	Control	30.14 ± 1.09^{ns}	$387 \pm 0.43^{**}$	$326 \pm 3.87^{**}$	160.5 ± 5.02^{ns}	$0.94 \pm 0.06^{**}$	$0.204 \pm 0.014^{*}$
	10 μM ABA	25.09 ± 2.82^{ns}	$390 \pm 1.08^{**}$	$269 \pm 19.28^{**}$	153.3 ± 7.89^{ns}	$0.38 \pm 0.07^{**}$	$0.316 \pm 0.045^{*}$
Populus	Control	16.82 ± 0.97^{ns}	393 ± 0.45^{ns}	$338 \pm 3.17^{*}$	208 ± 12.1^{ns}	0.58 ± 0.05^{ns}	$0.131 \pm 0.008^{**}$
	5 μM ABA	19.09 ± 1.22^{ns}	393 ± 0.20^{ns}	$320 \pm 4.91^{*}$	230 ± 13.2^{ns}	0.49 ± 0.04^{ns}	$0.219 \pm 0.022^{**}$

^{ns} Nonsignificant difference; * Differed at 0.05 level of significance; ** Differed at 0.01 level of significance

Nevertheless, insignificantly decreased g_s was able to significantly decrease C_i. However, photosynthetic assimilation rate decreased slightly in 10 μM ABA-plants of sunflower and was increased in 5 μM ABA-plants of poplar (**Tab. 4.1; Fig. 3.2 A, B**) although C_i was significantly lower than in control plants.

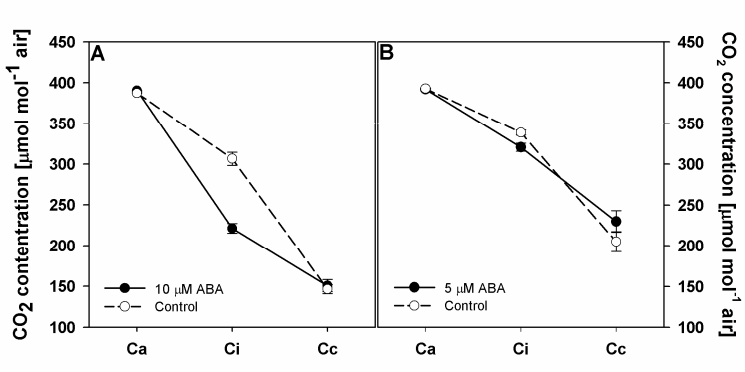


Figure 4.2. Drop of CO_2 concentration from ambient (C_a) to sub-stomatal (C_i), and chloroplastic (C_c) CO_2 concentration in controls and ABA-treated plants of sunflower (**A**) and poplar (**B**). Values are means ± SE of 9 (A, controls), 3 (A, 10 µM ABA) and 6 (B, both controls and 5 µM ABA) replicates.

Explanation for this provides comparison of g_m in controls with ABA-treated plants. g_m was significantly higher in presence of ABA. This led to unchanged (in sunflower) or slightly increased (in poplar) C_c (**Fig. 4.2**) and finally to almost unchanged or even slightly increased A_N **(Tab. 4.1)**. These results indicate that g_m could be somehow linked to g_s and that both of them could be regulated in a feedback way to keep the maximal CO_2 concentration in the site of carboxylation. This led us to the hypothesis illustrated on Fig. 4.3. When stomata are fully opened (**Fig. 4.3 A**), CO_2 uptake is sufficient, therefore C_i is high enough and g_m decreases since photosynthesis is not limited by CO_2 availability. On the other hand, when stomata are closed, e.g. after ABA addition or in drought stress (**Fig. 4.3 B**), CO_2 uptake is restricted and C_i decreases. As a reaction to decreased C_i, g_m increases to enhance the CO_2 flux into the chloroplast.

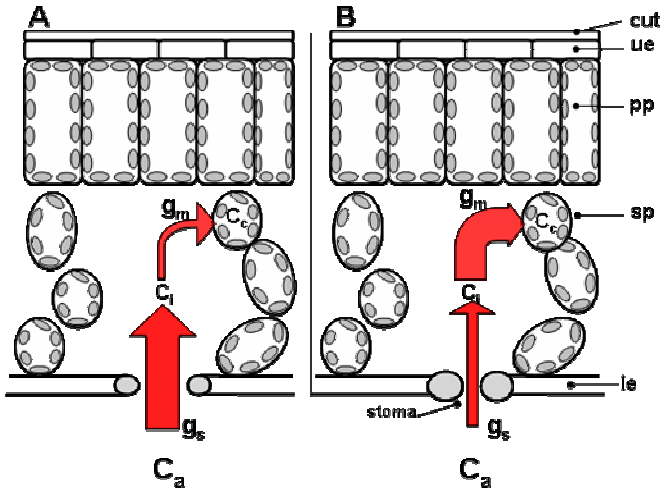


Figure 4.3. Simplified model of leaf section showing possible link between g_m and g_s in the case when stomata are fully opened as in controls (**A**) and closed as in ABA-treated plants (**B**). Red arrows represent a magnitude of CO_2 flux from ambient (C_a) to sub-stomatal cavity (C_i) and from sub-stomatal cavity to chloroplast stroma (C_c). The cuticle (cut), upper epidermis (ue), palisade parenchyma (pp), sponge parenchyma (sp), lower epidermis (lp) and stoma is shown.

g_m-C_i curves show that at low C_i, g_m increases (**Fig. 3.6 A, B**) but when C_i increases, g_m decreases as photosynthesis is not more limited by CO_2 availability. As discussed in Flexas *et al*. (2007a), a sustained high g_m at high C_i, when the CO_2 assimilation rate is saturated, would almost double the CO_2 concentration in the chloroplast stroma (C_c). Maintaining g_m high at high CO_2 concentrations would result in problems involving low stromal pH detrimental for photosynthetic enzymes (Berkowitz *et al*., 1983) or high energy requirement (Flexas *et al*., 2007a). Alternatively, g_m needs to be increased at low CO_2 concentrations where energy is in excess to that required for photosynthesis and increased CO_2 availability would be better for photosynthesis as Flexas *et al*. (2007a) speculated. Therefore, fine-tuned co-regulation of g_s and g_m is needed.

However, depression of C_i due to low g_s and high g_m may force the stomatal opening accompanying with consequent increase of C_i and this may lead to oscillation of photosynthesis (Šantrůček *et al*., 2003). Nevertheless, if g_m increases enough to remove CO_2 from sub-stomatal cavity, C_i can decrease enough to create a gradient of CO_2 concentrations between the ambient and sub-stomatal cavity. Hence, CO_2 flux would possibly be enhanced even through the half-closed stomata. As a result, rate of CO_2 assimilation does not change or is enhanced while water loss through stomata decreases. This will lead to enhancement of water use efficiency (WUE = A/E) without any depression of photosynthesis. In the presented study, increased WUE over the entire range of CO_2 concentrations was observed in ABA-treated plants in both sunflower and poplar (**Fig. 4.4**).

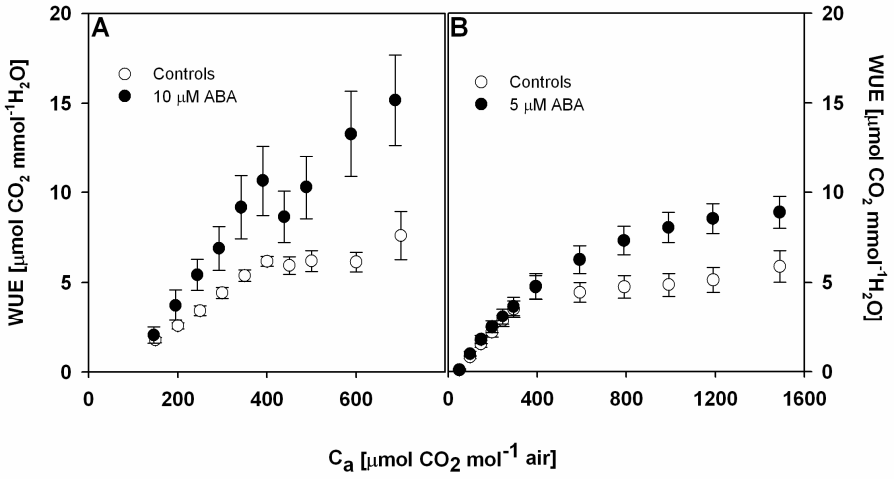


Figure 4.4. Response of water use efficiency of controls and ABA-treated plants to C_a in sunflower (A) and poplar (B). Values are means ± SE of 9 (A, controls), 3 (10, μM ABA) and 6 (B, both controls and 5 μM ABA) replicates.

Following the same experimental procedure as in presented study, Vrábl *et al*. (2009) did not find any increase of g_m in ABA-treated sunflower plants, although A_N was unaffected by ABA addition that led to decrease in g_s. The authors discussed that all values were close to the saturated part of the well-known curvilinear relationship between A_N and g_s, i.e. that at ambient CO_2, A_N in these plants was more limited by photosynthetic capacity than by CO_2 availability (Flexas *et al*., 2006b). In other words, although g_s was reduced after ABA-addition, it was not low enough to cause a high CO_2 limitation for photosynthesis. Therefore g_m remained unchanged. Using the same species and procedures, however, we were able to observe g_m enhancement described above. This was probably due to greater differences between the g_s of controls and ABA-treated plants (drop from 0.94 to 0.38 mol CO_2 m^{-2} s^{-1} at 400 μmol CO_2 mol^{-1} air) than in the study of Vrábl *et al*. (2009) (drop from 0.45 to 0.27 mol CO_2 m^{-2} s^{-1} at 400 μmol CO_2 mol^{-1} air). Therefore, in our case greater diffusional limitation was introduced which finally led to higher g_m found in ABA-treated plants.

4.4 Possible role of aquaporins in regulation of mesophyll conductance

Presented study proved significant enhancement of g_m in ABA-treated plants in comparison to controls. By studying temperature response of g_m, it was suggested that g_m is controlled by protein-facilitated process (Bernacchi *et al*., 2002) and aquaporins were proved to be connected with g_m (Flexas *et al*., 2006a; Hanba *et al*., 2004). These results suggest possible involvement of ABA in regulation of g_m through modulation of aquaporins activity since ABA was previously found to enhance AQP activity for water permeability (Wan *et al*., 2004). Therefore, regulation of mesophyll conductance by ABA could be either direct or indirect (see **Fig. 4.5**). Direct regulation could be possible if ABA enhances CO_2 permeability of aquaporins together with the water permeability. If so, CO_2 could permeate through the central or side pore of AQP tetramer (Wang *et al*., 2007) (**Fig. 1.9 A**). Indirect regulation of g_m by ABA is possible by enhancing flux of water through AQP, thus reducing the thickness of unstirred layers on the surface of the membrane that represents barrier for CO_2 and finally allowing CO_2 to permeate only through membrane.

In summary, it was proved that ABA affects g_m and probably is connected with its regulation. However, if ABA regulates g_m directly or indirectly should be further

tested.

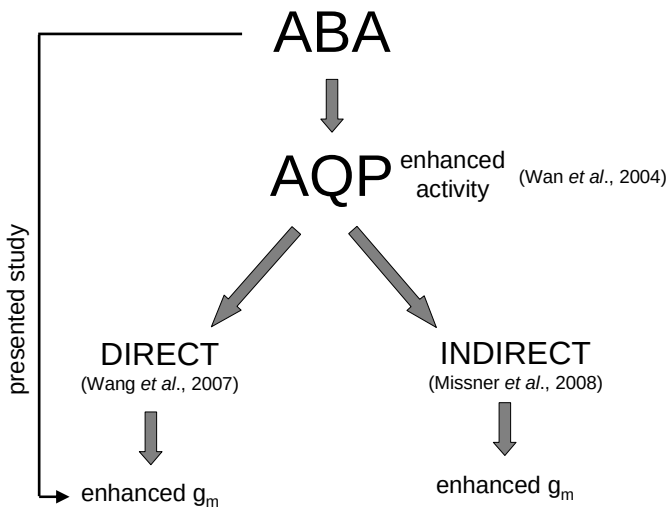


Figure 4.5. Schematic illustration of possible processes laying behind the regulation of g_m by ABA.

5. Conclusion

The assessment of mesophyll conductance (g_m) at different CO_2 concentration in absence and presence of abscisic acid conducted in presented study has provided an insight into diffusional limitations of photosynthesis. g_m was observed to respond rapidly to varying CO_2 concentration in both the controls and ABA-treated plants. For the first time, an increase of g_m over the range of CO_2 concentrations in presence of low concentration of exogenously added ABA was found. Based on obtained results, it was suggested that g_m could be somehow linked to g_s and that both of them could be regulated in a feedback way to keep the maximal CO_2 concentration in the sites of carboxylation even when stomata are partially closed. By doing this, plants can reduce water losses but keep the photosynthetic rate unchanged. Additionally, by using ABA-treated plants, possible way of g_m regulation by ABA through aquaporins was outlined with appeal for further testing.

5. References

Berkowitz GA, Chen C,Gibbs M. 1983. Stromal acidification mediates in vivo water stress inhibition of nonstomatal-controlled photosynthesis. *Plant Physiology* **72,** 1123–1126.

Bernacchi CJ, Portis AR, Nakano H, von Caemmerer S, Long SP. 2002. Temperature response of mesophyll conductance. Implications for the determination of Rubisco enzyme kinetics and for limitations to photosynthesis *in vivo*. *Plant Physiology* **130**, 1992-1998.

Bernacchi CJ, Morgan PB, Ort DR, Long SP. 2005. The growth of soybean under free air [CO_2] enrichment (FACE) stimulates photosynthesis while decreasing in vivo Rubisco capacity. *Planta* **220,** 434–446.

Bongi G and Loreto F. 1989. Gas exchange properties of salt stressed olive (*Olea europaea* L.) leaves. *Plant Physiology* **90**, 1408-1416.

Brugnoli E, Scartazza A, Lauteri M, Monteverdi MC, Máguas C. 1998. Carbon isotope discrimination in structural and nonstructural carbohydrates in relation to productivity and adaptation to unfavorable conditions. In *Stable Isotopes: Integration of Biological, Ecological and Geochemical Processes* (ed. H. Griffiths), pp. 133 144. BIOS Scientific Publishers, Oxford, UK.

Bruhn D, Wiskich JT, Atkin O.K. 2007. Contrasting response by respiration to elevated CO_2 in intact tissue and isolated mitochondria. *Functional Plant Biology* **34,** 112–117.

von Caemmerer S and Evans J.R. 1991 Determination of the average partial pressure of CO_2 in chloroplasts from leaves of several C_3 plants. *Australian Journal Plant Physiology* **18,** 287– 305.

von Caemmerer S. 2000. Biochemical models of leaf photosynthesis. CSIRO Publishing, Canberra, Australia.

Centritto M, Loreto F, Chartzoulakis K. 2003. The use of low [CO_2] to estimate diffusional and non diffusional limitations of photosynthetic capacity of salt-stressed olive samplings. *Plant, Cell & Environment* **26**, 585–594.

Chaumont F, Barrieu F, Wojcik E, Chrispeels MJ, Jung R. 2001. Aquaporins constitute a large and highly divergent protein family in maize. *Plant Physiology* **122**, 1025-1034.

Chaumont F, Moshelion M, Daniels MJ. 2005. Regulation of plant aquaporin activity. *Biology of the Cell* **97**, 749-764.

Cooper GJ and Boron WF. 1998. Effect of PCMBS on CO_2 permeability of *Xenopus* oocytes expressing aquaporin 1 or its C189S mutant. *American Journal of Physiology* **275**, C1481–C1486.

Denker BM, Smith BL, Kuhajda FP, Agre P. 1988. Identification, purification, and partial characterization of a novel Mr 28,000 integral membrane protein from erythrocytes and renal tubules. *Journal of Biological Chemistry* **263**, 15634-15642.

Di Marco G, Manes F, Tricoli D, Vitale E. 1990. Fluorescence parameters measured concurrently with net photosynthesis to investigate chloroplastic CO_2 concentration in leaves of *Quercus ilex* L.*Journal of Plant Physiology* **136,** 538-543.

Düring H. 2003. Stomatal and mesophyll conductances control CO_2 transfer to chloroplsts in leaves of grapevine (*Vitis vinifera* L.). *Vitis* **42**, 65-68.

Eckert M, Biela A, Siefritz F, Kaldenhoff R. 1999. New aspects of plant aquaporin regulation and specificity. *Journal of Experimental Botany* **50**, 1541–1545.

Eichelmann H, Oja A, Rasulov B, Padu E, Bichele I, Pettai H, Mols T, Kasparova I, Vapaavuori E, Laisk A. 2004. Photosynthetic parameters of birch (*Betula pendula* Roth) leaves growing in normal and in CO2-and O3-enriched atmospheres. *Plant, Cell and Environment* **27**, 479-495.

Ethier GH and Livingston NJ. 2004. On the need to incorporate sensitivity to CO_2 transfer conductance into the Farquhar–von Caemmerer–Berry leaf photosynthesis model. *Plant, Cell and Environment* **27**, 137–153.

Ethier G, Pepin S. 2010. Mesophyll conductance short-term sensitivity to CO_2 and light: Myth or reality? Presented at The 15th Internatiol Congress of Photosynthesis in Beijing, China, August 2010.

Evans JR, Sharkey TD, Berry JA, Farquhar GD. 1986. Carbon isotope discrimination measured concurrently with gas exchange to investigate CO_2 diffusion in leaves of higher plants. *Australian Journal of Plant Physiology* **13**, 281-292.

Evans JR and Terashima I. 1988. Photosynthetical characteristics of spinach leaves grown with different nitrogen treatments. *Plant and Cell Physiology* **29**, 157-165.

Evans JR and Loreto F. 2000. Acquisition and diffusion of CO_2 in higher plant leaves. In *Photosynthesis: Physiology and Metabolism* (eds R.C. Leegood, T.D. Sharkey & S. von Caemmerer), pp. 321–351. Kluwer Academic Publishers, Dordrecht, the Netherlands.

Evans JR, Terashima I, Hanba Y, Loreto F. 2004. CO_2 capture: chloroplast to leaf. In: Smith WK, Vogelmann TC, Critchley C, eds. *Photosynthetic adaptation: chloroplast to landscape*. New York: Springer.

Evans JR and von Caemmerer S. 1996. Carbon dioxide diffusion inside leaves. *Plant Physiology* **110**, 339-346.

Fetter K, Van Wilder V, Moshelion M, Chaumont F. 2004. Interactions between plasma membrane aquaporins modulate their water channel activity. *Plant Cell* **16**, 215–228.

Farquhar GD, von Caemmerer S, Berry JA. 1980. A biochemical model of photosynthetic CO_2 assimilation in leaves of C3 species. *Planta* **149**, 78-90.

Flexas J, Bota J, Escalona JM, Sampol B, Medrano H. 2002. Effects of drought on photosynthesis in grapevines under field conditions: an evaluation of stomatal and mesophyll limitations. *Functional Plant Biology* **29**, 461–471.

Flexas J, Bota J, Loreto F, Cornic G, Sharkey TD. 2004. Diffusive and metabolic limitations to photosynthesis under drought and salinity in C3 plants. *Plant Biology* **6**, 269–279.

Flexas J, Ribas-Carbó M, Hanson DT, Bota J, Otto B, Cifre J, McDowell N, Medrano H, Kaldenhoff R. 2006a. Tobacco aquaporin NtAQP1 is involved in mesophyll conductance to CO_2 *in vivo*. *The Plant Journal* **48,** 427-439.

Flexas J, Ribas-Carbó M, Bota J, Galmés J, Henkle M, Martínez-Cañellas S, Medrano H. 2006b. Decreased Rubisco aktivity during water stress is not induced by decreased relative water content but related to conditions of low stomatal conductance and chloroplast CO_2 concentration. *New Phytologist* **172,** 73–82.

Flexas J, Diaz-Espejo A, Galmés J, Kaldenhoff R, Medrano H, Ribas-Carbo M. 2007a. Rapid variations of mesophyll conductance in response to changes in CO_2 concentration around leaves. *Plant, Cell and Environment* **30**, 1284-1298.

Flexas J, Díaz-Espejo A, Berry JA, Galmés J, Cifre J, Kaldenhoff R, Medrano H, Ribas-Carbo M. 2007b. Leakage in leaf chambers in open gas exchange systems: quantification and its effects in photosynthesis parameterization. *Journal of Experimental Botany* **58**, 1533-1543.

Flexas J, Ribas-Carbó M, Díaz-Espejo A, Galmés J, Medrano H. 2008. Mesophyll conductance to CO_2: current knowledge and future prospects. *Plant, Cell and Environment* **31**, 602-621.

Galmés J, Pou A, Alsina MM, Tomás M, Medrano H, Flexas J. 2007a. Aquaporin expression in response to different water stress intensities and recovery in Richter-110 (*Vitis* sp.): relationship with ecophysiological status. *Planta* **226**, 671-681.

Galmés J, Abadía A, Medrano H, Flexas J. 2007b. Photosynthesis and photoprotection responses to water stressing the wild-extinct plant *Lysimachia minoricensis*. *Environmental and Experimental Botany* **60**, 308–317.

Genty B, Briantais JM, Baker NR. 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta* **990**, 87-92.

Gillon JS and Yakir D. 2000. Internal conductance to CO2 diffusion and C18OO discrimination in C3 leaves. *Plant Physiology* **123**, 201-213.

Gerbeau P, Amodeo G, Henzler T, Santoni V, Ripoche P, Maurel C. 2002 The water permeability of *Arabidopsis* plasma membrane is regulated by divalent cations and pH. *Plant Journal* **30**, 71–81

Gonzalez-Meler MA, Ribas-Carbo M, Siedow JN, Drake BG. 1996. Direct inhibition of plant mitochondrial respiration by elevated CO_2. *Plant Physiology* **112**, 1349–1355.

Gorton HL, Herbert SK, Vogelmann TC. 2003. Photoacoustic analysis indicates that chloroplast movement does not alter liquid-phase CO2 diffusion in leaves of *Alocasia brisbanensis*. *Plant Physiology* **132**, 1529–1539.

Grassi G and Magnani F. 2005. Stomatal, mesophyll conductance and biochemical limitations to photosynthesis as affected by drought and leaf ontogeny in ash and oak trees. *Plant, Cell and Environment* **28**, 834–849.

Hanba YT, Shibasaka M, Hayashi Y, Hayakawa T, Kasamo K, Terashima I, Katsuhara, M. 2004. Overexpression of the barley aquaporin HvPIP2;1 increases internal CO_2 conductance and CO_2 assimilation in the leaves of transgenic rice plants. *Plant Cell Physiology* **45**, 521–529.

Harley PC, Loreto F, Di Marco G, Sharkey TD. 1992. Theoretical considerations when estimating the mesophyll conductance to CO_2 flux by analysis of the response of photosynthesis to CO_2. *Plant Physiology* **98**, 1429-1436.

Hassiatou F, Ludwig M, Renton M, Veneklaas EJ, Evans JR. 2009. Influence of leaf dry mass per area, CO_2, and irradiance on mesophyll conductance in sclerophylls. *Journal of Experimental Botany* **60**, 2303-2314.

Jannaud D, Monnet F, Reiter IM, Costa M, Leitao L, Fabre N, Tournaire-Roux C, Ksas B, Rumeau D, Schaffner AR, Maurel C, Genty B. 2010. CO_2 transfer in leaves of: a role for carbonic anhydrases and aquaporins? Presented at The 15th Internatiol Congress of Photosynthesis in Beijing, China, August 2010.

Johansson I, Karlsson M, Johanson U, Larsson C, Kjellbom P. 2000. The role of aquaporins in cellular and whole plant water balance. *Biochimica et Biophysica Acta* **1465**, 324–342.

Johanson U, Karlsson M, Johansson I, Gustavsson S, Sjovall S, Fraysse L, Weig AR, Kjellbom P. 2001. The complete set of genes encoding major intrinsic proteins in *Arabidopsis* provides a framework for a new nomenclature for major intrinsic proteins in plants. *Plant Physiology* **126**, 1358-1369.

Laisk AK. 1977. Kinetics of photosynthesis and photorespiration in C3 plans (in Russian). Moscow, Russia: Nauka.

Laisk A and Loreto F. 1996. Determining photosynthetic parameters from leaf CO_2 exchange and chlorophyll fluorescence. Ribulose - 1,5 – bisphosphate carboxylase/oxygenase specificity factor, dark respiration in the light, excitation distribution between photosystems, alternative electron transport rate, and mesophyll diffusion resistance. *Plant Physiology* **110,** 903–912.

Laisk A, Eichelmann H, Oja V, Rasulov B, Padu E, Bichele I,Pettai H, Kull O. 2006. Adjustment of leaf photosynthesis to shade in a natural canopy: rate parameters. *Plant, Cell and Environment* **28,** 375–388.

Laisk A, Eichelmann H, Oja V, Rasulov B, Rämma H. 2006. Photosystem II cycle and alternative electron flow in leaves. *Plant and Cell Physiology* **47**, 972 -983.

Long SP and Bernacchi CJ. 2003. Gas exchange measurements, what can they tell us about the underlying limitations to photosynthesis? Procedures and sources of error. *Journal of Experimental Botany* **54**, 2393- 2401.

Loreto F. Harley PC, Di Marco G, Sharkey TD. 1992. Estimation of mespphyll conductance to CO_2 flux by three different methods. *Plant Physiology* **98**, 1437-1443.

Makino A, Sakashita H, HidemaJ, J, Mae T, Ojima K, Osmond B. 1992. Distinctive responses of ribulose-1,5-bisphosphate carboxylase and carbonic anhydrase in wheat leaves to nitrogen nNutrition and their possible relationships to CO_2-transfer resistance. *Plant Physiology* **100**, 1737-1743.

Maurel C, Reizer J, Schroeder JI, Chrispeels MJ. 1993. The vacuolar membrane protein □-TIP creates water specific channels in *Xenopus* oocytes. *The EMBO Journal* **12**, 2241-2247.

Maurel Ch and Chrispeels MJ. 2001. Aquaporins. A molecular entry into plant water relations. *Plant Physiology* **125**, 135-138.

Missner A, Kügler P, Saparov SM, Sommer K, Mathai JC, Zeidel ML, Pohl P. 2008. Carbon dioxide transport through membranes. *The Journal of Biological Chemistry* **283**, 25340-25347.

Miyake C and Yokota A. 2000. Determination of the rate of photoreduction of O2 in the water–water cycle in watermelon leaves and enhancement of the rate by limitation of photosynthesis. *Plant and Cell Physiology* **41,** 335–343.

Nakhoul NL, Davis BA, Romero MF, Boron WF. 1998. Effect of expressing the water channel aquaporin-1 on the CO_2 permeability of *Xenopus oocytes*. *American Journal of Physiology* **274 C**, 543–548.

Ögren E and Evans JR. 1993. Photosynthetic light response curves I. Influence of CO2 partial pressure and leaf inversion. *Planta* **189**, 182-190.

Parkhurst DF and Mott KA. 1990. Intercellular diffusion limits to CO_2 uptake in leaves. *Plant Physiology* **94**, 1024-1032.

Parkhurst DF. 1994. Diffusion of CO_2 and other gases inside leaves. *New Phytologist* **126**, 449–479.

Peeva V and Cornic G. 2009. Leaf photosynthesis of *Haberlea rhodopensis* before and during drought. *Environmental and Experimental Botany* **65**, 310-318.

Pinelli P and Loreto F. 2003. (CO_2)-C^{12} emission from different metabolit pathways measured in illuminated and darkened C3 and C4 leaves at low, atmospheric and elevated CO_2 concentration. *Journal of Experimental Botany* **54**, 1761–1769.

Prasad GV, Coury LA, Finn F, Zeidel ML. 1998. Reconstituted aquaporin 1 water channels transport CO_2 across membranes. *Journal of Biological Chemistry* **273**, 33123–33126.

Price D, von Caemmerer S, Evans JR, Yu JW, Lloyd J, Oja V, Kell P, Harrison K, Gallagher A, Badger M. 1994. Specific reduction of chloroplast carbonic anhydrase activity by antisense RNA in transgenic tobacco plants has a minor effect on photosynthetic CO_2 assimilation. *Planta* **193,** 331–340.

Raghavendra AS, Gonugunta VK, Christmann A, Grill E. 2010. ABA perception and signalling. *Trends in Plant Science* 15, 395-401.

Sasaki H, Semejima M, Ishii R. 1996. Analysis by delta- ^{13}C measurement on mechanism of cultivar difference in leaf photosynthesis on rize (*Oryza sativa* L.) *Plant and Cell Physiology* **37**, 1161-1166.

Šantrůček J, Hronková M, Květoň J, Sage RF. 2003. Photosynthesis inhibition during gas exchange oscillations in ABA-treated *Helianthus annuus*: relative role of stomatal patchiness and leaf carboxylation capacity. *Photosynthetica* **41**, 241 253.

Sharkey TD, Berry JA, Sage RF. 1988. Regulation of photosynthesis electron-transport in Phaseolus *vulgaris* L., as determined by room temperature chlorophyll fluorescence. *Planta* **176**, 415–424.

Sharkey TD, Vassey TL, Vanderveer PJ, Vierstra RD. 1991. Carbon metabolism enzymes and photosynthesis in transgenic tobacco (*Nicotiana tabacum* L.) having excess phytochrome. *Planta* **185**, 287–296.

Schäufele R, Šantrůček J, Schnyder H. 2011. Dynamic changes of canopy-scale mesophyll conductance to CO_2 diffusion of sunflower as affected by CO_2 concentration and abscisic acid. *Plant, Cell and Environment* **34**, 127-136.

Singsaas EL, Ort DR, De Lucia EH. 2004. Elevated CO_2 effects on mesophyll conductance and its consequences for interpreting photosynthetic physiology. *Plant, Cell and Environment* **27**, 41–50.

Steudle E. 1993. Pressure probe techniques: basic principles and application to studies of water and solute relations at the cell, tissue, and organ level. In: Smith JAC, Griffiths H, eds. Water deficits: plant responses from cell to community. Oxford: Bios Scientific Publishers Ltd. 5-36.

Tamimi A, Rinker EB, Sandall OC. 1994. Diffusion coefficient of hydrogen sulfide, carbon dioxide, and nitrous oxide in water over the temperature range 293-368 K. *Journal of Chemical and Engineering Data* **39**, 330-332.

Tazoe Y, von Caemmerer S, Badger MR, Evans JR. 2009. Light and CO_2 do not affect the mesophyll conductance to CO_2 diffusion in wheat leaves. *Journal of Experimental Botany* **60**, 2291- 2301.

Tazoe Y, von Caemmerer S, Evans JR. 2010. Measurements of mesophyll conductance in tobacco, *Arabidopsis* and wheat leaves with tunable diode laser absobtion spectroscopy (TDLAS). Presented at The 15th Internatiol Congress of Photosynthesis in Beijing, China, August 2010.

Terashima I. 1992. Anatomy of non-uniform leaf photosynthesis. *Photosynthesis Research* **31**, 195–212.

Terashima I and Ono K. 2002. Effects of $HgCl_2$ on CO_2 dependence of leaf photosynthesis: evidence indicating involvement of aquaporins in CO_2 diffusion across the plasma membrane. *Plant Cell Physiology* **43**, 70–78.

Tholen D, Boom C, Noguchi K, Terashima I. 2007. The effects of chloroplast movement on CO_2 transfer conductance in *Arabidopsis thaliana*. *Plant and Cell Physiology* **48,** S95.

Tiwari A, Kumar P, Chawhaan PH, Singh S, Ansari SA. 2006 Carbonic anhydrase in *Tectona grandis*: kinetics, stability, isozyme analysis and relationship with photosynthesis. *Tree Physiology* **26,** 1067–1073.

Tournaire-Roux C, Sutka M, Javot H, Gout E, Gerbeau P, Luu DT, Bligny R, Maurel C. 2003. Cytosolic pH regulates root water transport during anoxic stress through gating of aquaporins. *Nature* (London) **425**, 393–397.

Uehlein N, Lovisolo C, Siefritz F, Kaldenhoff R. 2003. The tobacco aquaporin NtAQP1 is a membrane CO_2 pore with physiological functions. *Nature* **425**, 734–737.

Valentini R, Epron D, De Angelis P, Matteuci G, Dreyer E. 1995. In situ estimation of net CO_2 assimilation, photosynthetic electron flow and photorespiration in Turkey oak (*Quercus cerris* L.) leaves: diurnal cycles under different leaves of water supply. *Plant, Cell and Environment* **18**, 631- 640.

Vrábl D, Vašková M, Hronková M, Flexas J, Šantrůček J. 2009. Mesophyll conductance to CO_2 transport estimated by two independent methods: effects of variable CO_2 concentration and abscisic acid. *Journal of Experimental Botany* **60**, 2315-2323.

Wan X, Steudle E, Hartung W. 2004. Gating of water channels (aquaporins) in cortical cells of young corn roots by mechanical stimuli (pressre pulses): effects of ABA and $HgCl_2$. *Journal of Experimental Botany* **55**, 411-422.

Wang Y, Cohen J, Boron WF, Schulten K, Tajkorshid E. 2007. Exploring gas permeability of cellular membranes and membrane channels with molecular dynamics. *Journal of Structural Biology* **157**, 534-544.

Warren CR, Livingston NJ, Turpin DH. 2004. Water stress decreases the transfer conductance of Douglas-fir (*Pseudotsuga menziesii*) seedlings. *Tree Physiology* **24**, 971-979.

Warren CR and Deyer E. 2006. Temperature response of photosynthesis and internal conductance co CO_2: results from two independent approaches. *Journal of Experimental Botany* **57**, 3057-3067.

Warren CR. 2006. Estimating the internal conductance to CO_2 movement. *Functional Plant Biology* **33**, 431-442.

Warren CR. 2008a. Stand aside stomata, another actor deserves centre stage: the forgotten role of the internal conductance to CO_2 transfer. *Journal of Experimental Botany* **59**, 1475-1487.

Warren CR. 2008b. Does growth temperature affect the temperature responses of photosynthesis and internal conductance to CO_2? A test with *Eucalyptus regnans*. *Tree Physiology* **28**, 11-19.

Williams TG, Flanagan LB, Coleman JR. 1996. Photosynthetic gas exchange and discrimination against $^{13}CO_2$, and $C^{18}O^{16}O$ in tobacco plants modified by an antisense construct to have low chloroplastic carbonic anhydrase. *Plant Physiology* **112,** 319–326.

Yamori W, Noguchi K, Hanba YT, Terashima I. 2006. Effects of internal conductance on the temperature dependence of the photosynthetic rate in spinach leaves from constraining growth temperatures. *Plant and Cell Physiology* **48**, 1069-1080.

Ye Q, Wiera B, Steudle E. 2004. A cohesion/tension mechanism explains the gating of water channels (aquaporins) in *Chara* internodes by high concentration. *Journal of Experimental Botany* **55**, 449–461.

Yin X, Struik PC, Romero P, Harbinson J, Evers JB, van der Putten PEL, Vos J. 2009. Using combined measurements of gas exchange and chlorophyll fluorescence to estimate parameters of a biochemical C3 photosynthesis model: a critical appraisal and a new integrated approach applied to leaves in a wheat (*Triticum aestivum*) canopy. *Plant, Cell and Environment* **32,** 448–464.

Zhang J, Davies WJ. 1990. Changes in the concentration of ABA in xylem sap as a function of changing soil water status can account for changes in leaf conductance. *Plant Cell Environment* **13**, 277–285

Appendix

Possible sources of errors of g_m estimation

Estimation of g_m by *variable J* method involves several parameters used in calculations. These parameters are: day respiration (R_d), CO_2 photocompensation point (Γ^*), leaf absorbtance (α), light partition between photosystems II and I (β) and the absence of alternative electron–consuming reactions, such as the Mehler reaction.

Parameter $\alpha^*\beta$ were not affected by CO_2 concentration since relationship between Φ_{PSII} and Φ_{CO2} estimated at variable CO_2 concentration under low O_2 was not affected as well (see **Fig. 3.1**). However, besides photosynthesis and photorespiration, other reactions such as Mehler reaction or nitrite reduction have been shown to consume as much as 10% of J_f (Miyake and Yokota, 2000; Laisk *et al*., 2006). In addition, R_d may be underestimated using the method of Laisk (Pinelli and Loreto, 2003). R_d and alternative electron consumption can be affected by CO_2 concentration (Miyake and Yokota, 2000; Gonzalez-Meler *et al*., 1996; Bruhn *et al*., 2007). Finally, using C_c^* as a proxy for Γ^* can also bring some uncertainty.

All these possible sources of errors were tested by simulation as described in Flexas *et al*. (2007a). An example of such simulation is shown in **Table A1** for a leaf of sunflower (simulation for poplar resulted in qualitatively similar results, not shown). At ambient CO_2 (400 μmol mol^{-1} air) and high CO_2 (700 μmol mol^{-1} air), leaf temperature was 23°C , A_N 30 and 35 μmol CO_2 m^{-2} s^{-1}, C_i was 326 and 591 μmol CO_2 mol^{-1} air, and J_f was 209.1 and 208.8 μmol m^{-2} s^{-1}. These data, together with estimations of R_d (-0.48 μmol CO_2 m^{-2} s^{-1}) and C_c^* (33 μmol CO_2 mol^{-1} air, used as a proxy for Γ^*) obtained form the Laisk method, resulted in g_m estimates of 0.194 and 0.101 mol CO_2 m^{-2} s^{-1} at ambient and high CO_2, respectively.

Two simulations were performed for each parameter tested. Alternative electron-consuming reactions were considered to consume 10% of total J_f. Secondly, these reactions were assumed to consume 10% of J_f only at ambient CO_2 concentration. Two possibilities were tested regarding R_d. Firstly, it was assumed to be twice the estimated one (i.e. close to R_N, as suggested by Pinneli and Loreto, 2003). Secondly, it was assumed to be reduced by 30% at high CO_2 concentration (Gonzalez-

Meler *et al*., 1996; Bruhn *et al*., 2007). Regarding Γ^*, two values for gm that could appear at compensation point were used for test, 0.3 mol CO_2 m^{-2} s^{-1} (as judged from **Fig. 3.5 A**) resulting in a Γ^* of 34.4 µmol CO_2 mol^{-1} air and value of g_m lowered by 10 %, i.e. 0.27 mol CO_2 m^{-2} s^{-1}, resulting in a Γ^* of 48.3 µmol CO_2 mol^{-1}. Γ^* may be equal at ambient and high CO_2 since it reflects an intrinsic property of Rubisco. At ambient CO_2, all simulated g_m values ranged between 0.201 and 0.270 mol CO_2 m^{-2} s^{-1} which is close to original value of 0.194 mol CO_2 m^{-2} s^{-1} except when Γ^* was 48.3 µmol CO_2 mol^{-1} air was considered which yielded in much higher g_m (0.399 mol CO_2 m^{-2} s^{-1}). In addition, at high CO_2, all simulated values ranged between 0.100 and 0.149 mol CO_2 m^{-2} s^{-1} which is, again, very close to original value of 0.101 mol CO_2 m^{-2} s^{-1}.

Therefore, these simulations proved that although three parameters tested can bring some variation in absolute value of g_m, especially Γ^*, none of them would impair the result that g_m responds to varying CO_2 concentration. Together, it provides certainty that decreased g_m at high CO_2 has a physiological basis, and does not result from artifacts in the methods (Flexas *et al*., 2007a).

Table A1. Simulation of the effect of possible errors in the parameters assumptions on mesophyll conductance (g_m) estimates using the chlorophyll fluorescence method by Harley *et al.* (1992) in a leaf of *Helianthus annuus* at 400 μmol CO_2 m^{-2} s^{-1}air (ambient CO_2) or 700 μmol CO_2 m^{-2} s^{-1}air (high CO_2).

A. Effect of possible alternative electron flow and its dependence on CO_2. In the first row, alternative electron-consuming reactions are assumed to use 10% of total electron transport rate (J_f), while in the second row alternative electron-consuming eractions are assumed to use 10% of total J_f only at ambient CO_2, being negligible at high CO_2.

J_f Ambient CO_2 (μmol m^{-2} s^{-1})	J_f High CO_2 (μmol m^{-2} s^{-1})	g_m Ambient CO_2 (mol CO_2 m^{-2} s^{-1})	g_m High CO_2 (mol CO_2 m^{-2} s^{-1})
188.3	187.9	0.270	0.138
188.3	208.8	0.270	0.101

B. Effect of possible misleading estimates of R_d and its dependence on CO_2. In the first row, R_d is assumed to be equal to respiration in the dark (R_N). In the second row, in addition to R_d at high CO_2 is assumed to be only 30% that at low CO_2.

R_d Ambient CO_2 (μmol CO_2 m^{-2} s^{-1})	R_d High CO_2 (μmol CO_2 m^{-2} s^{-1})	g_m Ambient CO_2 (mol CO_2 m^{-2} s^{-1})	g_m High CO_2 (mol CO_2 m^{-2} s^{-1})
0.96	0.96	0.201	0.103
0.96	0.288	0.201	0.100

C. Effect of using apparent CO_2 photocompensation point (C_c*) as a proxy for chloroplastic CO_2 photocompensation point (Γ*). Two estimations were made. In the first row, g_m near the compensation point was assumed to be 0.3 mol CO_2 m^{-2} s^{-1}, resulting in a Γ* of 37.2 μmol CO_2 m^{-2} s^{-1}air. In the second row, g_m near the compensation point was assumed to be 10% lower, i.e. 0.27 μmol CO_2 m^{-2} s^{-1}, resulting in 23% higher Γ* of 48.3 μmol CO_2 m^{-2} s^{-1}air.

Γ* Ambient CO_2 (μmol CO_2 mol^{-1}air)	Γ* High CO_2 (μmol CO_2 mol^{-1}air)	g_m Ambient CO_2 (mol CO_2 m^{-2} s^{-1})	g_m High CO_2 (mol CO_2 m^{-2} s^{-1})
37.2	37.2	0.226	0.110
48.3	48.3	0.399	0.149