Diffusive and Metabolic Limitations to Photosynthesis under Drought and Salinity in C_3 Plants

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Abstract: **Drought and salinity are two widespread environmental conditions leading to low water availability for plants. Low water availability is considered the main environmental factor limiting photosynthesis and, consequently, plant growth and yield worldwide. There has been a long-standing controversy as to whether drought and salt stresses mainly limit photosynthesis through diffusive resistances or by metabolic impairment. Reviewing *in vitro* and *in vivo* measurements, it is concluded that salt and drought stress predominantly affect diffusion of CO_2 in the leaves through a decrease of stomatal and mesophyll conductances, but not the biochemical capacity to assimilate CO_2, at mild to rather severe stress levels. The general failure of metabolism observed at more severe stress suggests the occurrence of secondary oxidative stresses, particularly under high-light conditions. Estimates of photosynthetic limitations based on the photosynthetic response to intercellular CO_2 may lead to artefactual conclusions, even if patchy stomatal closure and the relative increase of cuticular conductance are taken into account, as decreasing mesophyll conductance can cause the CO_2 concentration in chloroplasts of stressed leaves to be considerably lower than the intercellular CO_2 concentration. Measurements based on the photosynthetic response to chloroplast CO_2 often confirm that the photosynthetic capacity is preserved but photosynthesis is limited by diffusive resistances in drought and salt-stressed leaves.**

Key words: **Diffusive limitations to photosynthesis, metabolic limitations to photosynthesis, drought, salinity, photosynthetic metabolism, stomatal–mesophyll conductance.**

Introduction

Low water availability is considered the main environmental factor limiting plant growth and yield worldwide, especially in semi-arid areas (Boyer, 1982; Chaves et al., 2003). Global change will likely make water scarcity an even greater limitation to plant productivity across an increasing amount of land. Water availability is decreased under drought (Lawlor, 1995) and salinity stress (Munns, 1993), primarily due to the so-called osmotic or water deficit effect and, thus, reducing the ability of plants to take up water. It is well documented that one of the primary physiological impacts of drought and salinity is on photosynthesis (Lawlor, 1995; Munns, 2002).

However, there has been a long-standing controversy as to whether these stresses primarily limit photosynthesis through stomatal closure (Sharkey, 1990; Chaves, 1991; Ort et al., 1994; Cornic and Massacci, 1996; Loreto et al., 2003) and in general through diffusive resistances (Massacci and Loreto, 2001) or by metabolic impairment (Boyer, 1976; Lawlor, 1995). The suggestion that impaired ATP synthesis is the main factor limiting photosynthesis even under mild drought (Tezara et al., 1999), has further stimulated debate in recent years (Cornic, 2000; Cornic and Fresneau, 2002; Flexas and Medrano, 2002; Lawlor, 2002; Lawlor and Cornic, 2002). While some authors agree that impaired ATP is a likely explanation for decreased photosynthesis under water stress (Tezara et al., 1999; Lawlor, 2002; Tang et al., 2002), others found that this explanation fails to explain some other observations, such as the fact that under a light-limited condition O_2 uptake can replace entirely CO_2 uptake in drought-stressed plants (Cornic and Fresneau, 2002), or that removing diffusion limitations totally reverses the water stress-induced decline in photosynthesis (Kaiser, 1987; Centritto et al., 2003).

At least part of the above-mentioned controversy may be due to the fact that A_N-Ci analysis has been frequently used to describe non-stomatal limitations to photosynthesis. However, it is not clear whether this kind of analysis is reliable under drought or salinity, because two main problems have been described related to Ci calculations: patchy stomatal closure (Laisk, 1983; Buckley et al., 1997) and changes in the cuticular conductance to vapour pressure (Boyer et al., 1997). In addition, drought-induced changes in the mesophyll conductance to CO_2 may also invalidate the interpretation of A_N-Ci analysis (Flexas et al., 2002; Centritto et al., 2003).

We focus here on primary effects of water stress and salinity and not on secondary effects that may come about as a result of the stresses reducing growth or inducing senescence. Emphasis is given to the response of C_3 plants, which have been more studied regarding their photosynthetic response to both drought and salinity. A primary issue that would be addressed in the present review is the apparent contradiction among reports showing only diffusion limitations and those showing

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strong metabolic limitations under drought or salinity. Data from the literature will be put into a novel perspective to discuss this issue. A particular emphasis will be given to discuss the evidence for decreased ATP synthesis under stress. A second important issue that will be addressed is the reliability of A_N-Ci analysis under stress conditions. Another kind of analysis of A_N-Ci curves will be proposed to take into account not only variations of stomatal conductance but also possible variations of mesophyll conductance under stress.

Evidence for Stomatal Limitation of Photosynthesis in Leaves of Water-Stressed Plants

Because stomatal closure is among the early physiological events occurring in response to decreased water availability, and because a close relationship is usually found between stomatal conductance (g_s) and net CO_2 assimilation (A_N), it has been frequently assumed that stomatal closure reduces CO_2 uptake in drought and salt-stressed leaves. The argument is not always valid as g_s can also be decreased in response to decreased photosynthetic capacity (Wong et al., 1979). A model has been proposed linking stress-induced changes of adenylate charge in mesophyll and guard cells with stomatal closure (Buckley et al., 2003). However, this model does not explain stomatal closure in some physiological conditions such as in darkness. On the other hand, if decreased g_s is the factor limiting CO_2 assimilation, removing and/or overcoming the stomatal limitation should reverse the stress-induced decline of A_N. Several studies demonstrate, indeed, that this occurs under most drought stress conditions.

1. By stripping the epidermis from leaves, stomatal limitation is removed, permitting CO_2 to freely diffuse into the intercellular spaces. Using this approach, Dietz and Heber (1983) and Schwab et al. (1989) demonstrated in *Ramonda mykoni* and *Primula palinuri,* respectively, that most of the drought-induced decline in A_N was explained by stomatal closure, even when leaf relative water content was largely decreased (by as much as 50%). In another study, however, it was not possible to restore A_N in water-stressed, stripped sunflower (*Helianthus annuus* L.) leaves; presumably, relative water content of the studied leaves was lower than in the previous studies (Tang et al., 2002).
2. Cornic and Ghashghaie (1991) used a different approach, consisting of modulating g_s by changing leaf temperature in well-watered and water-stressed *Phaseolus vulgaris* L. These authors were able to induce stomatal opening in substantially water-stressed leaves (relative water content around 70%, A_N close to zero at 23 °C) by progressively reducing leaf temperature. A_N increased in parallel to g_s, in contrast to what should be expected if photosynthetic capacity had been impaired by drought. At 14 °C, well-watered and water-stressed plants presented identical A_N. It was therefore concluded that the entire photosynthetic reduction in water-stressed leaves was due to stomatal closure.
3. Applying large CO_2 concentrations around leaves increases the CO_2 gradient and overcomes diffusional limitations to CO_2. This has been done using oxygen electrodes. Many reports have shown that very high CO_2 fully restores maximum photosynthesis in water-stressed leaves (Kaiser, 1987; Cornic et al., 1989; Chaves, 1991; Cornic et al., 1992; Quick et al., 1992; Tourneux and Peltier, 1995). Other reports, however, have suggested that maximum photosynthesis is not totally recovered by high CO_2 in water-stressed plants (Graan and Boyer, 1990; Kanechi et al., 1998; Flexas et al., 1999a; Tezara et al., 1999). However, Cornic and Fresneau (2002) have shown that plotting together the results from Graan and Boyer (1990), Cornic et al. (1989), and Tourneux and Peltier (1995), a single relationship is obtained between the percentage reductions in A_N at normal CO_2 and maximum photosynthesis at saturating CO_2, so that no reduction in photosynthetic capacity is observed until A_N is reduced by more than 80%. The data of Kanechi et al. (1998) and Flexas et al. (1999a) also fit the above relationship so, up to now, the report by Tezara et al. (1999) remains the only one showing an earlier drought-induced reduction of photosynthetic capacity.
4. Finally, evidence for stomatal-dependent photosynthesis reduction in water-stressed leaves comes from analysis of the response of A_N to sub-stomatal CO_2 concentration (C_i) (i.e., A_N-C_i curves). From these curves, stomatal limitation (Ls) can be calculated, as well as metabolic limitation (Lm) and several components of the latter (Martin and Ruiz-Torres, 1992; Tezara et al., 2002). In most studies, Ls shows an increasing tendency with increasing water stress severity, and it is usually maintained at higher values than Lm or any of its components (Martin and Ruiz-Torres, 1992; Escalona et al., 1999; Gulías et al., 2002). Only a few reports have shown higher Lm than Ls in plants subjected to drought or salinity (Lawlor, 2002; Tezara et al., 2002, 2003). Nevertheless, as discussed in the next sections, analysis of A_N-C_i curves can be controversial under water stress conditions.

Taken together, the evidence clearly indicates that stomatal closure is the first event restricting photosynthesis at mild to moderate water stress. In most cases, photosynthesis is nearly completely stopped by stomatal closure before metabolism is affected. Given the effects of such severe water stress on other plant processes, it is possible that these responses of photosynthetic metabolism are not direct responses to water stress.

How Strong is the Evidence for Photosynthetic Metabolism Impairment under Drought and/or Salinity?

a) *In vitro* Measurements

The effects of drought and salt on metabolic processes are often assessed by *in vitro* measurements of both the activity of some enzymes and the size of some metabolite pools of intact leaves submitted to stress. This approach has led to different conclusions. For instance, Giménez et al. (1992) and Gunasekera and Berkowitz (1993) found strong reductions of leaf RuBP content, but Lal et al. (1996) reported that this was unaffected by drought. If photosynthetic metabolism is not impaired by drought, CO_2 molar ratio inside the leaves experiencing a water deficit is expected to decrease, causing in turn an increase of RuBP. However this expected rise in RuBP concentration could be obscured by a general depletion of PCR cycle intermediates due to a reduction of CO_2 uptake in this condition.

Similarly, some reports, have shown strong drought-induced reductions of Rubisco activity (Maroco et al., 2002; Parry et al., 2002), but other studies have observed no effect of moderate drought (Lal et al., 1996; Pankovic et al., 1999; Delfine et al., 2001) or salinity (Delfine et al., 1998). These apparent discrepancies may arise from the fact that the different studies have been performed under different environmental conditions, us-

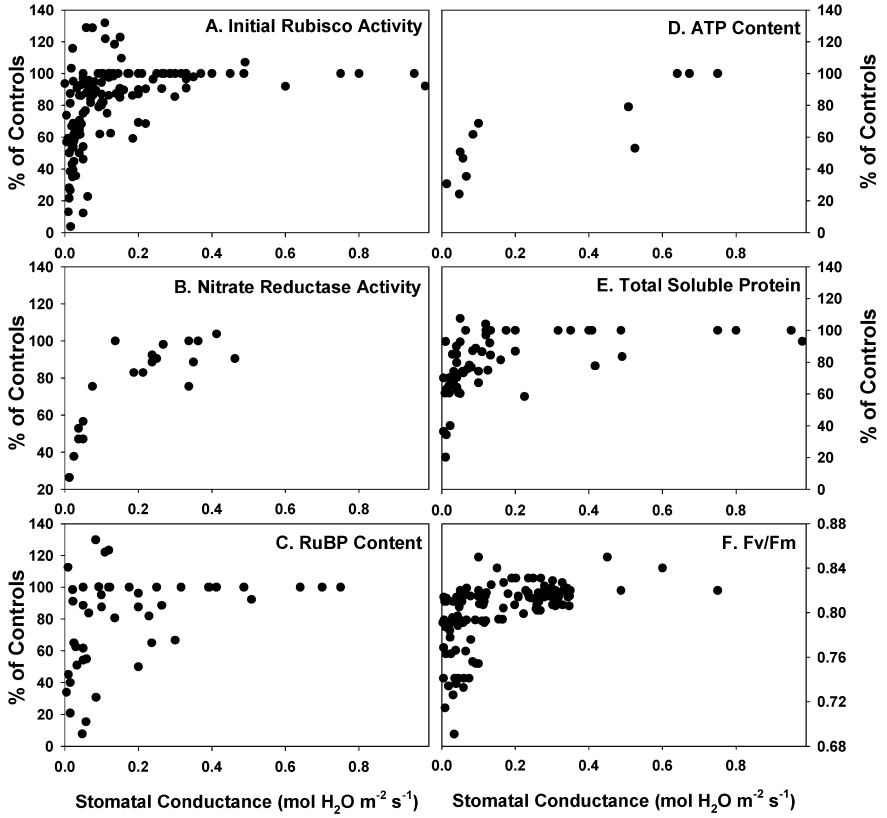

Fig. 1 The relationship of light-saturated stomatal conductance (g_s) and leaf initial Rubisco activity (**A**), nitrate reductase activity (**B**), RuBP content (**C**), ATP content (**D**), total soluble protein content (**E**), and Fv/Fm (**F**).

Data have been compiled from literature on either drought or salinity in which g_s was available, and include many different species, including different origins, life forms and leaf habits, as well as different environmental conditions during the experiments. Except for g_s (mol H_2O m^{-2} s^{-1}) and Fv/Fm (unitless), all parameters are expressed as % of maximum values to facilitate comparison, due to the large variability in the units given in the original references. The references from which data have been compiled are the following. (**A**) Jones, 1973; Castrillo and Calcagno, 1989; Holaday et al., 1992; Antolín and Sánchez-Díaz, 1993; Brestic et al., 1995; Lal et al., 1996; Medrano et al., 1997; Sánchez-Rodríguez et al., 1997, 1999; Delfine et al., 1998, 1999, 2001; Pankovic et al., 1999; Tezara et al., 1999, 2002; Wingler et al., 1999; Castrillo et al., 2001; Maroco et al., 2002; Bota et al., 2004, and unpublished results; Galmés et al., unpublished results. (**B**) Arndt et al., 2001. (**C**) Giménez et al., 1992; Gunasekera and Berkowitz, 1993; Lal et al., 1996; Tezara et al., 1999; Bota et al., 2004, and unpublished results. (**D**) Lawlor and Khanna-Chopra, 1984; Tezara et al., 1999, 2002. (**E**) Jones, 1973; Castrillo and Calcagno, 1989; Holaday et al., 1992; Antolín and Sánchez-Díaz, 1993; Moran et al., 1994; Iturbe-Ormaetxe et al., 1998; Castrillo et al., 2001; Maroco et al., 2002; Tezara et al., 2002; Bota et al., 2004, and unpublished results. (**F**) Pankovic et al., 1999; Flexas et al., 2002; Gulías et al., 2002; Tezara et al., 2002; Bota et al., 2004, and unpublished results. Data from Galmés et al. (unpublished) are from the following species: *Cistus albidus* L., *Hypericum balearicum* L., *Mentha aquatica* L., *Phlomis italica* L. In these experiments, potted plants growing in a controlled growth chamber (12 h photoperiod, 26 °C day/20 °C night, relative humidity around 50 %, and a photon flux density at the top of the leaves of about 600 – 800 µmol m^{-2} s^{-1} supplied by halogen lamps) were slowly dehydrated by withholding water (6 to 15 days). For details on plant material and experimental conditions of the other data, see the original references.

ing different species, and undergoing different drought intensities.

To try to normalize these factors we compared results on a stomatal conductance basis (Fig. **1**). As recently demonstrated by Flexas and Medrano (2002), drought-induced changes in different processes related to photosynthetic metabolism are strongly related to variations in light-saturated stomatal conductance (g_s). Data of Fig. **1** have been compiled from a large number of studies, undertaken under different conditions, using different rates of drought or salt stress imposition, and in different species. Clearly, the state of all these metabolic components remains unaffected within most of the g_s range, with a few exceptions. Below a certain g_s threshold (generally lower than 0.1 mol H_2O m^{-2} s^{-1}), the relationship changes steeply. As A_N is strictly related to g_s, this general picture is strongly consistent with the finding of Cornic and Fresneau (2002) that there is no change in the photosynthetic capacity until A_N at ambient CO_2 is depressed by 80 %. In the following sections we highlight results concerning biochemical factors putatively limiting photosynthesis in stressed leaves.

Activity of Rubisco and nitrate reductase activity

There is a large amount of data on initial Rubisco activity (Fig. **1 A**) and only one study in which nitrate reductase activity was followed concomitantly with g_s during a drought cycle (Fig. **1 B**). Even so, it seems clear that both enzymes share a common pattern of regulation with decreasing g_s. The fact that initial Rubisco activity remains unaffected from maximum g_s down to 0.1 mol H_2O m^{-2} s^{-1} implies that, within this range, photosynthesis is not impaired by the carboxylation capacity. Below that threshold, Rubisco activity eventually declines, and typically only a small fraction of this decline is due to de-

creased activation state (Lal et al., 1996; Parry et al., 2002; Bota et al., 2004). More likely, the reduction is due to a reduction in Rubisco content, as suggested by the general decline of total soluble protein at the same g_s threshold (Fig. **1 E**).

RuBP regeneration capacity

Leaf RuBP content variation with g_s is similar to that of Rubisco activity and nitrate reductase (Fig. **1 C**). This pattern could indicate that the capacity for RuBP regeneration is impaired early during water stress imposition. In fact, if stomatal closure limits A_N by limiting CO_2 availability, then RuBP content should increase, as it does under low CO_2 (von Caemmerer and Edmundson, 1986). Decreased capacity for RuBP regeneration can be due to (i) decreased electron transport rate and, thus, NADPH supply; (ii) activity of one or more of the enzymes involved in regeneration, (iii) decreased capacity for photophosphorylation; and (iv) decreased triose phosphate utilisation, which results in decreased Pi availability in the stroma (although this normally leads to high RuBP because of deactivation of Rubisco [Sharkey et al., 1986]). All of these have been indicated as possible sources of photosynthesis regulation under water stress (Lawlor and Cornic, 2002).

Leaf photochemistry has been shown to be extremely resistant to water stress (Cornic and Massacci, 1996) and, indeed, as water stress intensifies the ratio of electron transport rate to A_N progressively increases (Flexas et al., 1999 a, b; 2002). Therefore, decreased electron transport and NADPH synthesis can be discarded as limiting factors for RuBP regeneration.

The activities of several enzymes involved in regeneration, such as fructose-1,6-bisphosphatase (Sharkey and Seemann, 1989; Sánchez-Rodríguez et al., 1997, 1999; Maroco et al., 2002), glyceraldehyde-3-phosphate dehydrogenase (Maroco et al., 2002; Thimmanaik et al., 2002), ribulose-5-phosphate kinase (Maroco et al., 2002; Thimmanaik et al., 2002), or 3-phosphoglycerate kinase (Thimmanaik et al., 2002), have been analysed in response to water stress. Most of these studies have shown that these enzymes are not impaired by water stress at g_s higher than 0.1 mol H_2O m^{-2} s^{-1} (Sharkey and Seemann, 1989; Sánchez-Rodríguez et al., 1997, 1999), although they are impaired below that threshold (Sánchez-Rodríguez et al., 1999; Maroco et al., 2002). Only in the study by Thimmanaik et al. (2002) do all the studied enzymes decrease linearly with g_s, but in that study total chlorophyll content also decreased in parallel to g_s, suggesting the presence of additional stresses other than water shortage or that the changes reflect a general reduction in all aspects of photosynthesis, perhaps as a secondary response to the effects of water stress on growth or senescence. In conclusion, at present there is no clear evidence for a specific inhibition of the enzymes involved in RuBP regeneration under mild to moderate water stress.

Tezara et al. (1999) suggested that decreased coupling factor and photophosphorylation was the cause for decreased photosynthesis under water stress, but they stated in a later study using the same species that "decrease in net photosynthesis with water deficiency was related to lower Rubisco activity rather than to ATP and RuBP contents" (Tezara et al., 2002). Most of the previous studies suggesting water stress-induced decreased capacity for photophosphorylation were performed with chloroplasts or protoplasts extracted from stressed tissue (Flexas and Medrano, 2002; Lawlor and Cornic, 2002), and the only *in vivo* study suggested no impairment of photophosphorylation (Ortiz-Lopez et al., 1991). Plotting the few available data of leaf ATP content versus g_s (Fig. **1 D**), the pattern resembles that of enzyme activity and RuBP content, although there are very few points and one point behaving as an outlier at still high g_s. Still, this does not allow us to discern whether photophosphorylation is impaired. As in the case of RuBP, decreasing A_N due to stomatal closure may result in increased ATP content, but this may be partly counteracted by increased photorespiration, which occurs under mild to moderate stress (Wingler et al., 1999; Flexas et al., 1999 a, b, 2002; Cornic and Fresneau, 2002). Even the strong decreases of ATP content observed at low g_s may not necessarily imply decreased photophosphorylation. This is because a substantial amount of ATP (up to 40%) present in the leaf tissue may come from the mitochondria (Krömer and Heldt, 1991), and we have recently observed very strong increases of mitochondrial alternative oxidase activity (which produces much less ATP) at the expense of cytochrome pathway activity at low g_s in water stressed soybean (Flexas et al., 2004; Ribas-Carbó, pers. comm.). In summary, there is no clear evidence for decreased photophosphorylation in leaves from water-stressed plants.

Finally, a photosynthetic limitation by Pi under water stress has been suggested, and attributed to stress-induced decline of sucrose phosphate synthase (SPS) activity (Vassey and Sharkey, 1989; Cornic et al., 1992). The few data available show that SPS activity shares a similar pattern of regulation to other enzymes in response to decreasing g_s, although possibly starting a decline at some higher g_s (Vassey and Sharkey, 1989; Vassey et al., 1991). This is consistent with the fact that SPS activity is regulated by CO_2 availability. Using the A_N-C_i curves approach, this kind of limitation may be revealed by increased limitation by decreased triose phosphate utilization, and this is indeed the most important limitation usually observed (Maroco et al., 2002). The data of Tezara et al. (1999) indicate a reduction in triose phosphate use (e.g., sucrose synthesis) rather than impaired photophosphorylation (see below). This means that it is not the water stress that affects the photosynthetic metabolism, but the stomatal closure caused by water stress.

In summary, it is not clear what limits the capacity for RuBP regeneration at mild to moderate water stress, although Pi limitation is the most likely candidate. Whatever the mechanism involved, however, the constancy of RuBP content implies that it does not limit photosynthesis at normal CO_2 concentration. The large decreases of RuBP at lower g_s may be part of general metabolism impairment rather than a specific response to water stress.

Other metabolic components

Total soluble protein (Fig. **1 E**) content is also maintained at constant levels for the entire range of g_s higher than 0.1 mol H_2O m^{-2} s^{-1}. The fact that all these components, and even chlorophyll content (data not shown) and maximum photochemical efficiency (Fv/Fm, Fig. **1 F**), abruptly decline at lower g_s strongly suggests an orchestrated down-regulation of the whole photosynthetic metabolism at this stress level. Alternatively, because chloroplastic CO_2 concentration is quite low under these conditions (Cornic and Fresneau, 2002; Flexas et al., 2002; Loreto et al., 2003), this general depression of photo-

synthetic metabolism could be the result of increased oxidative stress in the cells, especially under high-light conditions, which has been described frequently as a result of water stress (Moran et al., 1994; Sgherri and Navari-Izzo, 1995). Together, these results support the notion that metabolism is not responsible for the decline of photosynthesis at mild to moderate water stress (i.e when g_s limits A_N to about 20% of that in well-watered plants and, in many species, at a leaf relative water content lower than 60–70%). Under these conditions, plants show a very rapid (less than 1 day) recovery of photosynthesis upon re-watering (Flexas et al., 1999b). At more severe stress, a general metabolic impairment occurs. At this stage, photosynthesis recovery upon re-watering is slow, and sometimes incomplete (Kirschbaum, 1987, 1988).

How Strong is the Evidence for Photosynthetic Metabolism Impairment under Drought and/or Salinity? b) *In vivo* Measurements

Many of the reports suggesting that photosynthesis is substantially limited by metabolism under drought and/or salinity stresses are based on the *in vivo* measurements of the relationship between A_N and the intercellular CO_2 concentration *(C_i)* (Flexas and Medrano, 2002; Lawlor and Cornic, 2002; Centritto et al., 2003). As often reviewed (e.g., Farquhar and Sharkey, 1982), this relationship allows estimation of Rubisco and RuBP limitations, as well as stomatal limitation of photosynthesis. Many studies based on A_N-C_i curves suggest that non-stomatal (i.e., metabolic) limitations to photosynthesis appear almost at the same time as stomatal limitations, i.e., at early stages of water stress (Martin and Ruiz-Torres, 1992; Escalona et al., 1999; Tezara et al., 1999, 2002, 2003). However, it is controversial whether A_N-C_i analysis is reliable under drought, since two main problems have been described related to C_i calculations in stressed leaves: patchy stomatal closure (Laisk, 1983; Buckley et al., 1997) and the increase of the relative importance of cuticular transpiration when stomata are closing in drying leaves (Boyer et al., 1997).

The effects of patchy stomatal closure on the calculation of C_i and non-stomatal limitations have been discussed (Terashima et al., 1988; Cheeseman, 1991; Buckley et al., 1997). Patchy stomatal closure has been specifically demonstrated in water-stressed leaves (Downton et al., 1988; Sharkey and Seemann, 1989). However, it has been shown that patchiness is not a universal phenomenon in water stress experiments (Giménez et al., 1992; Gunasekera and Berkowitz, 1992), and it has even been shown that heterogeneous photosynthesis in water-stressed *Potentilla* leaves is not correlated with stomatal closure (Osmond et al., 1999). Moreover, the effects of heterogeneous stomatal closure on the estimations of C_i are not necessarily as important as previously thought (Cheeseman, 1991), and they may affect C_i significantly only under certain patterns of heterogeneity distribution and/or at very low g_s (Buckley et al., 1997). Regarding cuticular conductance, Flexas et al. (2002) found that the patchy-induced and cuticular-associated errors in C_i calculation were not large until g_s was lower than 0.03 mol H_2O m^{-2} s^{-1}, a low value usually reached only under very severe drought. While patchiness and cuticular conductance may not totally prevent the usefulness of A_N-C_i analysis to determine *in vivo* the metabolic limitations of photosynthesis in stressed leaves, the impact of internal conductance to CO_2 may be somehow greater. We will discuss in the next section how internal conductance may affect A_N-C_i analyses.

Dynamic Variations of Mesophyll Conductance to CO_2 May Explain Non-Stomatal Effects of Drought and/or Salinity on Photosynthesis

It has been shown (e.g., Evans et al., 1986;Loreto et al., 1992) that the internal leaf conductance to CO_2 diffusion *(g_{mes})* is finite, thus C_i is not equal to the CO_2 concentration inside the chloroplasts (Cc), the actual concentration at the site of carboxylation (Bernacchi et al., 2002; Flexas et al., 2002). Finite g_{mes}, resulting in a reduction of the CO_2 available to photosynthesis, introduces another possible diffusive photosynthetic limitation in addition to stomatal conductance. If g_{mes} changes in stressed leaves compared to leaves in non-stressed conditions, then comparing A_N-C_i of the two specimens is conceptually wrong and may give misleading results. Indeed, there is an increasing body of evidence that g_{mes} decreases in response to drought (Cornic et al., 1989; Brugnoli et al., 1998; Flexas et al., 2002) and salinity (Bongi and Loreto, 1989; Delfine et al., 1998, 1999; Loreto et al., 2003). When A_N-Cc responses of stressed and non-stressed plants are compared, differences in A_N-C_i responses often disappear (Delfine et al., 1998). Diffusive (stomatal plus internal) limitations may therefore also explain effects that are erroneously attributed to metabolic limitations on the basis of A_N-C_i analysis.

This reduction of g_{mes} in stressed leaves has been long considered as irreversible, being related to changes in mesophyll structure (Bongi and Loreto, 1989) or to a possible rearrangement of intercellular spaces (Delfine et al., 1998). However, Delfine et al. (1999) demonstrated that alleviation of a salinity stress prior to irreversible biochemical damage also induced an increase of g_{mes}. g_{mes} plasticity may influence the extent to which leaves can recover photosynthetic capacity after a stress.

Centritto et al. (2003) measured A_N-C_i curves in control and salt-stressed olive plants, observing an often-reported reduction of both carboxylation efficiency and CO_2-saturated photosynthesis in salt-stressed leaves. After this, however, the authors pre-conditioned the leaves at low CO_2 concentration (50 µmol mol^{-1}) for 1 or 2 h, which induced stomatal opening in salt-stressed plants. Then A_N-C_i curves were repeated, and the obtained curves were identical in control and stressed leaves, demonstrating that there was no metabolic limitation to photosynthesis. In the same experiment it was clearly shown that these short-term changes in CO_2 not only affected g_s, but also affected g_{mes}. In fact, g_{mes} measured at CO_2 of 350 µmol mol^{-1} was not constant, but was linearly related ($r^2 = 0.68$) to g_s. This experiment also suggested that changes of g_{mes} can be as fast as those of g_s and also that the sum of the diffusional resistances sets the limit to photosynthesis rates in stressed leaves.

These findings are in agreement with the biphasic response of photosynthesis to increasing CO_2, with an unrealistic apparent plateau at intermediate CO_2 concentrations, that has been described in ABA-treated and/or drought-stressed plants using oxygen electrode systems and much larger CO_2 concentrations (Terashima et al., 1988; Cornic et al., 1992). These authors already suggested that A_{SAT}, estimated from A_N-C_i curves, does

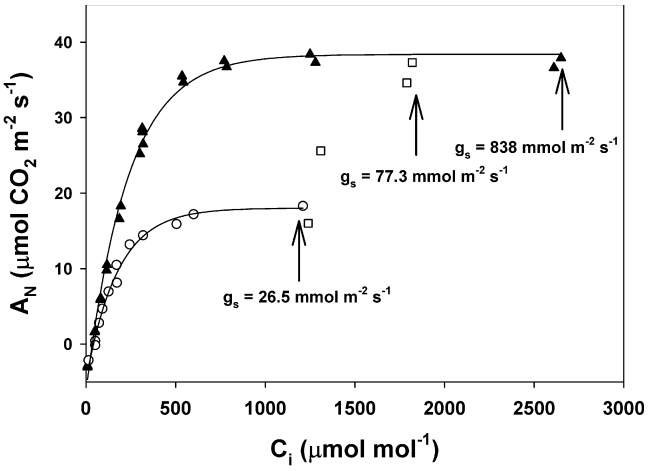

Fig. 2 The response of net CO_2 assimilation (A_N) to sub-stomatal CO_2 concentration (C_i) in *Helianthus annuus* L. plants. The conditions during all measurements were RH 50%, air temperature 25 °C, PAR 1500 μmol m^{-2} s^{-1}. Curves are shown for leaves from a well-watered plant (average leaf relative water content 81%, filled triangles) and for the same plant 10 days after withholding water in greenhouse conditions (relative water content 68%, open circles). g_s at the highest C_i attained is indicated in each case. Then, the water-stressed leaf was preconditioned for about 30 min at a CO_2 concentration of 50 μmol mol^{-1}, and then suddenly increased to 2700 μmol mol^{-1}. Four points were subsequently obtained (open squares) as the stomata rapidly closed again, from the value indicated to 0.018 mol H_2O m^{-2} s^{-1} (data from Flexas and Cornic, unpublished).

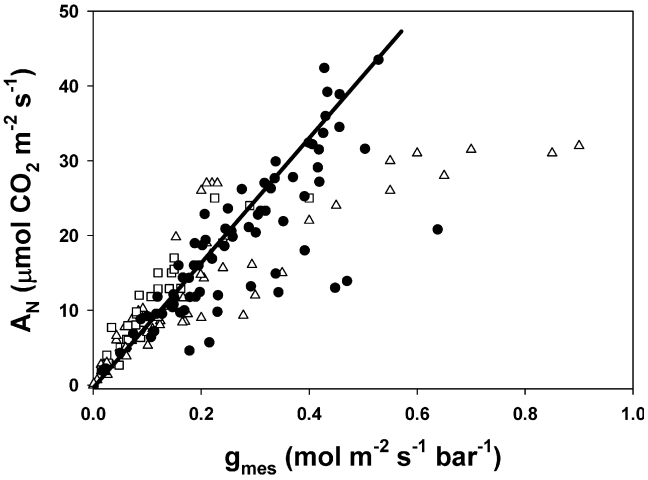

Fig. 3 The relationship between A_N and g_{mes} from different experiments comparing different species under non-stressing conditions (filled circles), one or a few species during drought (empty triangles, including control plants) and one or a few species during salinity (empty squares, including control plants). g_{mes} estimations are either from combined chlorophyll fluorescence and gas exchange measurements, gas exchange plus on-line isotope discrimination measurements, or isotope discrimination in recently assimilated sugars. Data on non-stressing conditions are as compiled by Evans and Loreto (2000), and come from the following references: von Caemmerer and Evans, 1991; Epron et al., 1995; Evans and Vellen, 1996; Roupsard et al., 1996; Lauteri et al., 1997 and Loreto et al., 1992. Data for drought experiments are from: Brugnoli et al., 1998; Delfine et al., 2001; Flexas et al., 2002; Bota et al., unpublished results; Nogués, Flexas and Cornic, unpublished results. Data for salinity experiments are from: Bongi and Loreto, 1989; Delfine et al., 1998, 1999; Centritto et al., 2003. Unpublished data from Bota et al., are from the following species: *Rhamnus alaternus* L., *Rhamnus ludovici-salvatoris* R. Chodat, *Nicotiana sylvestris* L., *Phaseolus vulgaris* L., *Vitis vinifera* L., and the experimental conditions were as described (Bota et al., 2004). In this experiment, g_{mes} estimations were made by gas exchange and fluorescence measurements. Unpublished data from Nogués, Flexas and Cornic are from potted *Phaseolus vulgaris* L. plants growing under natural greenhouse conditions in October at Orsay (France), and slowly dehydrating for 3 weeks. In this experiment, g_{mes} estimations were made by on-line carbon discrimination.

not reflect the actual photosynthetic capacity in drought-stressed plants. Indeed, to see whether phenomena like those observed by Centritto et al. (2003) also applied to drought, a similar experiment was performed in dehydrating sunflower plants (Fig. **2**). Typical A_N-C_i curves were performed in a well-irrigated plant, as well as in the same plant 10 days after withholding water. After finishing the curve in the stressed plant, the leaf was maintained for ca. 30 min at a CO_2 molar ratio of 50 μmol mol^{-1}, which forced stomatal opening, reaching a g_s of 0.257 mol H_2O m^{-2} s^{-1}. Then CO_2 molar ratio was suddenly raised to 2700 μmol mol^{-1}, and stomata started closing rapidly. When photosynthesis was stabilized and gas exchange system calibrated (i.e., ca. 5 min later), g_s was not fully reversed, as indicated in the figure, but A_N was as high as expected from the curve of the non-stressed plant. This effect may indicate that mesophyll conductance, in addition to stomatal conductance, was decreased in the stressed plant and reversed by low CO_2 (but could also indicate that photosynthesis was inhibited by end-product accumulation, see page 275). Low CO_2 in the light caused an increase of RuBP concentration in the leaf (Laisk and Oja, 1974). However, the fact that the enhancement effect was still observed more than 5 min after return of the leaf in air containing a high CO_2 molar ratio makes it unlikely that it was the result of the transient increase in A_N shown in Fig. **2**. Whatever the reason, these results clearly demonstrate that photosynthetic capacity was not impaired.

Therefore, there are some indications that g_s and g_{mes} are co-regulated under drought and salinity, and that the sum of both resistances (stomatal and mesophyll), and not metabolic impairment, sets the limit for photosynthesis under most water-stress conditions, although the mesophyll resistance could have a metabolic component, as suggested by Bernacchi et al. (2002). A strong dependency of A_N on g_{mes} was indeed found, pooling all available data together, and this relationship was identical to that shown by Evans and Loreto (2000) comparing different species under non-stressful conditions (Fig. **3**). Outlying data at high g_{mes}, where the model is more sensitive to small changes in dark respiration, CO_2 compensation point, are likely to be due to misleading estimations of g_{mes} (Harley et al., 1992). These results strongly suggest that A_N and g_{mes} are strongly co-regulated in a more dynamic way than previously thought, and just as A_N and g_s are.

The reasons why mesophyll conductance also recovers after pre-conditioning leaves at low CO_2, and is again reduced rapidly when CO_2 is increased (Centritto et al., 2003), are unknown and deserve further investigation. Similarly, the mechanisms that down-regulate g_{mes} under drought and salinity are also unknown. Bernacchi et al. (2002) have recently suggested that protein-facilitated diffusion of CO_2 might be a determinant of g_{mes}. The involvement of a protein in g_{mes} regulation supports

the possibility of tight and rapid co-regulation of photosynthesis and g_{mes}. Likely candidates are carbonic anhydrase and aquaporins. Carbonic anhydrase, indeed, is inhibited by progressive drought (Jones, 1973), but its activity is usually too large (some two orders of magnitude higher than A_N) to limit photosynthesis. The idea that aquaporins are involved in g_{mes} regulation is even more provocative since these are water channels, which would imply that internal diffusion of water and CO_2 share some common agent, as occurs at the stomatal level. Recently, Terashima and Ono (2002), using $HgCl_2$ to inhibit aquaporin function, suggested a role of aquaporins in mesophyll conductance to CO_2. More recently, Uehlein et al. (2003) have directly demonstrated that tobacco aquaporin NtAQP1 facilitates trans-membrane CO_2 transport by expressing its gene in *Xenopus* oocytes. The role of NtAQP1 on g_{mes} has been analysed *in vivo* using transgenic (anti-sense and over-express) tobacco plants, and it was observed that g_{mes} was as much as double in over-expressing than anti-sense plants (Flexas et al., unpublished).

It should also be mentioned that the observed plasticity of g_{mes} may be an artefact caused by unexpected variations of some parameters entering calculation of internal conductance (Harley et al., 1992). The most likely candidate is mitochondrial respiration in the light (Rd). Recent technical advances have made possible actual measurements of Rd (Loreto et al., 2001) and have shown that Rd is inversely associated to A_N. It may therefore be plausibly hypothesized that Rd is very low in non-stressed leaves but increases substantially with stress-induced reductions of photosynthesis. Harley et al. (1992), simulated the impact of a 10% variation of Rd on g_{mes} and concluded that this may not result in substantial errors in g_{mes} calculation. However, Loreto et al. (1999) have shown that Rd may vary up to 100% (from totally inhibited to totally emitted). We have simulated the influence of such a large variation of Rd on the data set of Centritto et al. (2003) and show here that Rd can account for a large part of the observed difference in g_{mes} before and after exposing leaves to the low CO_2 treatment and to elevated CO_2 (Fig. **4**). In particular, in control leaves of the two olive cultivars, g_{mes} differences are not statistically significant following the CO_2 treatments. This confirms data collected at different CO_2 in non-stressed leaves (Harley et al., 1992), and shows that g_{mes} may not change as rapidly as stomatal conductance and may therefore have a constitutive basis. However, g_{mes} of stressed leaves changes significantly following the treatments even after simulating different Rd emission. This confirms the interpretation of Centritto et al. (2003) that g_{mes} responds to stress and contributes to setting diffusive limitations to photosynthesis that may be partially or totally removed when the stress is alleviated.

Clearly, the precise steps and mechanisms by which g_{mes} is affected by stresses still remain to be elucidated, and this will likely be a very active research area in the near future.

Effects of Large Diffusion Resistances on Metabolic Processes

Stomatal closure and increased mesophyll diffusion resistance cause photosynthesis to be limited by the availability of CO_2. This low CO_2 has been shown to lead to changes in metabolism of the leaf. Both sucrose phosphate synthase and nitrate reductase have been shown to change their activity when leaves experience prolonged periods of low CO_2 (Kaiser and Forster, 1989; Vassey and Sharkey, 1989). In both cases, this may be caused by phosphorylation of the enzyme leading to binding of a regulatory 14-3-3 protein (Huber et al., 2002). Water stress *per se* can also activate sucrose phosphate synthase (Vassey et al., 1991), making it difficult to predict whether sucrose synthesis will be stimulated or inhibited by water stress. If carbon cannot be metabolized to sucrose it is often converted to starch. Vassey and Sharkey (1989) showed that starch synthesis was inhibited even more than sucrose synthesis in response to water stress. The reason for this is not clear.

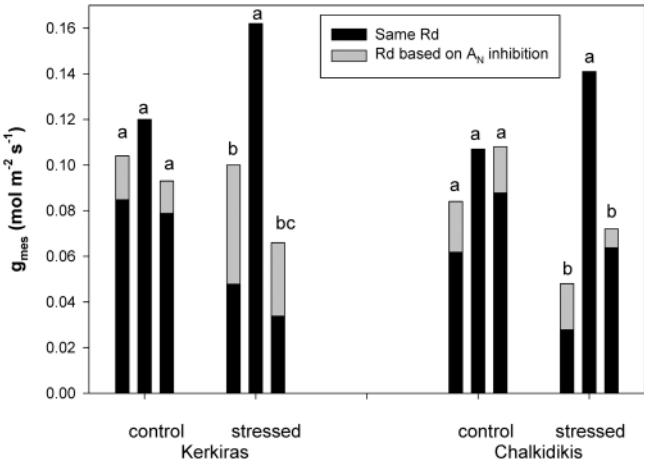

Fig. 4 Estimation of internal conductance to CO_2 diffusion (g_{mes}) in control and salt-stressed leaves of two olive cultivars (data from Centritto et al., 2003). The three columns of each data set show g_{mes} before and after exposure of leaves to low CO_2 (50 μmol mol^{-1}) and after exposure to very high CO_2 (1500 μmol mol^{-1}). Stacked bars show g_{mes} estimation considering the same mitochondrial respiration in the light (Rd) for the three treatments (black) or different Rd, i.e., Rd totally inhibited when photosynthesis was stimulated by removing diffusional limitations with the low CO_2 treatment (2nd bars), and Rd totally emitted when photosynthesis was not stimulated by this treatment (1st and 3rd bars) (grey). Means (n = 3) are statistically separated within treatments with a Tukey's test and differences at $p < 0.05$ are shown by different letters.

To examine whether the ability to make starch or sucrose affects metabolism in water-stressed leaves, we analysed the relationship between A_N and C_i published by Tezara et al. (1999). We fitted lines to the data assuming that Rubisco was limiting (dashed line), that RuBP regeneration was limiting (damage to photophosphorylation components would affect this line) (short dashed line) or that starch and sucrose synthesis were limiting (solid grey line). The lack of response to CO_2 at the higher CO_2 levels can only be explained by a functional limitation in starch plus sucrose synthesis. Setting the Rubisco or electron transport capacity high enough to account for the data at low CO_2 predicts a much higher maximum rate of photosynthesis at higher CO_2. The predicted rate of RuBP use (open circles in Fig. **5**) falls with increasing CO_2, a phenomenon associated with restricted starch and sucrose synthesis capacity (Sharkey, 1985). Thus, water stress can affect photosynthetic metabolism, but the effect is indirect and mediated by the stomatal closure and reduced CO_2 concentration inside the leaf.

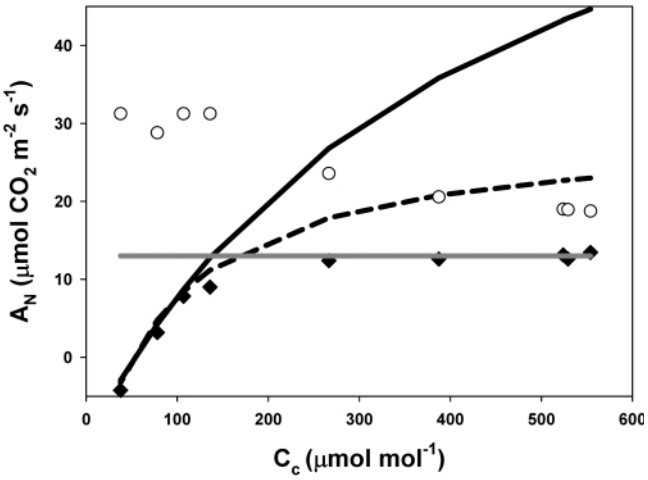

Fig. 5 The response of A_N to chloroplastic CO_2 concentration (C_c) in *Helianthus annuus* L. plants. Modelling was done using the Farquhar model (Farquhar et al., 1980) and the parameterisation of Bernacchi et al. (2001). V_{cmax} used to estimate Rubisco-limited rates (Ac) was set to 90 μmol m^{-2} s^{-1}, electron transport rate used to estimate RuBP regeneration-limited rates (Aj) was set to 125 μmol electrons m^{-2} s^{-1}, and the rate of triose phosphate use (to set At) was 5 μmol triose phosphate m^{-2} s^{-1}. Leaf temperature was assumed to be 25 °C and the mesophyll conductance was assumed to be 1 μmol m^{-2} s^{-1}. Data are taken from Tezara et al. (1999) for the leaf at – 1.5 MPa water potential.

Concluding remarks

In summary we have reviewed results showing that:

1. Salt and drought stress predominantly affect diffusion of CO_2 in the leaves through a decrease of stomatal and probably also of mesophyll conductances (g_s and g_{mes}, respectively), but not the biochemical capacity to assimilate CO_2. Therefore, in these conditions the sum of the diffusional resistances sets the limit to photosynthesis rates. This can in turn induce an indirect, secondary effect on photosynthetic metabolism.
2. In stressed leaves, changes of g_{mes} may occur as fast as those of g_s, and this may reflect a protein-dependent mechanism operating as a g_{mes} regulator.
3. The estimates of photosynthetic limitations based on A_N-C_i curves may lead to incorrect interpretations, as g_{mes} reduces CO_2 concentration at the chloroplasts and makes invalid the estimation of this concentration by C_i.

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