An introductory guide to gas exchange analysis of photosynthesis and its application to plant phenotyping and precision irrigation to enhance water use efficiency

Matthew Haworth, Giovanni Marino and Mauro Centritto

ABSTRACT

Leaf gas exchange is central to the analysis of photosynthetic processes and the development of more productive, water efficient and stress tolerant crops. This has led to a rapid expansion in the use of commercial plant photosynthesis systems which combine infra-red gas analysis and chlorophyll fluorescence (Chl-Flr) capabilities. The present review provides an introduction to the principles, common sources of error, basic measurements and protocols when using these plant photosynthesis systems. We summarise techniques to characterise the physiology of light harvesting, photosynthetic capacity and rates of respiration in the light and dark. The underlying concepts and calculation of mesophyll conductance of CO_2 from the intercellular air-space to the carboxylation site within chloroplasts using leaf gas exchange and Chl-Flr are introduced. The analysis of stomatal kinetic responses is also presented, and its significance in terms of stomatal physiological control of photosynthesis that determines plant carbon and water efficiency in response to short-term variations in environmental conditions. These techniques can be utilised in the identification of the irrigation technique most suited to a particular crop, scheduling of water application in precision irrigation, and phenotyping of crops for growth under conditions of drought, temperature extremes, elevated [CO_2] or exposure to pollutants.

Key words | chlorophyll fluorescence, mesophyll conductance, photorespiration, respiration, stomatal kinetics, water optimisation

Matthew Haworth
Giovanni Marino
Mauro Centritto (corresponding author)
The National Research Council of Italy – Trees and Timber Institute (CNR-IVALSA),
Via Madonna del Piano 10, Sesto Fiorentino, 50019 Florence,
Italy
E-mail: *mauro.centritto@cnr.it*

ABBREVIATIONS

C_a	atmospheric [CO_2] (μmol mol^{-1})
C_c	[CO_2] within the chloroplast envelope (μmol mol^{-1})
Chl-Flr	light absorbed by chlorophyll and then re-emitted as chlorophyll fluorescence
C_i	[CO_2] within the internal sub-stomatal air-spaces (μmol mol^{-1})
E	leaf level evapotranspiration of water from stomatal and non-stomatal sources (mol_{H2O} m^{-2} s^{-1})
F_m'	maximum fluorescence under steady state conditions in the light after exposure to a saturating pulse of light
F_s	steady state fluorescence under steady state conditions in the light
F_v/F_m	the maximum quantum efficiency of PSII determined by exposing the dark-adapted leaf to a saturating pulse of light
Γ	[CO_2] compensation point where P_N equals R_d and R_{PR} (μmol mol^{-1})
Γ^*	[CO_2] photo-compensation point where P_N equals R_d (μmol mol^{-1})
Gb_L	boundary layer conductance over a leaf (mol_{H2O} m^{-2} s^{-1})
G_m	conductance of CO_2 across the mesophyll layer (μmol_{CO2} m^{-2} s^{-1} bar^{-1})

doi: 10.2166/wcc.2018.152

G_s stomatal conductance to water vapour ($G_{s\ H2O}$ – $mol_{H2O}\ m^{-2}\ s^{-1}$) or CO_2 ($G_{s\ CO2}$ – $mol_{CO2}\ m^{-2}\ s^{-1}$)

G_{tot} total conductance to CO_2 ($mol_{CO2}\ m^{-2}\ s^{-1}$)

IRGA infra-red gas analysers used in the measurement of [CO_2] and water vapour

J_A electron transport rate associated with photochemical carboxylation/oxygenation of RubisCO determined from gas exchange ($\mu mol_{e-}\ m^{-2}\ s^{-1}$)

J_F photochemical electron transport rate determined from Chl-Flr parameters ($\mu mol_{e-}\ m^{-2}\ s^{-1}$)

J_o electron transport for photorespiration using gas exchange ($\mu mol_{e-}\ m^{-2}\ s^{-1}$)

J_{max} maximum rate of electron transport required for ribulose-1,5-bisphosphate (RuBP) regeneration ($\mu mol_{e-}\ m^{-2}\ s^{-1}$)

PAR photosynthetically active radiation within the 400–700 nm wavelength spectrum of light ($\mu mol_{photon}\ m^{-2}\ s^{-1}$)

PAR_{comp} the light compensation point ($\mu mol_{photon}\ m^{-2}\ s^{-1}$)

PAR_{sat} light saturation point, the intensity of PAR where P_N is no longer limited by PAR ($\mu mol_{photon}\ m^{-2}\ s^{-1}$)

P_N net photosynthesis ($\mu mol_{CO2}\ m^{-2}\ s^{-1}$)

P_{Nmax} the maximum rate of P_N at saturating levels of light and [CO_2] (the part of the P_N-C_i curve where P_N levels off) ($\mu mol_{CO2}\ m^{-2}\ s^{-1}$)

PPFD amount of photosynthetically active radiation ($\mu mol_{photon}\ m^{-2}\ s^{-1}$)

R_d rate of CO_2 efflux in the light taken to represent mitochondrial respiration ($\mu mol_{CO2}\ m^{-2}\ s^{-1}$)

RH relative humidity of water vapour within the atmosphere (%)

R_n respiration in the dark ($\mu mol_{CO2}\ m^{-2}\ s^{-1}$)

R_{PR} photorespiratory evolution of CO_2 in the light ($\mu mol_{CO2}\ m^{-2}\ s^{-1}$)

RubisCO ribulose-1,5-bisphosphate carboxylase/oxygenase

RuBP ribulose-1,5-bisphosphate

Φ_{CO2max} maximum quantum yield of P_N derived from gas exchange expressed as ($mol_{CO2}\ photon^{-1}$)

ΦPSII quantum efficiency of photosystem II determined using Chl-Flr

SR ratio of stomata on either surface of the leaf

TPU triose phosphate utilisation, a measure of phosphate limitations to the regeneration of RuBP in the P_N-C_i curve ($\mu mol_{CO2}\ m^{-2}\ s^{-1}$)

V_c rate of carboxylation ($\mu mol_{CO2}\ m^{-2}\ s^{-1}$)

Vc_{max} maximum carboxylation capacity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) ($\mu mol_{CO2}\ m^{-2}\ s^{-1}$)

V_o rate of oxygenation ($\mu mol_{O2}\ m^{-2}\ s^{-1}$)

VPD leaf to air vapour pressure deficit (KPa)

WUE_i instantaneous water use efficiency ($\mu mol_{CO2}\ mmol_{H2O}^{-1}$)

INTRODUCTION

Gas exchange analysis of photosynthesis *in vivo* has become a central tool in gauging the performance of plants. The increased availability of commercial plant photosynthesis systems has facilitated an expansion in the use of gas exchange to analyse plant responses to global environmental changes such as drought, temperature stress, pollution (e.g. increased tropospheric ozone), elevated concentrations of atmospheric carbon dioxide ([CO_2]) and biotic stresses that impact upon the sustainability of agriculture and/or natural ecosystems. We intend this review to serve as an introduction to the concepts, standard measurements and common sources of error involved in gas exchange analysis of plant photosynthetic physiology. We will endeavour to clarify the terminology utilised in this field to those unfamiliar with a phraseology that is inconsistently used and frequently oversteps into off-putting jargon. As many commercial plant photosynthesis gas exchange systems are now also capable of performing simultaneous measurement of chlorophyll fluorescence (Chl-Flr), we will provide an outline of how Chl-Flr parameters may be utilised alongside gas exchange to assess the status of photosynthesis. This work will focus on the potential application of leaf gas exchange analysis in plant phenotyping and precision irrigation to improve crop water use efficiency and drought tolerance. Quantification of the carbon and water fluxes of plants are central to phenotyping of tolerance to abiotic/biotic stresses, but also to efforts to enhance the productivity and water use efficiency of crops

(Marino *et al.* 2014). For example, leaf gas exchange has been utilised effectively in the phenotyping of rice varieties exposed to different levels of water availability provided by line (Centritto *et al.* 2009) and sprinkler (Lauteri *et al.* 2014) irrigation systems, and in the identification of genotypes with enhanced drought tolerance (Chakhchar *et al.* 2017; Haworth *et al.* 2017b; Killi *et al.* 2018). Moreover, a combination of gas exchange on a single leaf alongside wider-scale imaging of a plant canopy can be effective in irrigation scheduling to maximise crop water productivity particularly in protected horticulture systems.

Background and principles of gas exchange

All photosynthesis gas exchange systems work by enclosing an entire leaf, or part of a leaf, within a chamber or cuvette. Within the chamber the quantity and quality of the photosynthetic photon flux density (PPFD: the amount of photosynthetically active radiation, PAR, arriving at the leaf within the 400–700 nm wavelength spectrum of light), the wind speed or 'flow' of air, the relative humidity (RH), temperature (leaf temperature may only be controlled to a degree under variable field conditions where the ambient conditions may diverge considerably from internal conditions within the leaf cuvette), and concentration of atmospheric gases can be controlled to determine the photosynthetic response of the area of leaf contained within the chamber. The difference in the concentration of [CO_2] and water vapour between the 'reference' air flow that enters the cuvette and the 'sample' air flow that exits the cuvette (commonly referred to as ΔCO_2 and ΔH_2O) can be used to infer rates of photosynthesis, respiration and stomatal conductance using calculations based on the work of Farquhar *et al.* (1980) and Von Caemmerer & Farquhar (1981) that are automatically computed by commercial gas exchange systems. Values of ΔCO_2 and ΔH_2O are determined using infra-red gas analysers (IRGA). Carbon dioxide and water vapour absorb different wavelengths of infra-red radiation. The most recent 'open system' commercial photosynthesis gas exchange systems possess two pairs of IRGAs to concurrently measure the amount of CO_2 and H_2O in the reference and sample air streams. If the concentration of [CO_2] and [H_2O] within the air stream increases, a greater proportion of infra-red radiation is absorbed and less is received by the sensor of the IRGA. To control [CO_2] within the reference airstream, CO_2 is removed from the air entering the plant photosynthesis system by soda lime, pure CO_2 is then mixed with the reference air to a set concentration. Water vapour in the reference air stream can be regulated by being passed through desiccants (such as silica gel or gypsum based desiccants) or humidifiers (such as porous ceramic substrates that hold water).

When photosynthesis (P_N) occurs inside the cuvette, the leaf takes up CO_2 from the air within the cuvette resulting in a lower [CO_2] in the sample air in comparison with the reference air flow. Alongside the uptake of CO_2 for P_N, plants lose water via transpiration; this results in a high concentration of water vapour in the sample air flow that can be used to estimate the rate of stomatal conductance (G_s). Calculation of G_s to water vapour ($G_{s\ H2O}$) is fairly straight forward and performed instantly by commercial gas exchange systems as:

$$G_{sH2O} = \frac{1}{(1/G_{totH2O}) - (SR/Gb_L)} \quad (1)$$

where $G_{tot\ H2O}$ is the total conductance to water vapour; SR is the ratio of stomata on either surface of the leaf and Gb_L is the boundary layer conductance. Stomatal conductance to CO_2 ($G_{s\ CO2}$) can be estimated as:

$$G_{sCO2} = \frac{1}{(1.6/G_{sH2O}) + (1.37/Gb_L)} \quad (2)$$

where 1.6 is the ratio between the diffusivity of CO_2 and water vapour in air and 1.37 the equivalent in the boundary layer. If the leaf within the cuvette receives no illumination, then photosynthetic CO_2 uptake will not occur. In this case, the efflux of CO_2 from mitochondrial respiration will result in increased [CO_2] in the sample air flow and a positive ΔCO_2 can be used to determine the rate of respiration in the dark (R_n). As these parameters are strongly influenced by environmental conditions, it is necessary to maintain constant cuvette conditions of light, temperature, air flow and [CO_2] so that the true effect of the experimental factor under investigation on leaf P_N and G_s can be gauged. However, manipulation

of the conditions within the cuvette can provide information regarding the status of the underlying photosynthetic physiology and/or stomatal functionality.

Photosynthetic carbon fixation is regulated by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) which catalyses the carboxylation reaction between CO_2 and ribulose-1,5-bisphosphate (RuBP) to form two molecules of glycerate-3-phosphate that can be used to form more complex sugars. However, RubisCO also has an affinity for oxygen resulting in the oxidation of RuBP to produce phosphoglycolate and 3-phosphoglycerate during photorespiration (R_{PR}) (Bowes *et al.* 1972; Laing *et al.* 1974; Jordan & Ogren 1984). The rate of net CO_2 assimilation (P_N) within a leaf can be described as:

$$P_N = V_c - 0.5V_o - R_d \quad (3)$$

where V_c is the rate of carboxylation, V_o the rate of oxygenation and R_d the rate of mitochondrial respiration in the light. The net photosynthesis rate (P_N) differs from the gross rate of photosynthesis ($P_{N\ gross}$) that does not include the effect of R_d and R_{PR}. Each molecule of CO_2 involved in carboxylation requires the transport of four electrons. The electron transport for photosynthetic carboxylation (J_A) can be described as:

$$J_A = 4*(P_N + R_d + R_{PR}) \quad (4)$$

Each molecule of CO_2 released during R_{PR} requires the transport of eight electrons (J_o):

$$J_o = 8*R_{PR} \quad (5)$$

The processes described in Equations (1)–(5) form the theoretical basis of the method of photosynthetic gas analysis and estimation of physiological parameters outlined later in this review. Photosynthetic electron transport can be determined using gas exchange and Chl-Flr; moreover, the simultaneous use of these two methods can provide valuable insights into the uptake of CO_2 and the physiology of light capture and usage (e.g. Genty *et al.* 1989; Harley *et al.* 1992; Loreto *et al.* 1994).

Instantaneous point measurements of leaf gas exchange and chlorophyll fluorescence

Instantaneous point measurements of leaf gas exchange and chlorophyll fluorescence are powerful indicators of the physiological status of plants under experimental or natural growth conditions. These types of measurement are the most widely used gas exchange analyses when assessing the impact of abiotic or biotic stress factors on plants. These point measurements can be tied to environmental indicators such as soil water availability (e.g. soil water potential or the fraction of transpirable soil water content), leaf water potential, nutrient availability, temperature and the duration of growth at elevated [CO_2] or exposure to pollutants. As such, instantaneous point measurements can provide insights into the effect of environmental conditions on P_N, G_s, [CO_2] within the internal sub-stomatal air-spaces (C_i) and Chl-Flr parameters. This information can be highly insightful in terms of phenotyping and understanding the impacts of irrigation strategies on plant physiological status. Importantly, point measurements of gas exchange also provide an indication of the instantaneous water use efficiency (WUE_i): a ratio of CO_2-uptake relative to water loss:

$$WUE_i = \frac{P_N}{E} \quad (6)$$

where E indicates evapotranspiration of water and WUE_i is expressed as $\mu mol_{CO2}\, mmol^{-1}_{H2O}$. Instantaneous water use efficiency reflects WUE at that point in time and is not as representative as longer-term measures of WUE such as discrimination between stable carbon isotopes (e.g. Farquhar *et al.* 1989) or measures of the amount of biomass gained relative to water used by the plant (e.g. Morison *et al.* 2008). An analogous parameter is the intrinsic water use efficiency, which is the ratio of P_N to G_s (e.g. Flexas *et al.* 2013). However, the use of the term intrinsic is somewhat controversial because of the implication that this measure is a constitutive genetic trait, whereas in reality the intrinsic water use efficiency represents WUE at a single point in time in a manner similar to WUE_i.

Point measurements of leaf gas exchange can be performed under ambient or set conditions of [CO_2], light

intensity and temperature within the leaf cuvette. The use of ambient cuvette conditions (a transparent window is attached to the leaf cuvette instead of an LED light unit – this precludes the measurement of Chl-Flr alongside leaf gas exchange) in the field may be beneficial in terms of increased speed of measurement (as leaves do not need to adapt to vastly different conditions within the leaf cuvette) and extended battery life. However, variation in environmental conditions (e.g. changing cloud cover, temperature) may affect consistency between measurements; it is therefore preferable to conduct such measurements when possible at the same time of day on clear sunny days to minimise fluctuations in light intensity and temperature. In some cases, the impact of an experimental treatment will be evident under controlled cuvette settings (where all leaves are measured under identical conditions of [CO_2], temperature, RH and PAR), but not when ambient measurements are performed because of heterogeneity in conditions in the field (e.g. Haworth *et al.* 2016). Instantaneous point measurements under set controlled cuvette conditions generally take longer, as the leaf requires time to adjust to the new conditions within the leaf cuvette. The maintenance of temperature, [CO_2], RH and PPFD within the leaf cuvette when performing point measurements under set controlled cuvette conditions also places additional demands upon battery life if the plant photosynthesis system is not running from mains power.

The use of LED light units within the cuvette to maintain a constant PAR also enables the potential analysis of Chl-Flr parameters alongside leaf gas exchange. Extensive reviews of the principles and practicalities of the measurement of Chl-Flr under light and dark-adapted conditions are already available (e.g. Maxwell & Johnson 2000; Kalaji *et al.* 2016). We do not propose to repeat this material within this introductory guide, but instead to highlight the most common Chl-Flr measures to be conducted alongside leaf gas exchange. Under steady state conditions in the light, characteristic of instantaneous point measurements using set cuvette conditions, one of the most useful Chl-Flr measurements is the quantum efficiency of photosystem II (ΦPSII). This is a measure of the maximum fluorescence under steady state conditions in the light (F_m') following a saturating pulse of light (usually $>8{,}000\ \mu mol\ m^{-2}\ s^{-1}$, although over time LEDs can dim and it may not be possible to achieve such light intensities in older plant photosynthesis systems) relative to the steady state fluorescence (F_s) (see Equation (7)). The saturating pulse of light fills all of the available photosystem II (PSII) reaction centres, providing an indication of the efficiency of light harvesting (Genty *et al.* 1989):

$$\Phi PSII = \frac{F_m' - F_s}{F_m'} \tag{7}$$

The actual quantum efficiency of PSII is frequently more sensitive to the impact of abiotic stress (e.g. Haworth *et al.* 2017c) than the maximum quantum efficiency of PSII (expressed as the F_v/F_m ratio), determined by exposing the dark adapted leaf, where all of the PSII reaction centres are open, to a saturating pulse of light (Butler & Kitajima 1975). The ΦPSII parameter frequently correlates to gas exchange measurement of CO_2 assimilation (Genty *et al.* 1989; Loreto *et al.* 1994) and is used in the calculation of the electron transport rate (J_F) required for the determination of chloroplastic [CO_2] (C_c) and mesophyll conductance (G_m) (Harley *et al.* 1992). Dark adapting the leaf (exposure to no light for a minimum of 30 minutes is standard – although some researchers suggest a pre-dawn analysis of Chl-Flr is preferable) can provide a more in-depth analysis of PSII performance when utilised in conjunction with Chl-Flr parameters recorded under light adapted conditions (such as the dissipation of excess energy by xanthophylls during non-photochemical quenching). However, this greatly increases the time of measurement, particularly if undertaken in conjunction with leaf gas exchange analysis as full stomatal opening after exposure to darkness can take up to two to three hours in some plants (e.g. Doi *et al.* 2015). The type of point measurement of leaf gas exchange and Chl-Flr parameters must therefore reflect a compromise between the aims of the study and the availability of time and plant photosynthesis system equipment.

Photosynthetic light response curves

The basis of photosynthesis involves the capture of PAR to provide the chemical energy to produce sugars (Evans *et al.* 1993). Photosynthetic light response curves (commonly

termed P_N – PAR curves) characterise the relationship between P_N and increasing levels of PPFD. As PPFD is increased from zero, P_N rises rapidly (Kok 1948); the PAR level where P_N equals zero is termed the light compensation point (PAR_{comp}). The relationship between P_N and PPFD at low light intensity around PAR_{comp} can be used to determine respiration and is outlined in more detail in the section 'Respiration in the light and in the dark', below. Leaves that are efficient in converting low levels of PPFD such as shade leaves generally have lower PAR_{comp} than leaves adapted to intercept higher intensities of PPFD such as sun leaves (e.g. Demmig-Adams *et al.* 1989; Thornley 2002). At PPFD from 0 μmol m^{-2} s^{-1} to PAR_{comp}, and often above PAR_{comp}, P_N rises linearly with increasing PPFD (usually this linear relationship holds to a PAR level of 200 to 400 μmol m^{-2} s^{-1}); this is known as the maximum quantum yield as the availability of light limits the carboxylation of RuBP for P_N (Φ_{CO2max}, expressed as mol_{CO2} $photon^{-1}$). Beyond this point the relationship between P_N and PAR is no longer linear; the curve becomes convex and begins to plateau where P_N becomes limited by RubisCO (Ögren & Evans 1993); the stage at which the curve flattens represents the point where the availability of PAR no longer limits P_N and is termed 'light saturation point' (PAR_{sat}) (Figure 1) (Ögren 1993).

When performing a light response curve, we recommend starting at PAR_{sat} and declining to lower intensities of PPFD. In species such as grasses that possess highly functional stomata that close rapidly in darkness (e.g. Haworth *et al.* 2018b), it is necessary to start at full light intensity and then decrease PAR rather than placing the leaf into darkness and then increasing PPFD (this approach will take comparatively longer as it is necessary to wait for the stomata to open fully at each PPFD stage). The order of the PPFD stages will not affect the shape of the curve or the calculation of parameters from the P_N – PAR response curve. Cuvette temperature should be maintained at the growth temperature of the plant (normally a value in the range of 20 to 30 °C is used). As stomata open or close with the change of PAR intensity, the leaf to air vapour pressure deficit (VPD) alters; this effect should be minimised by adjusting the humidity of the input air stream, which can be controlled automatically (within certain ranges) or manually. Commercial gas exchange

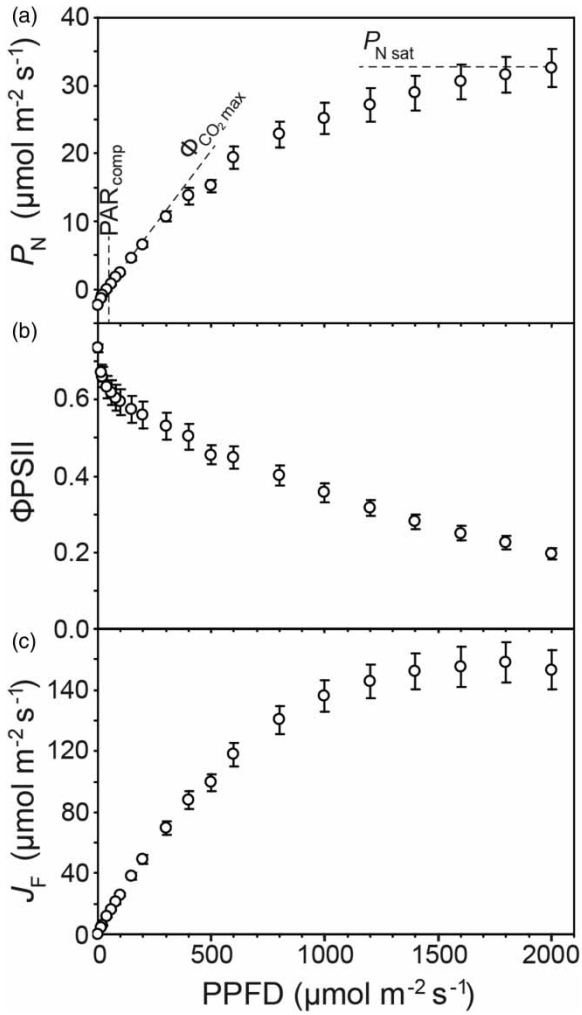

Figure 1 | (a) The response of photosynthesis (P_N), (b) the actual quantum efficiency of photosystem II (ΦPSII – Equation (7)), and (c) the electron transport rate determined by Chl-Flr (J_F – Equation (12)) of the fast growing grass species *Arundo donax* to increasing intensity of PPFD. The light saturated rate of photosynthesis ($P_{N\,sat}$), quantum efficiency of CO_2 assimilation (Φ_{CO2}) and light compensation point (PAR_{comp}) are marked in panel (a). Error bars indicate one standard deviation either side of the mean (n = 4).

systems control PAR using LEDs usually up to an intensity of 2,000–2,400 μmol m^{-2} s^{-1}, approximately equivalent to light intensity on a clear sunny day. Red and blue light can influence stomatal opening, so it is necessary to use both when performing a P_N/PAR curve (Mansfield & Meidner 1966; Zeiger & Hepler 1977; Sharkey & Raschke 1981; Loreto *et al.* 2009); although many plants also adsorb light in the yellow and green part of the spectrum (Terashima *et al.* 2009; Sakowska *et al.* 2018), so a more balanced quality of light is preferable. Standard steps for conducting a

P_N/PAR curve are: 2,000, 1,600, 1,200, 1,000, 800, 600, 400, 300, 200, 150, 100, 75, 50, 0 μmol m^{-2} s^{-1}. In species adapted to low PAR, such as many ferns, P_N will decline at the higher light intensities; this represents light inhibition and PAR levels above the PAR_{sat} point should not be used in photosynthetic response curves to [CO_2]. Moreover, in certain species such as some grasses with rapid growth rates that are adapted to high light environments PAR_{sat} occurs above 2,000 μmol m^{-2} s^{-1}. In these cases, a modelled value is assumed, but should be treated with a degree of caution. Lobo *et al.* (2013) provide an in-depth review of P_N/PAR models and Microsoft Excel solvers for these models that permit rapid and easy determination of physiological parameters from a P_N/PAR dataset.

Respiration in the light and in the dark

Respiration provides the energetic requirements for processes such as growth, repair, protective physiology and reproduction. Respiration involves the release of CO_2 and can therefore be measured using gas exchange techniques. However, the respiratory CO_2 release needs to be separated from the other processes involving CO_2 outlined in Equation (3). Carbon dioxide produced by mitochondrial respiration and R_{PR} is released into the cytosol as bicarbonate (HCO_3^-). As the concentration of [CO_2] within the cytosol is lower than that within the sub-stomatal internal leaf air-space or the external atmosphere and CO_2 released by photo- and mitochondrial respiration may be reabsorbed by P_N, it is challenging to accurately measure respiration. Gas exchange analysis of respiration is complex, and the techniques involved are each based on their own assumptions and subject to limitations. Moreover, the fluxes of CO_2 involved in gas exchange analysis of respiration are smaller than those involved in P_N; therefore, any sources of error associated with leakage, temperature control or calibration of the gas exchange system have a much greater proportional effect when calculating respiration. The simplest way to distinguish respiration is to switch off the light in the cuvette (and the surrounding leaf/plant) and thus shut off P_N and R_{PR}. When the efflux of CO_2 has stabilised (this will be expressed as a negative P_N value in the gas exchange system output) it can be recorded and considered to represent respiration in the dark (R_n: the 'n' in the acronym refers to night-time respiration) (e.g. Peisker & Apel 2001; Tjoelker *et al.* 2001). However, this may not reflect the true respiration in the dark (Atkin *et al.* 1998; Pinelli & Loreto 2003), but is widely accepted as the quickest and most straightforward method of gauging respiration in plants using commercial gas exchange systems, particularly when working in the field.

Respiration also takes place in the light alongside P_N and R_{PR} (Equation (4)). This is termed respiration in the light (R_d: the 'd' in the acronym refers to day-time respiration) and is more difficult to measure than R_n because of the recycling of respiratory CO_2 emission by P_N (Pinelli & Loreto 2003). In this context, the Kok (1948) and Laisk (1977) methods that utilise gas exchange to estimate R_d can be considered to be estimates of R_d that effectively analyse CO_2 evolution in the light (i.e. not 'true' R_d, as CO_2 may be re-adsorbed by P_N, as described above). By convention, respiration is sometimes expressed as a negative value, as in gas exchange systems it represents a loss of CO_2 from the leaf (in comparison to P_N that is positive and indicates an uptake or gain of CO_2). However, even though respiration is recorded as a negative value by gas exchange systems, it should be considered and reported as positive, particularly when used in physiological calculations such as those used to estimate mesophyll conductance of CO_2 (e.g. Equation (11)) (e.g. Harley *et al.* 1992).

Respiration in the light may be determined indirectly through gas exchange using the Kok (1948) and Laisk (1977) methods. The Kok (1948) method utilises the relationship between P_N and PAR at low light intensities close to PAR_{comp}. At PAR levels 0 to PAR_{comp}, P_N increases rapidly with PAR; however, above PAR_{comp} the relationship between P_N and PAR remains linear but is less steep. This disjunction in the P_N/PAR relationship around PAR_{comp} is known as the Kok effect and can be used to estimate R_d, as outlined in Figure 2(a), by extrapolation of the P_N/PAR relationship above the 'Kok effect' to the point where it intersects with the y-axis (dashed line in Figure 2(a)) (Wang *et al.* 2001). The disjunction in the linear response between P_N and PAR associated with the 'Kok effect' is caused by the elimination of light-induced suppression of respiration (Sharp *et al.* 1984). It is worth noting that in some cases the Kok effect does not occur and the relationship between P_N and PAR is the same either side of

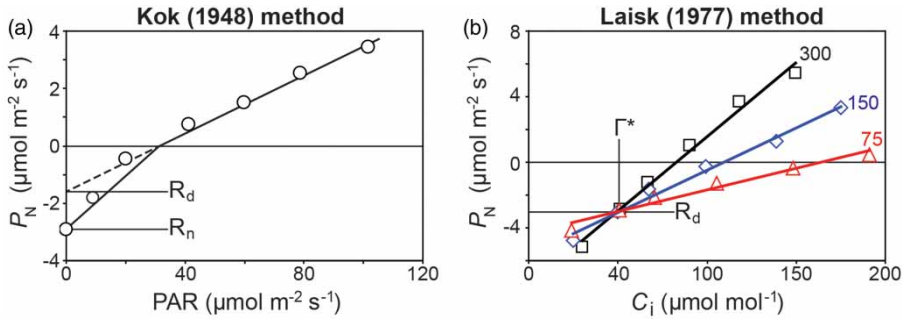

Figure 2 | Examples of the Kok (1948) (a) and Laisk (1977) (b) methods to estimate respiration in the light (R_d), respiration in the dark (R_n) and the CO_2-photocompensation point (Γ*) where P_N equals R_{PR} using leaf gas exchange. The P_N-C_i response undertaken for the Laisk protocol (b) was conducted at three PAR levels: 300 μmol m^{-2} s^{-1} (black), 150 μmol m^{-2} s^{-1} (blue) and 75 μmol m^{-2} s^{-1} (red).

PAR_{comp}; in this scenario the points above PAR_{comp} should still be used to extrapolate R_d, which should equal R_n. The Kok method can be used to determine R_d in both C3 and C4 plants (Bjorkman & Demmig 1987). The efficacy of the Kok method may be constrained by stomatal closure as PAR approaches zero, resulting in increased sub-stomatal $[CO_2]$ (C_i) (Crous *et al.* 2012). The effect of fluctuations in C_i can be compensated using the iterative approach of Kirschbaum & Farquhar (1987), which normally results in a slight increase in the level of R_d. However, this approach assumes that mesophyll conductance (G_m) is infinite, whereas experimental evidence suggests that this is not the case (e.g. Flexas *et al.* 2007b; Tholen *et al.* 2012). We would therefore advise against using such a correction, unless variations in C_i are comparatively large. The Kok method can be applied to plants grown at any $[CO_2]$ level (e.g. Haworth *et al.* 2016) or temperature (e.g. Haworth *et al.* 2018a). The intensity of PAR is decreased from a comparatively low level, with an increasing number of PAR steps as P_N approaches the PAR_{comp} point; normally PAR steps of 400, 300, 200, 150, 100, 75, 50, 30 and 0 μmol m^{-2} s^{-1} can be used but these should be manually adjusted in respect to P_N when around PAR_{comp}. Moreover, the latest generation of commercial exchange systems offer greater control of PAR intensity and quality (i.e. regulation of PPFD to ~5 μmol m^{-2} s^{-1}), permitting the construction of Kok P_N/PAR response curves at a higher resolution that affords increased accuracy in the estimation of R_d.

The Laisk (1977) method generally results in lower estimates of R_d than those produced by the Kok method (Villar *et al.* 1994). This approach uses the response of P_N to C_i taken at a number (usually 3–4) of relatively low levels of PAR (i.e. ≤400 μmol m^{-2} s^{-1}). Where the P_N-C_i curves intersect represents the point where P_N is equal to R_{PR}. The level of P_N represented by this point on the y-axis therefore indicates R_d, as all the CO_2 used by P_N is released by R_{PR} (see Equation (4)) (Figure 2(b)). As R_{PR} is extremely sensitive, the P_N – C_i curves undertaken as part of the Laisk (1977) protocol must be performed at the same temperature; this may preclude use of the method in the field where ambient temperatures may vary. The level of C_i at which this intersection occurs is known as the photo-compensation point (Γ*: pronounced *gamma star* – this value is different from the CO_2 compensation point, Γ, as it does not include the effect of R_{PR}). The photo-compensation point, Γ*, is an important parameter used in the modelling of P_N and the estimation of parameters such as mesophyll conductance (G_m). When performing the measurement of R_d and Γ* using the Laisk (1977) method, it is necessary to use a minimum of three PAR levels and to preferably use four. The first P_N – C_i curve should be performed at a PPFD of 300 and/or 400 μmol m^{-2} s^{-1}, most likely the slope of curves at these two PPFD intensities will be identical. The CO_2 concentration (C_a) used in the curve should be at ambient and sub-ambient level around the CO_2 compensation point (Γ), usually 400, 300, 200, 150, 100, 75, 50 and 30 μmol mol^{-1}. After each alteration in C_a, the levels of P_N and G_s should be allowed to stabilise (normally 2–5 minutes per step) before gas exchange parameters are recorded. When each sequence of C_a steps is complete, the level of light in the cuvette should be decreased and the P_N-C_i response curve repeated. Owing

to the CO_2 concentrating mechanism in the bundle sheath of C4 plants, it is not possible to use the Laisk (1977) method to estimate R_d in C4 species as the point where the lines of the P_N-C_i curves intersect often occurs above the x-axis, where P_N equals zero (i.e. the CO_2 compensation point, Γ); this would result in a negative R_d value (the expression of R_d as a positive or a negative is discussed earlier in this section). Likewise, it is not possible to utilise this approach for C3–C4 (also known as C2) species with photosynthetic physiologies intermediate between C3 and C4 where CO_2 is recaptured as part of a photorespiratory bypass.

Gas exchange measurement of R_d and Γ* using the Laisk method is fairly complex and very sensitive to errors such as variations in temperature or leaks from the gaskets that seal the leaves within a cuvette (Flexas *et al.* 2007a; Rodeghiero *et al.* 2007). Many commercial gas exchange systems are unable to maintain a constant cuvette temperature under field conditions; it is therefore extremely difficult to successfully perform measurement of R_d using the Laisk method in the field (an air-conditioned laboratory is preferable). However, analysis of R_d and R_n in plants using CO_2 composed of different stable isotopes of carbon (^{12}C and ^{13}C) suggests that in plants grown under optimal conditions levels of R_d and R_n are largely similar and that suppression of respiration in the light may be an artefact of the indirect method used to estimate R_d (Loreto *et al.* 2001). Under field conditions, where estimation of R_d may be impossible because of variations in ambient temperature, or impractical owing to time constraints, measurements of R_n may serve as an effective substitute. In fact, R_n measured under field conditions has been successfully used in place of R_d in the calculation of parameters such as G_m (e.g. Centritto *et al.* 2009; Lauteri *et al.* 2014) and other studies have used 0.5 R_n as a proxy for R_d (e.g. Niinemets *et al.* 2005).

The Kok (1948) and Laisk (1977) methods outlined above rely solely on gas exchange to extrapolate R_d. The majority of commercial gas exchange systems now possess the capacity to perform simultaneous measurement of Chl-Flr parameters alongside gas exchange permitting two estimates of the efficiency of CO_2 uptake via photochemistry. The method of Yin *et al.* (2009, 2011) exploits gas exchange and chlorophyll fluorescence measurements of electron transport for photosynthesis at low irradiances. This method follows the protocol outlined earlier for the Kok (1948) method, but incorporates measurement of a Chl-Flr parameter after a saturating pulse of light. The Kok (1948) method assumes that the transport efficiency of electrons required P_N under light conditions is constant, the Yin *et al.* (2009, 2011) method suggests that this is not the case and that electron transport efficiency declines as PAR increases (Figure 1(b) and 1(c)). Incorporation of the quantum efficiency of photosystem II (this is outlined in more detail in the section 'Instantaneous point measurements of leaf gas exchange and chlorophyll fluorescence', above) with gas exchange parameters produces a R_d value that is generally lower than that found using gas exchange alone in the Kok (1948) method. The Excel spreadsheet of Bellasio *et al.* (2016) can be used to calculate R_d based on the Yin method following the input of P_N, ΦPSII and PAR parameters. One disadvantage of the Yin method is that typical commercial plant photosynthesis systems require the use of a smaller cuvette size when performing simultaneous measurements of gas exchange and Chl-Flr, whereas the Kok (1948) and Laisk (1977) approaches require only gas exchange parameters, and therefore can use larger cuvettes that incorporate a larger area of leaf, giving a more representative analysis of gas exchange and minimising errors associated with the analysis of small areas of leaf.

CO_2 response curves

The response of P_N to increasing [CO_2] provides valuable insights into the biochemistry of photosynthesis (and photorespiration when combined with simultaneous measurement of Chl-Flr) and the diffusive limitations imposed on CO_2-uptake (mainly at the stomata and mesophyll). The response of P_N to [CO_2] is normally expressed in terms of the relationship of P_N to the sub-stomatal concentration of CO_2 (C_i). This measurement is referred to as a P_N-C_i curve (or more commonly as an A/C_i curve, as A is also widely used as an acronym for photosynthetic assimilation of CO_2) and is one of the most common gas exchange analyses undertaken to determine the response of photosynthetic biochemistry to factors such as drought, [CO_2], light intensity, temperature, nutrient status or exposure to pollution. The photosynthesis model of Farquhar *et al.* (1980) is used to calculate the parameters describing the

biochemistry of CO_2 uptake. Figure 3(a) shows a typical P_N-C_i curve of a C3 species (*Olea europaea*).

The C_i level where P_N is zero (i.e. $P_N = R_d + R_{PR}$, see Equation (3)) is known as the CO_2 compensation point (Γ); this differs from the CO_2-photocompensation point (Γ^*) where P_N equals R_{PR}. At 'low' sub-ambient [CO_2], the rate of P_N is limited by the availability of CO_2; in effect, there is insufficient CO_2 to be used by RubisCO and any rise in C_i leads to increased availability of CO_2 for carboxylation. This part of the curve is sometimes referred to as being CO_2 (Wullschleger 1993) or RubisCO (e.g. Long & Bernacchi 2003) limited. As this part of the curve is limited by the availability of substrate, the relationship between P_N and C_i is at its steepest – this slope can be used to calculate the maximum carboxylation rate of RuBP (V_{cmax}). At higher levels of C_i, where the curve begins to level off, the availability of CO_2 as a substrate no longer limits P_N; instead the rate of P_N is limited by the electron transport required for the regeneration of RuBP (J_{max}). The inflection point in the P_N-C_i curve represents the transition from V_{cmax} to J_{max} limited. Many studies regard the value of P_N recorded in this stage of the P_N-C_i curve at saturating levels of light and [CO_2] to be the maximum rate of P_N (P_{Nmax}) (e.g. Heath *et al.* 2005), whereas some studies define P_{Nmax} as the P_N at PAR_{sat} under ambient [CO_2] (e.g. Marshall & Biscoe 1980), highlighting the frequent lack of consistent use of terminology between studies. In the latter part of the P_N-C_i curve it may be possible to observe evidence of phosphate limitations to the regeneration of RuBP in a reduction in P_N (P_N is limited by the availability of phosphate due to high concentrations of triose phosphate sugars). This part of the curve is characterised by the parameter triose phosphate utilisation (TPU) (Sharkey & Vanderveer 1989; Ellsworth *et al.* 2015).

The P_N-C_i response curve is produced by altering the level of atmospheric [CO_2] (C_a) within the leaf cuvette; as long as stomata are sufficiently open, this results in C_i following C_a (a C_i to C_a ratio of 0.7 is normally observed but values within the range of 0.5 to 0.8 can be found in plants grown under favourable conditions). The parameters from a P_N-C_i response curve are reported at a standard temperature of 25 °C; however, it is not necessary to perform the P_N-C_i curves at 25 °C as the effect of leaf temperature on P_N is normally compensated in the Excel spreadsheets and R-statistics templates used to determine V_{cmax}, J_{max} and TPU. Temperature (normally between 20 and 30 °C), VPD and a saturating PAR intensity (derived from the P_N/PAR response of the plant used) should be maintained constant throughout the P_N-C_i curve. To ensure that the plant is fully acclimatised to the conditions within the leaf cuvette, it is standard to initially expose the leaf to ambient [CO_2] for 20–30 minutes before declining in stages to 50 ppm. In some plants with less robust photoprotective mechanisms, it may not be advisable to leave [CO_2] below 50 ppm for a sustained period of time as the cuvette is a high energy environment at PAR_{sat} and at a standard temperature of 20 to 30 °C, and without the substrate for P_N the leaf may incur oxidative damage as

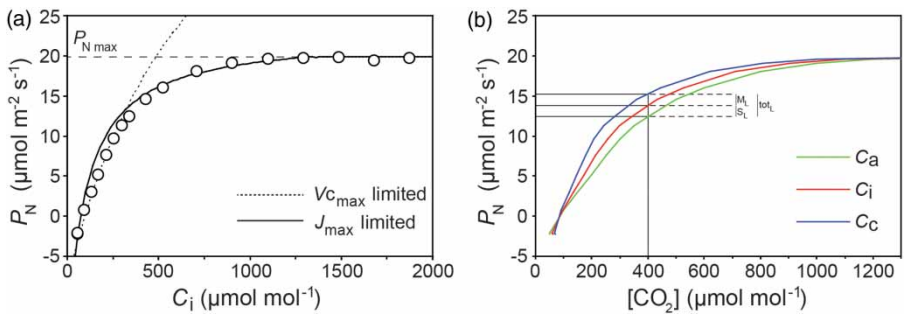

Figure 3 | (a) An example P_N-C_i response curve performed on olive (*Olea europaea*). The dashed line indicates the part of the curve limited by the carboxylation capacity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) (Vc_{max}). The solid line indicates the part of the curve limited by the rate of electron transport required for ribulose-1,5-bisphosphate (RuBP) regeneration (J_{max}). The maximum rate of photosynthesis at PAR_{sat} and high [CO_2] (P_{Nmax}) is marked by a dashed horizontal line. (b) The impact of stomatal (S_L), mesophyll (M_L) and total (tot_L) diffusive limitations to CO_2 transport on P_N at a given [CO_2] (in this case ambient [CO_2] of 400 μmol mol^{-1}) during a P_N-C_i response curve. The [CO_2] in the atmosphere within the cuvette surrounding the leaf (C_a) is represented by the green line, [CO_2] in the sub-stomatal internal leaf air-space (C_i) is indicated by the red line and [CO_2] within the chloroplast (C_c) by the light grey line.

photochemistry is reduced (e.g. Daniel 1997; Durchan *et al.* 2001). At 50 ppm [CO_2] the stomata should open fully and maximum rates of G_s be observed (e.g. Centritto *et al.* 2003; Haworth *et al.* 2013).

After full stomatal opening, levels of [CO_2] should then be increased in steps with close attention to declines in G_s that may limit P_N at higher C_a levels. Gas exchange parameters should be recorded when P_N is relatively stable (±5%). Each step should take 2 to 5 minutes, and the stages at C_a levels above ambient should be fairly rapid. Standard C_a levels used in a P_N-C_i response curve are 400, 350, 250, 150, 50, 100, 200, 300, 400, 600, 800, 1,000, 1,400, 1,800 and 2,000; however, these can be altered to suit the type of plant and/or experimental treatment under investigation. During drought stress, plants close their stomata to reduce transpirative water loss (e.g. Flexas *et al.* 2002; Lauteri *et al.* 2014). If G_s is too low, it is not possible to produce a meaningful P_N-C_i curve as insufficient CO_2 enters the sub-stomatal cavity (Lovelli & Perniola 2014). However, by extending the duration of the exposure to low C_a of 50 ppm for approximately 1 hour to fully open stomata before rapidly increasing C_a (and using fewer above-ambient C_a levels to minimise the impact of stomatal closure) it is often possible to remove the effect of stomatal diffusive limitations on P_N, and therefore assess the true impact of drought stress on the physiological status of photosynthetic CO_2 assimilation (Centritto *et al.* 2003). This approach appears to be effective in instances where V_{cmax} is unaffected by drought stress but J_{max} may be reduced (Aganchich *et al.* 2009; Killi & Haworth 2017). However, in some cases the speed of stomatal closure may be too rapid to permit completion of a full P_N-C_i response curve (Haworth *et al.* 2018b). Moreover, to accurately assess G_m using the curve fitting method, stomata must be open to minimise stomatal limitations to CO_2-uptake. In well-watered plants, with stomata that do not respond to above-ambient increases in [CO_2] (e.g. Haworth *et al.* 2015), it may be possible to take more time between C_a steps and/or add more C_a levels to enhance the resolution of the P_N-C_i curve. Moreover, the number of C_a steps may depend upon the specific factors under investigation; for instance, if the area of study is the efficiency of carboxylation then an increased number of data points within the C_a range of 50 to 300 ppm [CO_2] would be desirable. However, if the aim of the study is to assess possible phosphate nutrient limitations on photosynthetic physiology (e.g. Yang *et al.* 2016), then more data points in the higher C_a range such as 1,600 to 2,000 would be beneficial. The latest generation of plant photosynthesis systems can perform a rapid P_N-C_i response curve by tracking the dynamic response of P_N to [CO_2] (Stinziano *et al.* 2017; Bunce 2018). In theory, this approach could significantly reduce the length of time required to characterise the photosynthetic physiology of plants. However, the effectiveness of this approach in removing diffusive limitations to P_N in plants with low G_s or plants experiencing stress such as drought where diffusive limitations to CO_2-uptake are significant is not yet apparent.

It is possible to assess more accurately the biochemistry of photosynthetic physiology by recalculating the P_N-C_i relationship as the relationship of P_N to the concentration of CO_2 within the chlorophyll envelope (C_c) (the light grey line in Figure 3(b)). To calculate C_c the following formula is used:

$$C_c = C_i - \left(\frac{P_N}{G_m}\right) \tag{8}$$

To do this requires knowledge of the mesophyll conductance to CO_2 (G_m). The most common gas exchange methods to determine G_m are outlined in the section 'Determination of mesophyll conductance to CO′$_2$, below. It is possible to quantify the impact of diffusive limitations to CO_2 (L) on P_N from the P_N-C_i curve. The proportional difference between the P_N at an ambient C_a level of 400 ppm ($P_{N\ ACTUAL}$) and the P_N at a C_i or (C_c) of 400 ppm CO_2 ($P_{N\ HYP}$) where C_i would hypothetically be equal to C_a (Farquhar & Sharkey 1982) can be used to quantify L as:

$$L = \frac{P_{N\,HYP} - P_{N\,ACTUAL}}{P_{N\,HYP}} \tag{9}$$

Since its inception, the photosynthetic model of Farquhar *et al.* (1980) has been elaborated (e.g. Ethier & Livingston 2004; Yin *et al.* 2009). This has allowed the incorporation of the role of finite G_m within the P_N-C_i curve and therefore estimation of G_m from the P_N-C_i response (Figure 3(b)) (Ethier & Livingston 2004). A recent derivation of the model has incorporated the use of synchronous

Chl-Flr and gas exchange to provide further insight into CO_2 assimilation. At each C_a step of the P_N-C_i curve, a saturating pulse of light is applied and the quantum efficiency of PSII (ΦPSII) is determined; however, the P_N-C_i curve should be performed at ambient and low (~1% $[O_2]$ i.e. non-photorespiratory conditions) levels of $[O_2]$ and at different light levels (Yin *et al.* 2009). Excel (Sharkey *et al.* 2007; Bellasio *et al.* 2016) and R-Statistics (Duursma 2015) templates are available to calculate parameters from P_N-C_i curves using gas exchange or combined gas exchange and Chl-Flr data.

One of the most common sources of error associated with gas exchange measurements of P_N is caused by leaks and/or diffusion of gases through the gaskets. The gaskets are usually made from neoprene rubber material, and are used to seal the upper and lower surfaces of the leaf within the cuvette. If the gaskets are not changed regularly, or the leaf cuvette is stored in the closed position, the gasket material may become compressed and no longer seal the leaf effectively. During measurements that take a long time, the gaskets may retain an impression of the leaf within their surface (sometimes referred to as a 'memory'); this may lead to leaks during successive measurements as the gaskets will not seal subsequent leaves to the same degree. Leaks and diffusion of gases through the gaskets are a particular issue for the P_N-C_i response curve as the concentration gradient of gases within and outside the cuvette are more pronounced. One method to counter this is to complete the P_N-C_i response curve and then quantify the leaks by killing the leaf (usually by heating it for a short period within a microwave) and then returning the leaf to exactly the same position within the cuvette and repeating the C_a steps used in the P_N-C_i curve (Flexas *et al.* 2007a). Correcting for the effect of leaks should increase P_N at sub-ambient C_a and reduce P_N at super-ambient C_a. We recommend an approach whereby the diffusion gradient between the air within and outside the cuvette is minimised by feeding the exhaust air (that should more closely match the air within the leaf cuvette than the ambient atmosphere) from the plant photosynthesis system into a supplementary gasket surrounding the primary gasket or a bag placed over the cuvette head (although depending upon the time to replenish the air within the bag, this may increase the duration of each C_a step in the P_N-C_i curve) (Rodeghiero *et al.* 2007).

Determination of mesophyll conductance to CO_2

The movement of CO_2 from the external atmosphere to the site of carboxylation within the chloroplasts experiences two main resistances, firstly at the stomata and then from the intercellular air-space through the mesophyll (Loreto *et al.* 1992; Flexas *et al.* 2008; Centritto *et al.* 2011a, 2011b). Mesophyll conductance has become an increasingly important parameter in the identification and development of more productive (i.e. higher rates of CO_2 movement) and stress resistant plants (e.g. Hanba *et al.* 2004; Adachi *et al.* 2013; Sorrentino *et al.* 2016). An area of particular focus is the G_s to G_m ratio. In theory, because P_N and G_m/G_s have a typical hyperbolic relationship, plants with a lower G_s:G_m ratio would exhibit enhanced rates of carbon gain relative to water-loss and improved growth under limited water availability (Flexas *et al.* 2013). Stomatal conductance to water vapour can be determined by measurement of the diffusion of water vapour from the internal leaf air-space to the external atmosphere, as outlined in Equation (1). Mesophyll conductance to CO_2 (G_m) cannot be measured directly, but is instead approximated from calculation of $[CO_2]$ in the internal sub-stomatal leaf air-space (C_i) and inside the chloroplast envelope (C_c) (a re-working of Equation (8)):

$$G_m = \frac{P_N}{C_i - C_c} \tag{10}$$

A number of methodologies have been developed to quantify G_m:

i. simultaneous measurement of gas exchange and Chl-Flr parameters (the constant and variable J approaches: Bongi & Loreto 1989; Harley *et al.* 1992);
ii. curve fitting of the P_N-C_i response curve (Ethier & Livingston 2004);
iii. the response of P_N to $[O_2]$ (Bunce 2009, 2018); and
iv. analysis of leaf gas exchange and discrimination between stable ^{12}C and ^{13}C isotopes (Evans *et al.* 1986; Lloyd *et al.* 1992) in the gas stream passing over a leaf (Vrábl *et al.* 2009; Douthe *et al.* 2012), recently synthesised sugars (Lauteri *et al.* 1997, 2014; Centritto *et al.* 2009) or leaf structural tissue (Lauteri *et al.* 1997).

All of these methodologies involve specific weaknesses and/or assumptions. It is therefore preferable to utilise two

methodologies when determining G_m under experimental conditions (Pons *et al.* 2009). Nevertheless, it is necessary to note that all of the current methods employed to quantify G_m require leaf gas exchange parameters, and so are not truly independent of one another (e.g. Lauteri *et al.* 2014). These ambiguities associated with the determination of G_m may have contributed to observations that G_m alters rapidly to conditions such as [CO_2] (Flexas *et al.* 2007b) or light (Douthe *et al.* 2012), or being reported as a fixed constant (Ethier & Livingston 2004). However, G_m can be conceptualised as a 'flux-weighted quantity' (Tholen *et al.* 2012), determined by the interaction of CO_2 assimilation by P_N, availability of CO_2 within the internal sub-stomatal airspace (Sorrentino *et al.* 2016) and the biochemistry (Hanba *et al.* 2004; Flexas *et al.* 2006) and/or physical structure (Adachi *et al.* 2013) of the mesophyll layer.

In this introduction to gas exchange, we shall restrict our discussion to the techniques utilised for the measurement of G_m that do not require additional equipment (e.g. online isotope ratio mass spectrometers) and can be performed with a stand-alone plant photosynthesis system. More detailed review of techniques for the measurement of G_m can be found in Flexas *et al.* (2008) and Pons *et al.* (2009). The curve fitting approach of Ethier & Livingston (2004) allows the calculation of G_m from the P_N-C_i response curve by assuming that G_m is not infinite (see Equation (9)). Mesophyll conductance can be calculated from the P_N-C_i response curve using the Excel templates and R-scripts listed in the section 'CO_2 response curves', above.

The variable J approach is the most widely applied methodology to estimate G_m (Pons *et al.* 2009). The variable J approach utilises simultaneous measurement of leaf gas exchange and Chl-Flr parameters to calculate C_c and then determine G_m as (Harley *et al.* 1992):

$$G_m = \frac{P_N}{C_i - ((\Gamma*[J_F + 8*(P_N + R_d)])/(J_F - 4*(P_N + R_d)))} \quad (11)$$

Quantification of the parameters R_d and Γ^* are outlined in the section entitled 'Respiration in the light and in the dark', above. The theoretical basis for the use of Chl-Flr to estimate C_c alongside gas exchange is the linear relationship between ΦPSII from Chl-Flr and the quantum efficiency of CO_2 uptake (Φ_{CO2}) measured using gas exchange under conditions of low [O_2] and high [CO_2] conducive to the suppression of R_{PR} (Genty *et al.* 1989). The electron transport rate determined from Chl-Flr (J_F) is:

$$J_F = PPFD*\Phi PSII*\alpha*\beta \quad (12)$$

where β is the partitioning of light energy between photosystems I and II which is frequently assumed to be 0.5 and constant across species and/or treatments (Krall & Edwards 1992). A standard value of 0.85 is commonly used for the leaf absorbance (α), but this can be measured directly using an integrating sphere (e.g. Olascoaga *et al.* 2016). The actual quantum efficiency of photosystem II in the light adapted state (ΦPSII) (Genty *et al.* 1989) is:

$$\Phi PSII = \frac{F_m' - F_s}{F_m'} \quad (13)$$

where F_m' is the maximal fluorescence following a saturating pulse of light and F_s is the steady state fluorescence under light adapted conditions. In many plant photosynthesis systems with combined leaf gas exchange and Chl-Flr capabilities, measurement of the 'true' value of F_m' is not possible as the intensity of the saturating pulse of light is insufficient. This effect is particularly evident in fast growing species with high photosynthetic capacities. Loriaux *et al.* (2013) developed a technique whereby a series of light pulses of varying intensities allows extrapolation of the likely true F_m'. The operating systems of most plant photosynthesis systems now include the capacity to perform such a 'multi-phase flash', and we recommend its use in preference to a single saturating pulse of light when measuring ΦPSII to calculate G_m. The electron transport rate associated with carboxylation/oxygenation of RubisCO can also be determined from gas exchange (J_A) as outlined in Equation (4) (Harley *et al.* 1992; Loreto *et al.* 1994). However, the variable J approach is sensitive to the impact of alternative electron sinks associated with photorespiration and mitochondrial respiration (Harley *et al.* 1992; Gilbert *et al.* 2012) as the electron transport rate determined by Chl-Flr (J_F) incorporates these alternative electron sinks (Laisk & Loreto 1996):

$$J_F = J_A + J_o \quad (14)$$

where J_o represents alternative electron sinks. The effect of alternative electron sinks can be assessed by analysis of J_F under ambient and non-photorespiratory conditions (i.e. ~1.0% [O_2] and high [CO_2]) (Genty *et al.* 1989; Di Marco *et al.* 1990; Harley *et al.* 1992).

The overall impact of diffusive limitations on P_N can be represented by calculation of the total conductance to CO_2 (G_{tot}) (e.g. Lauteri *et al.* 2014):

$$G_{tot\,CO2} = \frac{G_{s\,CO2} * G_{m\,CO2}}{G_{s\,CO2} + G_{m\,CO2}} \quad (15)$$

Stomatal conductance to CO_2 ($G_{s\ CO2}$) is a standard data output of most commercial plant photosynthesis systems. Nevertheless, $G_{s\ CO2}$ can be calculated from the stomatal conductance to water vapour ($G_{s\ H2O}$) outlined in Equation (2). This approach is particularly important in the characterisation of environmental stresses such as drought where diffusive limitations to CO_2-uptake are a major component in the plant response (an example is provided in Figure 4).

Knowledge of C_c (see Equation (8)) values derived from G_m allows the calculation of R_{PR} as (for a review of the limitations of gas exchange analysis of photorespiration see Sharkey 1988):

$$R_{PR} = \frac{P_N + R_d}{(C_c/\Gamma*) - 1} \quad (16)$$

Moreover, this then allows the determination of the oxygenation rate (V_o) of RubisCO involved in photochemistry (Von Caemmerer 2000):

$$V_o = \frac{P_N + R_d}{(C_c/(2*\Gamma*)) - 0.5} \quad (17)$$

The rate of carboxylation can then be calculated as (Von Caemmerer 2000):

$$V_c = \frac{V_o}{(2*\Gamma*)/C_c} \quad (18)$$

Stomatal kinetics

Stomata are the interface between the external atmosphere and the internal leaf environment. By regulating the uptake of CO_2 for photosynthesis against the loss of water vapour as transpiration, stomata play a key role in maintaining leaf homeostasis (e.g. Haworth *et al.* 2010, 2011; Centritto *et al.* 2011a, 2011b). Changes in the turgor of the two guard cells determine the aperture of the stomatal pore. The importance of stomata in the adaptation of plants to their environment is well established (e.g. Heath 1950; Mansfield & Majernik 1970; Schulze *et al.* 1975; Woodward 1987). The examination of stomatal kinetics has become increasingly important in understanding plant responses to environmental change (Kardiman & Ræbild 2018; Gerardin *et al.* 2018; Haworth *et al.* 2018b), plant evolutionary history (McAdam & Brodribb 2012; Haworth *et al.* 2013; Doi *et al.* 2015) and the interaction of physiological control of guard cell turgor and stomatal morphology in regulating leaf gas exchange (Haworth *et al.* 2015, 2018c). Stomatal kinetics

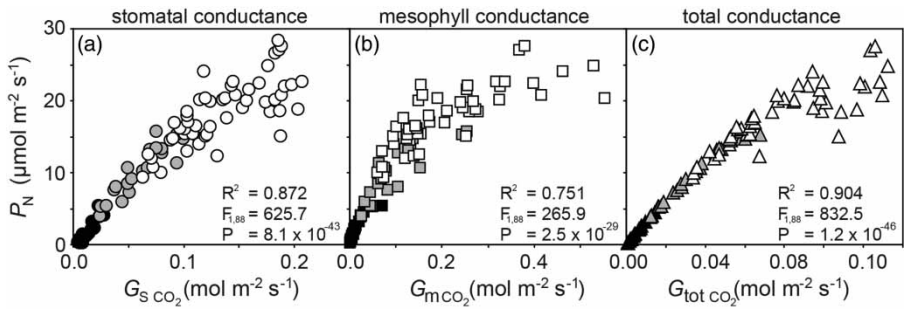

Figure 4 | (a) The relationship between photosynthesis (P_N) and stomatal conductance to CO_2 ($G_{s\ CO2}$; circle symbols); (b) mesophyll conductance to CO_2 ($G_{m\ CO2}$; square symbols); and (c) total conductance to CO_2 ($G_{tot\ CO2}$; triangle symbols) of *Populus nigra* grown under full irrigation (white fill symbols), moderate drought stress (grey fill symbols) and strong drought stress (black fill symbols). Linear regression was used to calculate the significance of these relationships.

involves analysis of the dynamic response of G_s to a change in environmental conditions. In effect, the alteration of G_s serves as a proxy for the opening/closing of the stomatal pore. When measuring stomatal kinetics, it is important to maintain constant conditions within the leaf cuvette with the exception of the specific factor under consideration.

To assess stomatal opening it is standard to keep the plant in the dark for at least 12 hours prior to measurement, and perform the stomatal kinetic response at a time when the illumination of the plant normally begins (i.e. dawn for plants grown outdoors or coinciding with the time at which lights are switched on for plants grown under artificial illumination). The wavelength of light used in a stomatal opening kinetic response is critical, as blue initiates the pumping of ions across the guard cell membrane, while P_N in the mesophyll associated with red light plays a role in maintaining stomatal opening (Mansfield & Meidner 1966; Zeiger & Hepler 1977; Sharkey & Raschke 1981; Doi *et al.* 2015). The latest plant photosynthesis systems contain green and white LEDs in addition to the standard red-blue. Green light has been shown to play an important role in driving efficient P_N (Terashima *et al.* 2009) and is strongly absorbed in many green plants (Sakowska *et al.* 2018). A more balanced spectrum of light may strongly influence G_s during stomatal opening (Mansfield & Meidner 1966; Doi *et al.* 2006, 2015) (a similar effect may be observed in mesophyll conductance: Loreto *et al.* 2009; Pallozzi *et al.* 2013), and should be utilised where possible.

The reduction in G_s associated with stomatal closure following a complete cessation of illumination (i.e. a light to dark transition) or reduction in the intensity of PAR has been shown to be more effective than the stomatal opening kinetic in differentiating evolutionary plant groups (McAdam & Brodribb 2012; Elliott-Kingston *et al.* 2016; Xiong *et al.* 2018) or plants grown under different water availability (Haworth *et al.* 2018b). This stomatal closure kinetic approach also has the benefit of not being so closely constrained to a particular time of day to be most effective (e.g. Haworth *et al.* 2015) as found when characterising stomatal opening. The response of stomata to fluctuations in the light environment and the optimisation of P_N to rapidly changing conditions is a major factor in improving the carbon and water efficiencies of crop plants. Such stomatal kinetic measurements to varying light quantities/qualities are becoming increasingly important to the development of more productive crop varieties (Lawson & Blatt 2014).

The kinetics of the G_s response to $[CO_2]$ (e.g. Heath & Meidner 1957; Haworth *et al.* 2013), separation of the leaf petiole from the supporting stem tissue as a proxy for desiccation (e.g. Brodribb & McAdam 2011) or leaf to air VPD (Brodribb & McAdam 2011; Haworth *et al.* 2018b) can also be measured. The response of stomata to a change in leaf to air VPD and increase in $[CO_2]$ above ambient levels requires the presence of light (Shimazaki *et al.* 2007). However, the response of stomata to a decrease in $[CO_2]$ below ambient levels occurs in both darkness and light (Mansfield *et al.* 1981). When measuring the kinetics of G_s to a change in conditions the 'timed-recording' or 'auto-log' function of the gas exchange system should be used. Stomatal conductance should be stable for at least 20–30 minutes prior to any change in conditions within the cuvette. When altering light intensity and/or $[CO_2]$ within the cuvette it is necessary to pay close attention to the effect of any change in G_s on leaf to air VPD. Any variation in leaf to air VPD should be minimised by adjusting the RH of the reference air-stream entering the leaf cuvette. These G_s kinetic measurements are often lengthy, and therefore consideration should be given to the performance of the two pairs of IRGAs within the plant photosynthesis system. If the time interval between measurements is sufficient, it may be possible to utilise each plant photosynthesis system's protocols to minimise drift between and/or zero the two IRGAs. However, if the frequency of measurements precludes this, it is necessary to match and/zero the IRGAs at the start and end of the stomatal kinetic response and then correct for the average drift between the reference and sample IRGAs over the duration of the measurement (e.g. Haworth *et al.* 2018b). Example G_s kinetics for $[CO_2]$, light intensity and leaf to air VPD are shown in Figure 5.

Application to plant phenotyping and precision irrigation

Plant phenotyping, or phenomics, is the study of morphological, biochemical and physiological characteristics of a plant under specific environmental growth conditions. A plant's phenotype is determined by the interaction of its genotype with the environment. The converging pressures

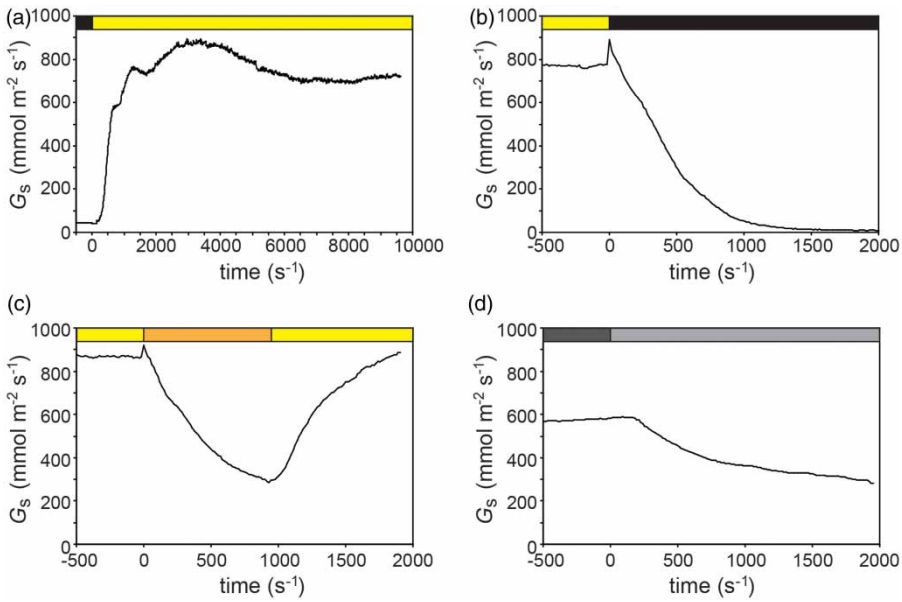

Figure 5 | Stomatal kinetic responses of the fast growing grass species *Arundo donax* to: (a) a transition from dark to light inducing stomatal opening; (b) a transition from light to dark inducing stomatal closure; (c) a reduction in the intensity of PAR followed by a return to full illumination; and (d) an increase in [CO_2] from 50 to 2,000 μmol mol^{-1}. The initial change in conditions within the leaf cuvette occurs at time 0 and is marked by colour changes in the horizontal bands running along the top of each graph.

of climate change, loss of productive land and population growth necessitate greater production of food and biofuel crops using fewer resources through precision agriculture techniques. However, the identification of more productive and stress resistant phenotypes has been constrained by the so-called 'phenotyping bottleneck' (Furbank & Tester 2011). Leaf gas exchange can provide direct measurement of photosynthetic CO_2-uptake and water-loss in addition to detailed characterisation of the underlying photosynthetic physiology and stomatal behaviour. This information is highly valuable for the identification of crop varieties with desirable characteristics such as tolerance to drought (e.g. Centritto *et al.* 2009; Haworth *et al.* 2017a), and also in the identification of climate-resilient plants with high agro-ecological potential to make improved use of areas affected by erratic rainfall, drought and other associated environmental stresses (Chakhchar *et al.* 2017; Zegada-Lizarazu *et al.* 2018).

Despite the detailed information that leaf gas exchange and Chl-Flr analysis can provide regarding photosynthetic (e.g. Loreto & Centritto 2008; Centritto *et al.* 2009; Lauteri *et al.* 2014) and stomatal physiology (e.g. Killi *et al.* 2016), the techniques outlined above are time-consuming and constitute a significant component of the phenotyping bottleneck. Nevertheless, given the potential application of gas exchange to understanding the CO_2 and water fluxes of plants, efforts to improve the capacity for gas exchange analysis in high throughput phenotyping are being undertaken (Bellasio *et al.* 2014). The use of leaf gas exchange analysis of a sub-set of plants alongside high throughput screening techniques such as spectroradiometry of a wider range of crop varieties may be effective in supplying both detailed physiological information and rapid high frequency analysis of plant status (e.g. Munns *et al.* 2010). Spectroradiometry indices such as the photochemical reflectance index (PRI) which measures the epoxidation state of xanthophylls (Gamon *et al.* 1992) have been shown to track photosynthetic parameters (e.g. Marino *et al.* 2014; Sun *et al.* 2014). The correlation of leaf gas exchange parameters such as P_N or G_s with remote sensing may be effective in alleviating the bottleneck while adding value and validation to high throughput imaging techniques (Figure 6).

Gas exchange is one of the most effective methods to detect the early impact of water stress on plants; for example, a reduction in G_s may occur prior to any change in leaf water status (Zhang & Davies 1990; Tardieu & Simonneau 1998). However, due to the complexities associated with gas exchange analysis it is not a viable methodology to be utilised in long-term monitoring of plant water status to schedule

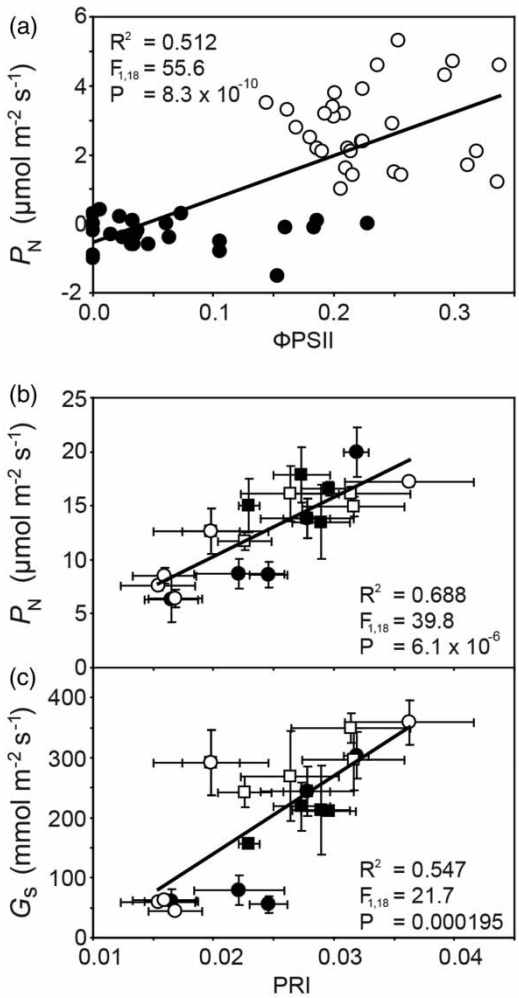

Figure 6 | Example correlations of leaf gas exchange with high throughput screening parameters: (a) the quantum efficiency of photosynthesis in the light (ΦPSII – see Equation (7)) and photosynthesis (P_N) in *Ginkgo biloba* grown at 25 (white fill symbols) and 35 °C (black fill symbols) (Haworth *et al.* 2018a); and the relationships between the photochemical reflectance index (PRI) and (b) P_N and (c) stomatal conductance (G_s) in olive (*Olea europaea*) grown with supplementary nitrogen fertilisation (black fill symbols) or 50% of adequate nitrogen fertilisation (white fill symbols) under full irrigation (square symbols) or water deficit (circle symbols) conditions. The PRI is an index of reflectance at certain wavelengths of light ($PRI = [R_{531}\text{-}R_{570}]/[R_{531} + R_{570}]$). Error bars indicate one standard error either side of the mean. Linear regression was used to determine the significance of these relationships.

irrigation. Nonetheless, in conjunction with wide-scale imaging, particularly canopy-scale reflectance and/or infra-red thermography, leaf gas exchange can be useful in developing calibration datasets (see Figure 6(c)) to enable effective irrigation scheduling to maximise the water productivity of a given crop (Jones 2004; Cifre *et al.* 2005; Patakas *et al.* 2005; Baker *et al.* 2007; Marino *et al.* 2014; Gago *et al.* 2015). Moreover, the detailed physiological information available from leaf gas exchange analysis can be vital in identifying the appropriate irrigation technique for a given crop. For example, partial root-zone drying is a common approach based on laboratory split-root studies where half of the root-zone receives full irrigation while the remainder is allowed to dry, thus generating a root-to-shoot signal from the dry half of the root-zone that promotes stomatal closure and enhances WUE (Davies *et al.* 2002; Romero *et al.* 2010). However, gas exchange analysis of a Tunisian olive variety indicated that root-zone drying was not suitable to its underlying stomatal physiology and reduced the water productivity of that specific variety (Dbara *et al.* 2016). Gas exchange analysis has also shown that improved P_N through irrigation is only beneficial to crop yield during the specific period of fruit development. Supplemental irrigation during episodes of vegetative growth or 'off-years' is unlikely to enhance water productivity (Sun *et al.* 2014; Dbara *et al.* 2016). Exploitation of gas exchange analysis of photosynthetic and stomatal physiology can therefore enable the identification of the most efficient precision agriculture approaches to be utilised in the context of a given crop or the prevailing environmental conditions.

CONCLUSION

Leaf gas exchange and Chl-Flr have dramatically expanded our understanding of plant photosynthetic physiology and stomatal regulation. This is critical to the assessment of the carbon and water balance of plants and their environmental adaptation. However, these approaches are sensitive to errors and require expertise and care when conducting these measurements and interpreting the resulting data. We intend the above review to act as an introductory guide to these techniques and not to serve as an exhaustive assessment. We hope that this guide can offer a foundational starting point for researchers interested in simultaneous leaf gas exchange and Chl-Flr analysis towards plant phenotyping and optimisation of plant water use.

ACKNOWLEDGEMENTS

We are grateful for funding from the Agreement of Scientific Cooperation 2017–2019 between the National Research

Council of Italy and Chinese Academy of Sciences, RGV-FAO Programma triennale 2017–2019 – Ministero delle Politiche Agricole e Forestali, and Regione Toscana PIF 'Ottimizzazione ed Innovazione della Filiera Olivicola ed Accrescimento della Qualità dell'Olio Toscano' Misura 16.2 Progetto 'SEMIA'.

REFERENCES

Adachi, S., Nakae, T., Uchida, M., Soda, K., Takai, T., Oi, T., Yamamoto, T., Ookawa, T., Miyake, H., Yano, M. & Hirasawa, T. 2013 The mesophyll anatomy enhancing CO_2 diffusion is a key trait for improving rice photosynthesis. *Journal of Experimental Botany* **64**, 1061–1072.

Aganchich, B., Wahbi, S., Loreto, F. & Centritto, M. 2009 Partial root zone drying: regulation of photosynthetic limitations and antioxidant enzymatic activities in young olive (*Olea europaea*) saplings. *Tree Physiology* **29**, 685–696.

Atkin, O. K., Evans, J. R. & Siebke, K. 1998 Relationship between the inhibition of leaf respiration by light and enhancement of leaf dark respiration following light treatment. *Functional Plant Biology* **25**, 437–443.

Baker, J. T., Gitz, D., Payton, P., Wanjura, D. & Upchurch, D. 2007 Using leaf gas exchange to quantify drought in cotton irrigated based on canopy temperature measurements. *Agronomy Journal* **99**, 637–644.

Bellasio, C., Burgess, S. J., Griffiths, H. & Hibberd, J. M. 2014 A high throughput gas exchange screen for determining rates of photorespiration or regulation of C4 activity. *Journal of Experimental Botany* **65**, 3769–3779.

Bellasio, C., Beerling, D. J. & Griffiths, H. 2016 An Excel tool for deriving key photosynthetic parameters from combined gas exchange and chlorophyll fluorescence: theory and practice. *Plant Cell and Environment* **39**, 1180–1197.

Björkman, O. & Demmig, B. 1987 Photon yield of O2 evolution and chlorophyll fluorescence characteristics at 77 K among vascular plants of diverse origins. *Planta* **170**(4), 489–504.

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

Bowes, G., Ogren, W. & Hageman, R. 1972 Light Saturation, photosynthesis rate, RuDP carboxylase activity, and specific leaf weight in soybeans grown under different light intensities 1. *Crop Science* **12**, 77–79.

Brodribb, T. J. & McAdam, S. A. M. 2011 Passive origins of stomatal control in vascular plants. *Science* **331**, 582–585.

Bunce, J. A. 2009 Use of the response of photosynthesis to oxygen to estimate mesophyll conductance to carbon dioxide in water-stressed soybean leaves. *Plant Cell and Environment* **32**, 875–881.

Bunce, J. 2018 Three methods of estimating mesophyll conductance agree regarding its CO_2 sensitivity in the RubisCO-limited C_i range. *Plants* **7**, 62.

Butler, W. L. & Kitajima, M. 1975 Fluorescence quenching in Photosystem II of chloroplasts. *Biochimica et Biophysica Acta (BBA) – Bioenergetics* **376**, 116–125.

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 saplings. *Plant Cell and Environment* **26**, 585–594.

Centritto, M., Lauteri, M., Monteverdi, M. C. & Serraj, R. 2009 Leaf gas exchange, carbon isotope discrimination, and grain yield in contrasting rice genotypes subjected to water deficits during the reproductive stage. *Journal of Experimental Botany* **60**, 2325–2339.

Centritto, M., Brilli, F., Fodale, R. & Loreto, F. 2011a Different sensitivity of isoprene emission, respiration, and photosynthesis to high growth temperature coupled with drought stress in black poplar (*Populus nigra*). *Tree Physiology* **31**, 275–286.

Centritto, M., Tognetti, R., Leitgeb, E., Střelcová, K. & Cohen, S. 2011b Above ground processes – anticipating climate change influences. In: *Forest Management and the Water Cycle: An Ecosystem-Based Approach* (M. Bredemeier, S. Cohen, D. L. Godbold, E. Lode, V. Pichler & P. Schleppi, eds). Springer, London, pp. 31–64.

Chakhchar, A., Haworth, M., El Modafar, C., Lauteri, M., Mattioni, C., Wahbi, S. & Centritto, M. 2017 An assessment of genetic diversity and drought tolerance in argan tree (*Argania spinosa*) populations: potential for the development of improved drought tolerance. *Frontiers in Plant Science* **8**, 276.

Cifre, J., Bota, J., Escalona, J. M., Medrano, H. & Flexas, J. 2005 Physiological tools for irrigation scheduling in grapevine (*Vitis vinifera* L.): an open gate to improve water-use efficiency? *Agriculture, Ecosystems and Environment* **106**, 159–170.

Crous, K. Y., Zaragoza-Castells, J., Ellsworth, D. S., Duursma, R. A., Löw, M., Tissue, D. T. & Atkin, O. K. 2012 Light inhibition of leaf respiration in field-grown *Eucalyptus saligna* in whole-tree chambers under elevated atmospheric CO_2 and summer drought. *Plant Cell and Environment* **35**, 966–981.

Daniel, E. 1997 The temperature dependence of photoinhibition in leaves of *Phaseolus vulgaris* (L.) Influence of CO_2 and O_2 concentrations. *Plant Science* **124**, 1–8.

Davies, W. J., Wilkinson, S. & Loveys, B. 2002 Stomatal control by chemical signalling and the exploitation of this mechanism to increase water use efficiency in agriculture. *New Phytologist* **153**, 449–460.

Dbara, S., Haworth, M., Emiliani, G., Mimoun, M. B., Gómez-Cadenas, A. & Centritto, M. 2016 Partial root-zone drying of olive (*Olea europaea* var. 'Chetoui') induces reduced yield under field conditions. *PLoS One* **11**, e0157089.

Demmig-Adams, B., Winter, K., Krüger, A. & Czygan, F.-C. 1989 Light response of CO_2 assimilation, dissipation of excess excitation energy, and zeaxanthin content of sun and shade leaves. *Plant Physiology* **90**, 881–886.

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.

Doi, M., Wada, M. & Shimazaki, K.-i. 2006 The fern *Adiantum capillus-veneris* lacks stomatal responses to blue light. *Plant and Cell Physiology* **47**, 748–755.

Doi, M., Kitagawa, Y. & Shimazaki, K.-i. 2015 Stomatal blue light response is present in early vascular plants. *Plant Physiology* **169**, 1205–1213.

Douthe, C., Dreyer, E., Brendel, O. & Warren, C. R. 2012 Is mesophyll conductance to CO_2 in leaves of three *Eucalyptus* species sensitive to short-term changes of irradiance under ambient as well as low O_2? *Functional Plant Biology* **39**, 435–448.

Durchan, M., Vacha, F. & Krieger-Liszkay, A. 2001 Effects of severe CO_2 starvation on the photosynthetic electron transport chain in tobacco plants. *Photosynthesis Research* **68**, 203–213.

Duursma, R. A. 2015 Plantecophys – an R package for analysing and modelling leaf gas exchange data. *PLoS One* **10**, e0143346.

Elliott-Kingston, C., Haworth, M., Yearsley, J. M., Batke, S. P., Lawson, T. & McElwain, J. C. 2016 Does size matter? Atmospheric CO_2 may be a stronger driver of stomatal closing rate than stomatal size in taxa that diversified under low CO_2. *Frontiers in Plant Science* **7**, 1253.

Ellsworth, D. S., Crous, K. Y., Lambers, H. & Cooke, J. 2015 Phosphorus recycling in photorespiration maintains high photosynthetic capacity in woody species. *Plant Cell and Environment* **38**, 1142–1156.

Ethier, G. J. & Livingston, N. J. 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.

Evans, J. R., Sharkey, T. D., Berry, J. A. & Farquhar, G. D. 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, J. R., Jakobsen, I. & Ogren, E. 1993 Photosynthetic light-response curves 0.2. Gradients of light-absorption and photosynthetic capacity. *Planta* **189**, 191–200.

Farquhar, G. D. & Sharkey, T. D. 1982 Stomatal conductance and photosynthesis. *Annual Review of Plant Physiology* **33**, 317–345.

Farquhar, G. D., Caemmerer, S. & Berry, J. A. 1980 A biochemical model of photosynthetic CO_2 assimilation in leaves of C3 species. *Planta* **149**, 78–90.

Farquhar, G., Hubick, K., Condon, A. & Richards, R. 1989 Carbon isotope fractionation and plant water-use efficiency. In: *Stable Isotopes in Ecological Research* (P. W. Rundel, J. R. Ehleringer & K. A. Nagy, eds). Ecological Studies (Analysis and Synthesis), vol. 68. Springer, New York, NY, pp. 21–40.

Flexas, J., Bota, J., Escalona, J. M., 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., Ribas-Carbo, M., Hanson, D. T., Bota, J., Otto, B., Cifre, J., McDowell, N., Medrano, H. & Kaldenhoff, R. 2006 Tobacco aquaporin NtAQP1 is involved in mesophyll conductance to CO_2 in vivo. *Plant Journal* **48**, 427–439.

Flexas, J., Díaz-Espejo, A., Berry, J., Cifre, J., Galmés, J., Kaldenhoff, R., Medrano, H. & Ribas-Carbó, M. 2007a Analysis of leakage in IRGA's leaf chambers of open gas exchange systems: quantification and its effects in photosynthesis parameterization. *Journal of Experimental Botany* **58**, 1533–1543.

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

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

Flexas, J., Niinemets, Ü., Gallé, A., Barbour, M., Centritto, M., Diaz-Espejo, A., Douthe, C., Galmés, J., Ribas-Carbo, M., Rodriguez, P., Rosselló, F., Soolanayakanahally, R., Tomas, M., Wright, I., Farquhar, G. & Medrano, H. 2013 Diffusional conductances to CO_2 as a target for increasing photosynthesis and photosynthetic water-use efficiency. *Photosynthesis Research* **117**, 45–59.

Furbank, R. T. & Tester, M. 2011 Phenomics–technologies to relieve the phenotyping bottleneck. *Trends in Plant Science* **16**, 635–644.

Gago, J., Douthe, C., Coopman, R. E., Gallego, P. P., Ribas-Carbo, M., Flexas, J., Escalona, J. & Medrano, H. 2015 UAVs challenge to assess water stress for sustainable agriculture. *Agricultural Water Management* **153**, 9–19.

Gamon, J., Penuelas, J. & Field, C. 1992 A narrow-waveband spectral index that tracks diurnal changes in photosynthetic efficiency. *Remote Sensing of Environment* **41**, 35–44.

Genty, B., Briantais, J.-M. & Baker, N. R. 1989 The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta (BBA) – General Subjects* **990**, 87–92.

Gerardin, T., Douthe, C., Flexas, J. & Brendel, O. 2018 Shade and drought growth conditions strongly impact dynamic responses of stomata to variations in irradiance in *Nicotiana tabacum*. *Environmental and Experimental Botany* **153**, 188–197.

Gilbert, M. E., Pou, A., Zwieniecki, M. A. & Holbrook, N. M. 2012 On measuring the response of mesophyll conductance to carbon dioxide with the variable J method. *Journal of Experimental Botany* **63**, 413–425.

Hanba, Y. T., 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 and Cell Physiology* **45**, 521–529.

Harley, P. C., Loreto, F., Dimarco, G. & Sharkey, T. D. 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.

Haworth, M., Heath, J. & McElwain, J. C. 2010 Differences in the response sensitivity of stomatal index to atmospheric CO_2 among four genera of Cupressaceae conifers. *Annals of Botany* **105**, 411–418.

Haworth, M., Fitzgerald, A. & McElwain, J. C. 2011 Cycads show no stomatal-density and index response to elevated carbon dioxide and subambient oxygen. *Australian Journal of Botany* **59**, 629–638.

Haworth, M., Elliott-Kingston, C. & McElwain, J. 2013 Co-ordination of physiological and morphological responses of stomata to elevated [CO_2] in vascular plants. *Oecologia* **171**, 71–82.

Haworth, M., Killi, D., Materassi, A. & Raschi, A. 2015 Co-ordination of stomatal physiological behavior and morphology with carbon dioxide determines stomatal control. *American Journal of Botany* **102**, 677–688.

Haworth, M., Catola, S., Marino, G., Brunetti, C., Michelozzi, M., Riggi, E., Avola, G., Cosentino, S. L., Loreto, F. & Centritto, M. 2017a Moderate drought stress induces increased foliar dimethylsulphoniopropionate (DMSP) concentration and isoprene emission in two contrasting ecotypes of *Arundo donax*. *Frontiers in Plant Science* **8**, 1016.

Haworth, M., Centritto, M., Giovannelli, A., Marino, G., Proietti, N., Capitani, D., De Carlo, A. & Loreto, F. 2017b Xylem morphology determines the drought response of two *Arundo donax* ecotypes from contrasting habitats. *GCB Bioenergy* **9**, 119–131.

Haworth, M., Cosentino, S. L., Marino, G., Brunetti, C., Scordia, D., Testa, G., Riggi, E., Avola, G., Loreto, F. & Centritto, M. 2017c Physiological responses of *Arundo donax* ecotypes to drought: a common garden study. *GCB Bioenergy* **9**, 132–143.

Haworth, M., Belcher, C. M., Killi, D., Dewhirst, R. A., Materassi, A., Raschi, A. & Centritto, M. 2018a Impaired photosynthesis and increased leaf construction costs may induce floral stress during episodes of global warming over macroevolutionary timescales. *Scientific Reports* **8**, 6206.

Haworth, M., Cosentino, S. L., Marino, G., Brunetti, C., Riggi, E., Avola, G., Loreto, F. & Centritto, M. 2018b Increased free abscisic acid during drought enhances stomatal sensitivity and modifies stomatal behaviour in fast growing giant reed (*Arundo donax* L.). *Environmental and Experimental Botany* **147**, 116–124.

Haworth, M., Moser, G., Raschi, A., Kammann, C., Grünhage, L. & Müller, C. 2016 Carbon dioxide fertilisation and supressed respiration induce enhanced spring biomass production in a mixed species temperate meadow exposed to moderate carbon dioxide enrichment. *Functional Plant Biology* **43**, 26–39.

Haworth, M., Scutt, C. P., Douthe, C., Marino, G., Gomes, M. T. G., Loreto, F., Flexas, J. & Centritto, M. 2018c Allocation of the epidermis to stomata relates to stomatal physiological control: stomatal factors involved in the diversification of the angiosperms and development of amphistomaty. *Environmental and Experimental Botany* **151**, 55–63.

Heath, O. V. S. 1950 Studies in stomatal behaviour. V. The role of carbon dioxide in the light response of stomata. *Journal of Experimental Botany* **1**, 29–62.

Heath, O. & Meidner, H. 1957 Effects of carbon dioxide and temperature on stomata of *Allium cepa* L. *Nature* **180**, 180–182.

Heath, J., Ayres, E., Possell, M., Bardgett, R. D., Black, H. I. J., Grant, H., Ineson, P. & Kerstiens, G. 2005 Rising atmospheric CO_2 reduces sequestration of root-derived soil carbon. *Science* **309**, 1711–1713.

Jones, H. G. 2004 Irrigation scheduling: advantages and pitfalls of plant-based methods. *Journal of Experimental Botany* **55**, 2427–2436.

Jordan, D. B. & Ogren, W. L. 1984 The CO_2/O_2 specificity of ribulose 1,5-bisphosphate carboxylase/oxygenase: dependence on ribulosebisphosphate concentration, pH and temperature. *Planta* **161**, 308–313.

Kalaji, H. M., Jajoo, A., Oukarroum, A., Brestic, M., Zivcak, M., Samborska, I. A., Cetner, M. D., Łukasik, I., Goltsev, V. & Ladle, R. J. 2016 Chlorophyll *a* fluorescence as a tool to monitor physiological status of plants under abiotic stress conditions. *Acta Physiologiae Plantarum* **38**, 1–11.

Kardiman, R. & Ræbild, A. 2018 Relationship between stomatal density, size and speed of opening in Sumatran rainforest species. *Tree Physiology* **38**(5), 696–705. doi: 10.1093/treephys/tpx149.

Killi, D. & Haworth, M. 2017 Diffusive and metabolic constraints to photosynthesis in quinoa during drought and salt stress. *Plants* **6**, 49–64.

Killi, D., Bussotti, F., Raschi, A. & Haworth, M. 2016 Adaptation to high temperature mitigates the impact of water deficit during combined heat and drought stress in C3 sunflower and C4 maize varieties with contrasting drought tolerance. *Physiologia Plantarum* **159**, 130–147.

Killi, D., Bussotti, F., Gottardini, E., Pollastrini, M., Mori, J., Tani, C., Papini, A., Ferrini, F. & Fini, A. 2018 Photosynthetic and morphological responses of oak species to temperature and [CO_2] increased to levels predicted for 2050. *Urban Forestry and Urban Greening* **31**, 26–37.

Kirschbaum, M. U. & Farquhar, G. D. 1987 Investigation of the CO_2 dependence of quantum yield and respiration in *Eucalyptus pauciflora*. *Plant Physiology* **83**, 1032–1036.

Kok, B. 1948 A critical consideration of the quantum yield of *Chlorella* photosynthesis. *Enzymologia* **13**, 1–56.

Krall, J. P. & Edwards, G. E. 1992 Relationship between photosystem II activity and CO_2 fixation in leaves. *Physiologia Plantarum* **86**, 180–187.

Laing, W. A., Ogren, W. L. & Hageman, R. H. 1974 Regulation of soybean net photosynthetic CO_2 fixation by the interaction of CO_2, O_2, and ribulose 1, 5-diphosphate carboxylase. *Plant Physiology* **54**, 678–685.

Laisk, A. 1977 Kinetics of photosynthesis and photorespiration in C3 plants. *Nauka Moscow* (in Russian).

Laisk, A. & 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.

Lauteri, M., Scartazza, A., Guido, M. C. & Brugnoli, E. 1997 Genetic variation in photosynthetic capacity, carbon isotope discrimination and mesophyll conductance in provenances of *Castanea sativa* adapted to different environments. *Functional Ecology* **11**, 675–683.

Lauteri, M., Haworth, M., Serraj, R., Monteverdi, M. C. & Centritto, M. 2014 Photosynthetic diffusional constraints affect yield in drought stressed rice cultivars during flowering. *PLoS One* **9**, e109054.

Lawson, T. & Blatt, M. R. 2014 Stomatal size, speed, and responsiveness impact on photosynthesis and water use efficiency. *Plant Physiology* **164**, 1556–1570.

Lloyd, J., Syvertsen, J. P., Kriedemann, P. E. & Farquhar, G. D. 1992 Low conductances for CO_2 diffusion from stomata to the sites of carboxylation in leaves of woody species. *Plant Cell and Environment* **15**, 873–899.

Lobo, F. d. A., De Barros, M., Dalmagro, H., Dalmolin, Â., Pereira, W., de Souza, É., Vourlitis, G. & Ortíz, C. R. 2013 Fitting net photosynthetic light-response curves with Microsoft Excel – a critical look at the models. *Photosynthetica* **51**, 445–456.

Long, S. P. & Bernacchi, C. J. 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. & Centritto, M. 2008 Leaf carbon assimilation in a water-limited world. *Plant Biosystems* **142**, 154–161.

Loreto, F., Harley, P. C., Dimarco, G. & Sharkey, T. D. 1992 Estimation of mesophyll conductance to CO_2 flux by three different methods. *Plant Physiology* **98**, 1437–1443.

Loreto, F., Dimarco, G., Tricoli, D. & Sharkey, T. D. 1994 Measurements of mesophyll conductance, photosynthetic electron transport and alternative electron sinks of field grown wheat leaves. *Photosynthesis Research* **41**, 397–403.

Loreto, F., Velikova, V. & Di Marco, G. 2001 Respiration in the light measured by $^{12}CO_2$ emission in $^{13}CO_2$ atmosphere in maize leaves. *Functional Plant Biology* **28**, 1103–1108.

Loreto, F., Tsonev, T. & Centritto, M. 2009 The impact of blue light on leaf mesophyll conductance. *Journal of Experimental Botany* **60**, 2283–2290.

Loriaux, S., Avenson, T., Welles, J., McDermitt, D., Eckles, R., Riensche, B. & Genty, B. 2013 Closing in on maximum yield of chlorophyll fluorescence using a single multiphase flash of sub-saturating intensity. *Plant Cell and Environment* **36**, 1755–1770.

Lovelli, S. & Perniola, M. 2014 Low CO_2 does not remove diffusional limitation to photosynthesis in salt stressed tomato during osmotic phase. *Acta Physiologiae Plantarum* **36**, 1953–1956.

Mansfield, T. A. & Majernik, O. 1970 Can stomata play a part in protecting plants against air pollutants? *Environmental Pollution* **1**, 149–154.

Mansfield, T. A. & Meidner, H. 1966 Stomatal opening in light of different wavelengths: effects of blue light independent of carbon dioxide concentration. *Journal of Experimental Botany* **17**, 510–521.

Mansfield, T. A., Travis, A. J. & Jarvis, R. G. 1981 Responses to light and carbon dioxide. In: *Stomatal Physiology. Society for Experimental Biology (Seminar Series)* (P. G. Jarvis & T. A. Mansfield, eds). Cambridge University Press, Cambridge, p. 295.

Marino, G., Pallozzi, E., Cocozza, C., Tognetti, R., Giovannelli, A., Cantini, C. & Centritto, M. 2014 Assessing gas exchange, sap flow and water relations using tree canopy spectral reflectance indices in irrigated and rainfed *Olea europaea* L. *Environmental and Experimental Botany* **99**, 43–52.

Marshall, B. & Biscoe, P. V. 1980 A model for C3 leaves describing the dependence of net photosynthesis on irradiance. *Journal of Experimental Botany* **31**, 29–39.

Maxwell, K. & Johnson, G. N. 2000 Chlorophyll fluorescence – a practical guide. *Journal of Experimental Botany* **51**, 659–668.

McAdam, S. A. M. & Brodribb, T. J. 2012 Stomatal innovation and the rise of seed plants. *Ecology Letters* **15**, 1–8.

Morison, J. I. L., Baker, N. R., Mullineaux, P. M. & Davies, W. J. 2008 Improving water use in crop production. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* **363**, 639–658.

Munns, R., James, R. A., Sirault, X. R. R., Furbank, R. T. & Jones, H. G. 2010 New phenotyping methods for screening wheat and barley for beneficial responses to water deficit. *Journal of Experimental Botany* **61**, 3499–3507.

Niinemets, Ü., Cescatti, A., Rodeghiero, M. & Tosens, T. 2005 Leaf internal diffusion conductance limits photosynthesis more strongly in older leaves of Mediterranean evergreen broad-leaved species. *Plant Cell and Environment* **28**, 1552–1566.

Ögren, E. 1993 Convexity of the photosynthetic light-response curve in relation to intensity and direction of light during growth. *Plant Physiology* **101**, 1013–1019.

Ögren, E. & Evans, J. R. 1993 Photosynthetic light-response curves1. The influence of CO_2 partial pressure and leaf inversion. *Planta* **189**, 182–190.

Olascoaga, B., MacArthur, A., Atherton, J. & Porcar-Castell, A. 2016 A comparison of methods to estimate photosynthetic light absorption in leaves with contrasting morphology. *Tree Physiology* **36**, 368–379.

Pallozzi, E., Tsonev, T., Marino, G., Copolovici, L., Niinemets, Ü., Loreto, F. & Centritto, M. 2013 Isoprenoid emissions, photosynthesis and mesophyll diffusion conductance in response to blue light. *Environmental and Experimental Botany* **95**, 50–58.

Patakas, A., Noitsakis, B. & Chouzouri, A. 2005 Optimization of irrigation water use in grapevines using the relationship

between transpiration and plant water status. *Agriculture, Ecosystems and Environment* **106**, 253–259.

Peisker, M. & Apel, H. 2001 Inhibition by light of CO_2 evolution from dark respiration: comparison of two gas exchange methods. *Photosynthesis Research* **70**, 291–298.

Pinelli, P. & Loreto, F. 2003 $^{12}CO_2$ emission from different metabolic pathways measured in illuminated and darkened C-3 and C-4 leaves at low, atmospheric and elevated CO_2 concentration. *Journal of Experimental Botany* **54**, 1761–1769.

Pons, T. L., Flexas, J., von Caemmerer, S., Evans, J. R., Genty, B., Ribas-Carbo, M. & Brugnoli, E. 2009 Estimating mesophyll conductance to CO_2: methodology, potential errors, and recommendations. *Journal of Experimental Botany* **60**, 2217–2234.

Rodeghiero, M., Niinemets, Ü. & Cescatti, A. 2007 Major diffusion leaks of clamp-on leaf cuvettes still unaccounted: how erroneous are the estimates of Farquhar *et al.* model parameters? *Plant Cell and Environment* **30**, 1006–1022.

Romero, P., Fernández-Fernández, J. I. & Martinez-Cutillas, A. 2010 Physiological thresholds for efficient regulated deficit-irrigation management in winegrapes grown under semiarid conditions. *American Journal of Enology and Viticulture* **61**, 300–312.

Sakowska, K., Alberti, G., Genesio, L., Peressotti, A., Vedove, G. D., Gianelle, D., Colombo, R., Rodeghiero, M., Panigada, C., Juszczak, R., Celesti, M., Rossini, M., Haworth, M., Campbell, B. W., Mevy, J.-P., Vescovo, L., Cendrero-Mateo, M. P., Rascher, U. & Miglietta, F. 2018 Leaf and canopy photosynthesis of a chlorophyll deficient soybean mutant. *Plant Cell and Environment* **41**, 1427–1437.

Schulze, E.-D., Lange, O., Kappen, L., Evenari, M. & Buschbom, U. 1975 The role of air humidity and leaf temperature in controlling stomatal resistance of *Prunus armeniaca* L. under desert conditions: II the significance of leaf water status and internal carbon dioxide concentration. *Oecologia* **18**, 219–233.

Sharkey, T. D. 1988 Estimating the rate of photorespiration in leaves. *Physiologia Plantarum* **73**, 147–152.

Sharkey, T. D. & Raschke, K. 1981 Effect of light quality on stomatal opening in leaves of *Xanthium strumarium* L. *Plant Physiology* **68**, 1170–1174.

Sharkey, T. D. & Vanderveer, P. J. 1989 Stromal phosphate concentration is low during feedback limited photosynthesis. *Plant Physiology* **91**, 679–684.

Sharkey, T. D., Bernacchi, C. J., Farquhar, G. D. & Singsaas, E. L. 2007 Fitting photosynthetic carbon dioxide response curves for C-3 leaves. *Plant Cell and Environment* **30**, 1035–1040.

Sharp, R. E., Matthews, M. A. & Boyer, J. S. 1984 Kok effect and the quantum yield of photosynthesis light partially inhibits dark respiration. *Plant Physiology* **75**, 95–101.

Shimazaki, K.-i., Doi, M., Assmann, S. M. & Kinoshita, T. 2007 Light regulation of stomatal movement. *Annual Review of Plant Biology* **58**, 219–247.

Sorrentino, G., Haworth, M., Wahbi, S., Mahmood, T., Zuomin, S. & Centritto, M. 2016 Abscisic acid induces rapid reductions in mesophyll conductance to carbon dioxide. *PLoS One* **11**, e0148554.

Stinziano, J. R., Morgan, P. B., Lynch, D. J., Saathoff, A. J., McDermitt, D. K. & Hanson, D. T. 2017 The rapid A–C_i response: photosynthesis in the phenomic era. *Plant Cell and Environment* **40**, 1256–1262.

Sun, P., Wahbi, S., Tsonev, T., Haworth, M., Liu, S. & Centritto, M. 2014 On the use of leaf spectral indices to assess water status and photosynthetic limitations in *Olea europaea* L. during water-stress and recovery. *PLoS One* **9**, e105165.

Tardieu, F. & Simonneau, T. 1998 Variability among species of stomatal control under fluctuating soil water status and evaporative demand: modelling isohydric and anisohydric behaviours. *Journal of Experimental Botany* **49**, 419–432.

Terashima, I., Fujita, T., Inoue, T., Chow, W. S. & Oguchi, R. 2009 Green light drives leaf photosynthesis more efficiently than red light in strong white light: revisiting the enigmatic question of why leaves are green. *Plant and Cell Physiology* **50**, 684–697.

Tholen, D., Ethier, G., Genty, B., Pepin, S. & Zhu, X.-G. 2012 Variable mesophyll conductance revisited: theoretical background and experimental implications. *Plant Cell and Environment* **35**, 2087–2103.

Thornley, J. H. M. 2002 Instantaneous canopy photosynthesis: analytical expressions for sun and shade leaves based on exponential light decay down the canopy and an acclimated non-rectangular hyperbola for leaf photosynthesis. *Annals of Botany* **89**, 451–458.

Tjoelker, M., Oleksyn, J., Lee, T. & Reich, P. 2001 Direct inhibition of leaf dark respiration by elevated CO_2 is minor in 12 grassland species. *New Phytologist* **150**, 419–424.

Villar, R., Held, A. A. & Merino, J. 1994 Comparison of methods to estimate dark respiration in the light in leaves of two woody species. *Plant Physiology* **105**, 167–172.

Von Caemmerer, S. 2000 *Biochemical Models of Leaf Photosynthesis*. CSIRO Publishing, Collingwood, Australia.

Von Caemmerer, S. & Farquhar, G. D. 1981 Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. *Planta* **153**, 376–387.

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

Wang, X., Lewis, J. D., Tissue, D. T., Seemann, J. R. & Griffin, K. L. 2001 Effects of elevated atmospheric CO_2 concentration on leaf dark respiration of *Xanthium strumarium* in light and in darkness. *Proceedings of the National Academy of Sciences* **98**, 2479–2484.

Woodward, F. I. 1987 Stomatal numbers are sensitive to increases in CO_2 from preindustrial levels. *Nature* **327**, 617–618.

Wullschleger, S. D. 1993 Biochemical limitations to carbon assimilation in C_3 plants – a retrospective analysis of the A/Ci

curves from 109 species. *Journal of Experimental Botany* **44**, 907–920.

Xiong, D., Douthe, C. & Flexas, J. 2018 Differential coordination of stomatal conductance, mesophyll conductance, and leaf hydraulic conductance in response to changing light across species. *Plant Cell and Environment* **41**, 436–450.

Yang, J. T., Preiser, A. L., Li, Z., Weise, S. E. & Sharkey, T. D. 2016 Triose phosphate use limitation of photosynthesis: short-term and long-term effects. *Planta* **243**, 687–698.

Yin, X., Struik, P. C., Romero, P., Harbinson, J., Evers, J. B., Van Der Putten, P. E. L. & Vos, J. A. N. 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.

Yin, X., Sun, Z., Struik, P. C. & Gu, J. 2011 Evaluating a new method to estimate the rate of leaf respiration in the light by analysis of combined gas exchange and chlorophyll fluorescence measurements. *Journal of Experimental Botany* **62**, 3489–3499.

Zegada-Lizarazu, W., Della Rocca, G., Centritto, M., Parenti, A. & Monti, A. 2018 Giant reed genotypes from temperate and arid environments show different response mechanisms to drought. *Physiologia Plantarum* **163**, 490–501.

Zeiger, E. & Hepler, P. 1977 Light and stomatal function: blue light stimulates swelling of guard cell protoplasts. *Science* **196**, 887–889.

Zhang, J. & Davies, W. 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 and growth. *Plant Cell and Environment* **13**, 277–285.

First received 18 June 2018; accepted in revised form 13 August 2018. Available online 24 September 2018