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REVIEW

The ratio of electron transport to assimilation (ETR/A_N): underutilized but essential for assessing both equipment's proper performance and plant status

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Abstract

Main conclusion **ETR/A_N ratios should be in the range 7.5–10.5 for non-stressed C_3 plants. Ratios extremely out of this range can be reflecting both uncontrolled plant status and technical mistakes during measurements. We urge users to explicitly refer to this ratio in future studies as a proof for internal data quality control.**

Abstract For the last few decades, the use of infra-red gas-exchange analysers (IRGAs) coupled with chlorophyll fluorometers that allow for measurements of net CO_2 assimilation rate and estimates of electron transport rate over the same leaf area has been popularized. The evaluation of data from both instruments in an integrative manner can result in additional valuable information, such as the estimation of the light respiration, mesophyll conductance and the partitioning of the flux of electrons into carboxylation, oxygenation and alternative processes, among others. In this review, an additional and more 'straight' use of the combination of chlorophyll fluorescence and gas exchange-derived parameters is presented, namely using the direct ratio between two fully independently estimated parameters, electron transport rate (ETR)—determined by the fluorometer—and net CO_2 assimilation rate (A_N)—determined by the IRGA, i.e., the ETR/A_N ratio, as a tool for fast detection of incongruencies in the data and potential technical problems associated with them, while checking for the study plant's status. To illustrate this application, a compilation of 75 studies that reported both parameters for a total of 178 species under varying physiological status is presented. Values of ETR/A_N between 7.5 and 10.5 were most frequently found for non-stressed C_3 plants. C_4 species showed an average ETR/A_N ratio of 4.7. The observed ratios were larger for species with high leaf mass per area and for plants subjected to stressful factors like drought or nutritional deficit. Knowing the expected ETR/A_N ratio projects this ratio as a routinary and rapid check point for guaranteeing both the correct performance of equipment and the optimal/stress status of studied plants. All known errors associated with the under- or overestimation of ETR or A_N are summarized in a checklist that aims to be routinely used by any IRGA/fluorometer user to strength the validity of their data.

Keywords Absorptance · Chlorophyll fluorescence · Electron transport rate · Gas exchange · Leaf mass per area (LMA) · Photosynthesis

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Abbreviations

A_N	Net CO_2 assimilation
ETR	Electron transport rate
IRGA	Infra-red gas-exchange analyser

Introduction

Instantaneous and non-destructive photosynthetic gas-exchange measurements are a frequent procedure followed by many researchers for assessing plant productivity and ecophysiology. During the 50 s, several studies (Rabinowitch

1951; Steeman-Nielsen 1952; Parker 1953; Decker 1955; Gaastra 1959) started the measurement of photosynthesis and respiration by the use of self-build instruments equipped with infra-red gas analysers (IRGAs). Although the infra-red spectrographic method for determining gases concentration was developed starting in the 30 s (McAlister 1936; Nelson 1945), the very first commercial portable photosynthesis systems were not released until the early 1980s, with LCA-1 (ADC BioScientific Ltd, Hoddesdon, UK), CIRAS-1 (PP Systems, Hitchin, UK) and LI-6000 (Li-Cor Inc., Lincoln, NE, USA) being among the pioneers. In the meanwhile, several research groups kept on developing and using their own custom-built instruments, not just for laboratory uses but even for notorious first incursions in field measurements (reviewed by Field et al. 1989). During the same period, another powerful technique for the assessment of photosynthesis was developed, termed the chlorophyll *a* fluorescence. The chlorophyll fluorescence was described by Müller (1874), after its earlier discover by Brewster (1834), although it was Kautsky and Hirsch (1931) who described a variation of this fluorescence with light that was sensitive to cyanide, an inhibitor of the electron transport chain, later stablishing a relationship between this phenomenon and carbon assimilation (Kautsky et al. 1960). However, the nature of the fluorescence signal allowed for its detection only under dark conditions for decades. Since the mid 80s, the development of the so-called pulse amplitude modulation (PAM) approach, which allows for inferring fluorescence under ambient light conditions, fuelled its use in photosynthesis studies (Schreiber 1986; Schreiber et al. 1986). Since then, multiple commercial fluorometers have been developed, following the trail of PAM-101 (Heinz Walz GmbH, Effeltrich, Germany). Although fluorometers typically have been coupled with oxygen electrodes (Walker 1985; Björkman and Demming 1987), it was not until the 90s that the conventional gas-exchange measurements were supplemented with chlorophyll fluorescence over the same leaf area by the coupling of different devices (Peterson 1989, 1990, 1994; Cornic and Briantais 1991). It was not until 1999 that the first commercial attempt of simultaneous measurement over the same leaf area was made by LICOR (Li-Cor Ltd.), by developing an adapter for the PAM-2000 fluorometer (Heinz Walz GmbH) onto their gas-exchange system LI-6400. Nowadays, most companies that manufacture portable photosynthesis systems (Li-Cor Biosciences, Heinz Walz GmbH, Hansatech Instruments Ltd, ADC Bioscientific, CID-Bioscience, etc.) include in their catalogue an IRGA version equipped with a fluorometer on a single device, something not conceived in the past (see, for instance, the list of the equipment detailed by Coombas et al. 1985).

The current combination of both techniques responds to the need of assessing aspects related to photosynthetic integrity and stoichiometry. Most of these applications require a modelling approach to the photosynthetic process, resulting in the acquisition of very valuable information, including the estimation of the light absorption and partitioning between PSII and PSI (Valentini et al. 1995), leaf respiration in the light (Yin et al. 2009a), estimating mesophyll conductance to CO_2 (Bongi and Loreto 1989; Harley et al. 1992) and the partitioning of the flux of electrons into carboxylation, oxygenation (photorespiration) and alternative processes (Miyake and Yokota 2000; Yin et al. 2006; Suggett et al. 2009; Yiotis and Manetas 2010; Driever and Baker 2011), among others. Furthermore, the simple ratio between net CO_2 assimilation (A_N)—obtained by gas-exchange measurements—and electron transport rate (ETR, also frequently referred as J_F or J_{FLU})—calculated from chlorophyll fluorescence measurements—has been proposed as a meaningful physiological parameter by itself during the 90s of the past century and early 2000s (Krall and Edwards 1992; Green et al. 1998; Flexas et al. 2002) but, surprisingly, not frequently used afterwards. The stoichiometry of photosynthetic process can be theorized: four electrons need to be mobilized through the linear electron transport for the generation of two molecules of NADPH, which are required for the fixation of one molecule of CO_2 through the Calvin–Benson cycle (Farquhar et al. 1980; von Caemmerer 2000). Thus, a theoretical minimum value of 4 can be established for ETR/A_N as a quick approximation of the ratio between the linear electron transport (see the next section) and gross CO_2 assimilation (A_N + mitochondrial respiration and photorespiration). The value of ETR/A_N tends to increase due to the contribution of the mitochondrial respiration to the total A_N, the concurrence of photorespiration under normal conditions, especially in C_3 plants, the concurrence of electron flow alternative to carboxylation and oxygenation of ribulose 1,5-bisphosphate, and the existence of stress factors that limit CO_2 fixation beyond electron transport, such as stomatal closure and other diffusional limiting processes during drought (Flexas et al. 2002). In non-stressed plants, the reliable range of ETR/A_N values has been suggested to range 8–10 (Flexas et al. 2002; Medrano et al. 2002). This expected range of ratios is based on the known photorespiratory component on C_3 species, i.e., (photo)respiration is between 1/3 and 1/2 of total photosynthesis (e.g., Farazdaghi and Edwards 1988; Van Oijen et al. 2010), so that measurements of plants growing under non-optimal conditions or any technical pitfall regarding gas exchange or chlorophyll fluorescence could be easily detected. However, no previous paper has deepened in such a useful application of this ratio.

It has to be considered that, during measurements of gas exchange and chlorophyll fluorescence, several biases can be introduced from a misuse of the techniques. The combination of a high technical complexity with a user-friendly

software unavoidably resulted in the popularization of the method concomitant with a relevant increase in the number of users with a diverse expertise in photosynthesis. In this context, the manuals of each commercial device include simple tests for routinely checking both gas-exchange measurements—calibration of the IRGAs, checking for leakage of CO_2, the proper functioning of the flow meter and the thermocouple, light sensors, test for CO_2 and water stability, etc.—as well as the proper recording of fluorescence—checking the pulse chart of fluorescence to assure the saturation of PSII and the use of standards of fluorescence for assuring the proper recording of the fluorescence signal. Some of the potential pitfalls of the standard measurements and proposes for avoiding them have been also summarized in several reviews (Long and Bernacchi 2003; Bernacchi et al. 2011; Haworth et al. 2018), as well as in the user guides of each commercial instrument. However, a procedure for checking the coherence between ETR and A_N values has not been established, yet it was already mentioned briefly in Douthe et al. (2018).

The aim of this review is (1) to highlight the advantages of assessing photosynthesis by making the most of both gas exchange and chlorophyll fluorescence measurements, evaluating both sources of data in an integrative way, and (2) to determine the reliable range of ETR/A_N ratio for developing a simple, rapid and routine method for checking both the adequate functioning of the equipment and the physiological status of the control plants, to ensure reliable conditions prior to starting analytical measurements.

On the accuracy of estimates of the electron transport rate

The chloroplast electron transport rate refers to the movement of electrons through the thylakoid membrane of chloroplasts that results upon light harvesting in photosystem complexes (Fig. 1). Most electrons are originated in the photolysis of water flow through the membrane by numerous reduction–oxidation reactions until reaching the final acceptor of electrons of the chain, i.e., ferredoxin (Fd). Following Yin et al. (2009a), three pathways can be defined at this point. The main one involves the generation of NADPH and its subsequent utilization by the rubisco enzyme in the carboxylation of ribulose 1,5-bisphosphate (RuBP) (Calvin–Benson cycle) or its oxygenation (photorespiration), with the additional consumption of ATP. The sum of ETR that generates the NADPH that is used for carboxylation (J_c) and oxygenation (J_o) pathways has received the name of linear electron transport (f_{linear} or LET). In addition, the NADPH and/or ATP produced by electron transport can be consumed by other numerous processes involved in the metabolism of nitrogen, fatty acids, lipids and in the scavenging of reactive oxygen species. The ETR fraction that leads to such processes alternative to J_c and J_o (J_a) is termed pseudo-cyclic flow ($f_{pseudocyclic}$). Finally, a third pathway, termed cyclic electron flow (f_{cyclic} or CEF), consists in the transference of electrons from Fd back to the cytochrome complex, so that the movement of electrons is restarted at that point. Notice that f_{cyclic} only requires the involvement of PSI and contributes to the generation of transthylakoid proton gradient and production of ATP without concomitant production of NADPH.

While the gas-exchange parameter A_N is calculated using a simple equation based only on parameters properly measured, i.e., CO_2 concentration, flow rate and leaf area, the ETR is instead estimated based on a model relying on several assumptions and parameters that are often estimated rather than measured. First, the quantum efficiency of PSII (ϕ_{PSII}) is assessed by chlorophyll fluorescence as $(F_m' - F_s)/F_m'$, with F_s and F_m' being the steady-state (under illumination) and maximum (after applying a short saturating light pulse) fluorescence levels, respectively, assumed to be emitted mainly by chlorophyll *a* of PSII (Genty et al. 1989). ϕ_{PSII} can be linked directly to the O_2 released from the photolysis of water (Björkman and Demming 1987) but not so straightforward to the assimilation of CO_2, due to the putative variable contribution of all the processes contributing to linear and pseudo-cyclic ETR described above (Genty et al. 1989; Seaton and Walker 1990; Krall and Edwards 1992; Oberhuber et al. 1993; Fryer et al. 1998; Flexas et al. 1998, 2002; Baker 2008), as well as the contribution of processes other than photosynthesis in the net balance of CO_2 gas exchange: photorespiration and mitochondrial respiration. Despite this, the ETR calculated from chlorophyll fluorescence is a good approximation to the photosynthetic capacity of plants and, empirically, it is highly associated with A_N across land plants' phylogeny (Fig. 2a). This relationship improves when gross assimilation (i.e., the sum of estimated photorespiration and mitochondrial respiration in the light) is considered instead of A_N (Flexas et al. 2002, 2022), although the latter requires easier and faster measurements. The equation for ETR was first developed by Krall and Edwards (1992)

$$\mathrm{ETR} = \mathrm{PPFD} \cdot \Phi_{PSII} \cdot \alpha \cdot \beta,$$

where PPFD is the photosynthetic photon flux density, α is the leaf absorptance and β is the partitioning of photons between PSII and PSI. The measurement of each component can represent a source of error. α can be measured by the use of a spectroradiometer coupled to an integrating sphere. However, very frequently, this parameter is simply assumed to be 0.84–0.85, which corresponds to the averaged absorptance of 44 species of diverse origin, life form and metabolism (Björkman and Demmig 1987).

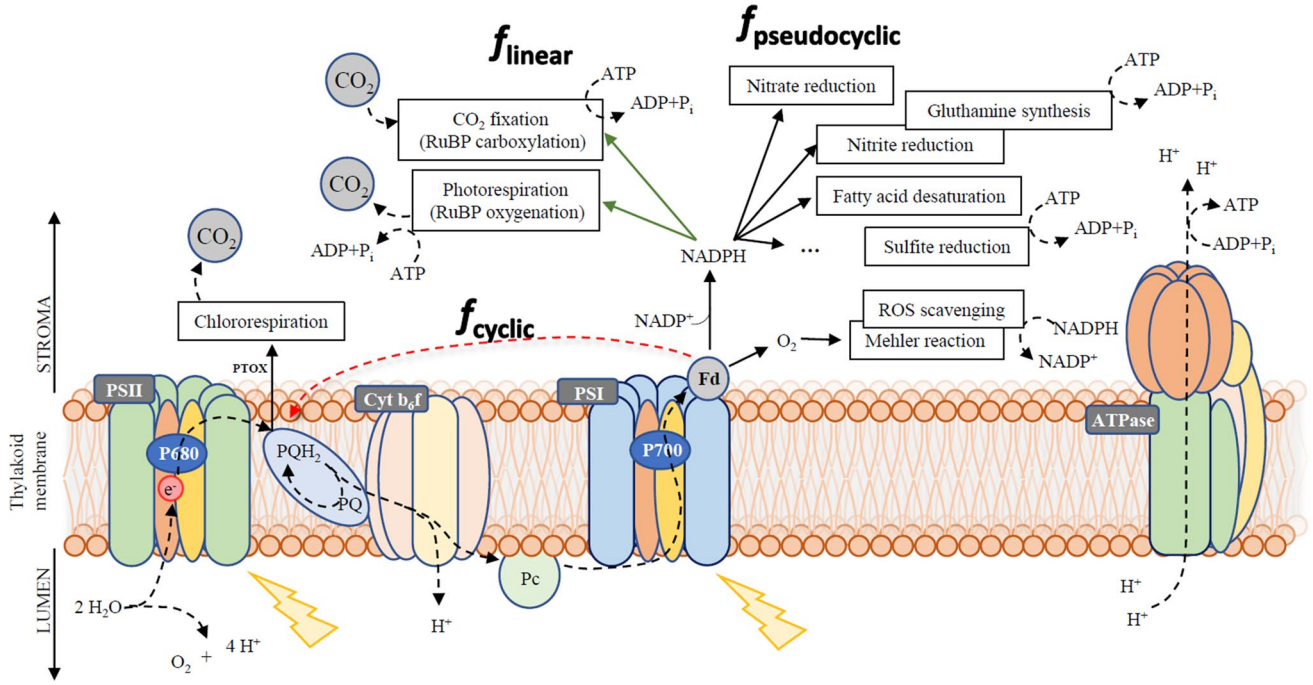


Fig. 1 Diagram illustrating the electron transport chain and sinks for the produced NADPH and ATP. The linear electron transport rate requires the dual photoexcitation of photosystem II (PSII) and photosystem I (PSI). When one photon reaches the pigments located in light harvesting complexes, its energy of excitation is transferred to the chlorophylls of the reaction centre of PSII (P680), which allows the transference of electrons from water (after its photolysis) to the pool of plastoquinones (PQH_2) and the consequent release of H^+ in the thylakoid lumen. The PQH_2 is oxidated in the cytochrome b_6f complex (Cyt b_6f), releasing H^+ in the lumen and finally reducing plastocyanin (Pc). Such accumulation of H^+ in the lumen constitutes the proton force that generates ATP through their movement to the stroma across the ATPase complex. In PSI, the Pc is oxidated, and the electrons are finally transferred to ferredoxin (Fd). At this point, several pathways can be followed by the transference of electrons. Fd can reduces $NADP^+$ to NADPH, which can be used together with ATP in the Calvin-Benson cycle for CO_2 fixation or photorespiration (f_{linear}), as well as in many other reductive reactions involving nitrogen, lipids, sulphur metabolism and reactive oxygen species (ROS) scavenging (as a whole, referred as $f_{pseudocyclic}$). Alternatively, Fd can reduce plastoquinones and reset the electron transference, with the consequent releasing of H^+ and generation of ATP (f_{cyclic}), balancing the ratios among ATP and NADPH needed for the metabolism. A sink of electrons alternative to the defined f_{linear}, f_{cyclic} and $f_{pseudocyclic}$ is also represented between PSII and PSI. This processed are termed chlororespiration and include, for instance, the oxidation of plastoquinone by the enzyme plastid terminal oxidase (PTOX)

Especially, in studies where irradiance is used as a treatment and where a variation of photosynthetic pigments is observed, the assumption of a constant α of 0.84–0.85 represents an important source of error for ETR (Blache et al. 2011; Stemke and Santiago 2011). Furthermore, a certain percentage of α corresponds to non-photosynthetic pigments that would overestimate ETR, especially if it is measured for blue light (McClain and Sharkey 2020). Another source of error derives from any discrepancy between the spectral quality of the light used for determining absorptance and that of the actinic light used during measurements. Therefore, some authors have measured absorptance as a function of wavelength (Björkman and Demmig 1987; Evans et al. 2017; McClain and Sharkey 2020). We strongly recommend determining leaf absorptance simply using exactly the same light source as for the actinic light during photosynthetic measurements (see, e.g., Flexas et al. 2007a).

β is also frequently assumed to equal 0.5, which theoretically implies a null involvement of f_{cyclic} in the total ETR and the same absorption of light between photosystems. This must be far from reality, since (1) the amount of PSII and PSI is not equal (Anderson et al. 1988), (2) their pigments and spectra of absorption are not identical (McClain and Sharkey 2020), (3) the capacity for energy dissipation of PSI may not be equal to PSII (Ort 2001), and (4) their absorption of light can experience a redistribution due to state transitions, a process in which the phosphorylated LHCII disconnects from PSII and migrates to PSI for promoting their absorption of light (Ruban and Johnson 2009). This latter process is linked with the occurrence of certain f_{cyclic} even during the normal performance of the photosynthetic process, to balance the production of ATP and NADPH that are required for CO_2 fixation, something that f_{linear} does not fully

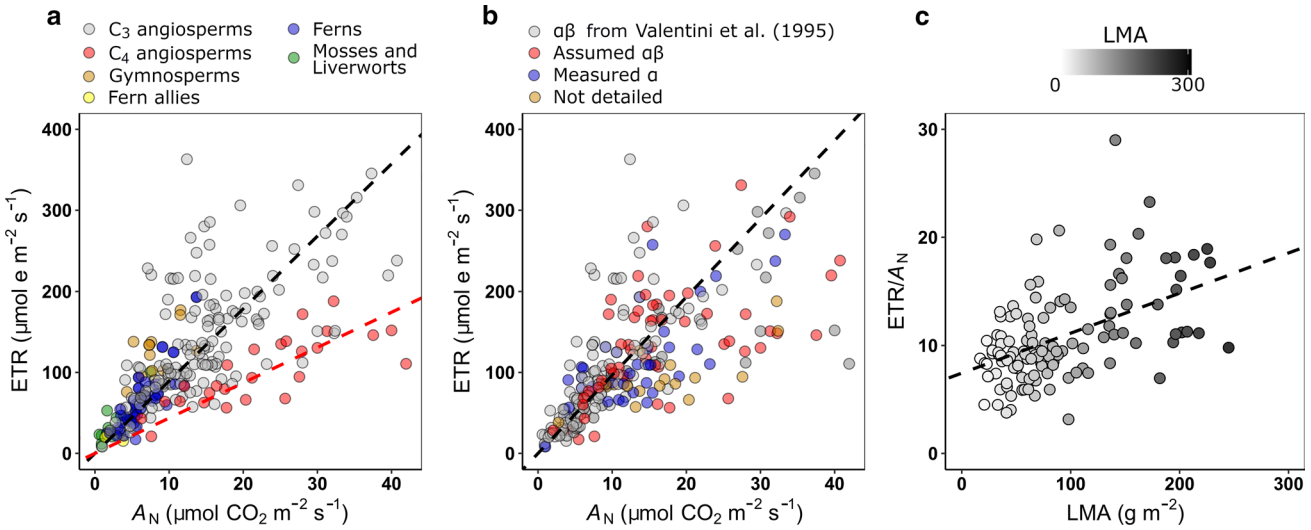


Fig. 2 Relationship between net CO_2 assimilation (A_N) and electron transport rate (ETR) for 253 entries corresponding to 178 species compiled form 75 studies (see Supplementary Excel file), differentiated by their taxonomical group and metabolism type (**a**) or by the method for obtaining the value of $\alpha\beta$ used in the calculation of ETR (**b**). Black dashed line of **a**: ETR = 8.93·A_N, excluding C_4 plants ($R^2 = 0.860$, $P < 0.001$). Red dashed line of **a** ETR = 4.35·A_N, for C_4 angiosperms ($R^2 = 0.934$, $P < 0.001$). Dashed line of **b** ETR = 9.65·A_N, using data for C_3 species whose ETR were calculated from a non-assumed α (blue and grey points, $R^2 = 0.868$, $P < 0.001$). **c** Relationship between the ratio ETR/A_N and the leaf mass area (LMA) averaged for each angiosperm species ($R^2 = 0.261$, $P < 0.001$). Dashed line of **c** ETR/A_N = 0.038·LMA + 7.29

achieve. β have been suggested to range between 0.45 and 0.5 (von Caemmerer 2000), although empirical values up to 0.6 have also been reported (Strasser and Butler 1977; Laisk and Loreto 1996), which could only be explained by a sink of electrons between PSII and PSI. Although infrequently alluded, all electron transfer reactions from stromal reductants to O_2 through the plastoquinone pool (termed chlororespiration) may explain values of β higher than 0.5. This is the case of the oxidation of plastoquinone by the enzyme plastid terminal oxidase (PTOX) (Havaux 2020; Peltier and Cournac 2002; Rumeau et al. 2007), which works homologous to the mitochondrial alternative oxidase (AOX) and may constitute a sink of electrons alternative to the defined f_{linear}, f_{cyclic} and $f_{pseudocyclic}$.

To avoid assumptions on α and β, several approaches have been proposed for estimating the product $\alpha\beta$ by the combination of measurements of gas exchange and chlorophyll fluorescence under non-photorespiratory conditions (Valentini et al. 1995; Yin et al. 2009b; Martins et al. 2013; Théroux-Rancourt et al. 2014). These estimations of $\alpha\beta$ are done under the assumption of a null $f_{pseudocyclic}$ and chlororespiration at non-photorespiratory conditions (i.e., low O_2), so that J_c and ETR can be directly linked. When such a procedure is not possible, at least an evaluation of leaf properties that would affect their absorption should be done, such as assessing chlorophyll content (Morales et al. 1991; Syvertsen et al. 1995; Cheng et al. 2000; Evans and Poorter 2001) or considering the presence of abundant pubescence (Ehleringer and Björkman 1978). Here, we have compiled data for A_N and ETR from 191 species corresponding to 77 published studies (see Supplementary Excel file). The method used for calculating α and β is considered, so that, in Fig. 2a, the relationship between A_N and ETR is shown for all C_3 species, while in Fig. 2b for the same relationship is shown for C_3 species whose ETR was calculated using non-assumed α (Fig. 2b). In the second case, the coefficient of determination was only slightly higher despite the lower number of entries (from $R^2 = 0.604$ in Fig. 2a and $R^2 = 0.633$ in Fig. 2b). This suggests that, in general, the typically assumed values of leaf absorptance are close to the actual ones but, of course, this can still be severely challenged under particular conditions (e.g., chlorosis, pubescence, etc.). Examples of the variation of ETR/A_N ratio due to errors in the estimation of $\alpha\beta$ are shown for real data obtained for an angiosperm (Fig. 3a), a fern (Fig. 3b) and a moss (Fig. 3c). Assuming an $\alpha\beta$ value of 0.42 would make ETR/A_N to increase on average from 7.82 to 9.28 in the angiosperm, from 8.69 to 9.62 in the fern and from 9.74 to 23.4 in the moss. Notice that the moss and the fern do not show very different A_N, but the actual $\alpha\beta$ of the moss is significantly lower than the other two species, probably due to a lower concentration of chlorophyll (Perera-Castro et al. 2022), emerging as a factor that can increase the sensitivity of ETR/A_N to $\alpha\beta$.

Additional factors can also affect the value of ϕ_{PSII} and, therefore, add additional sources of variation for the estimated ETR. The contribution of fluorescence emission of PSI in the measurement of ϕ_{PSII} is usually neglected, since at room temperature, it is thought not to exceed 0.8% of

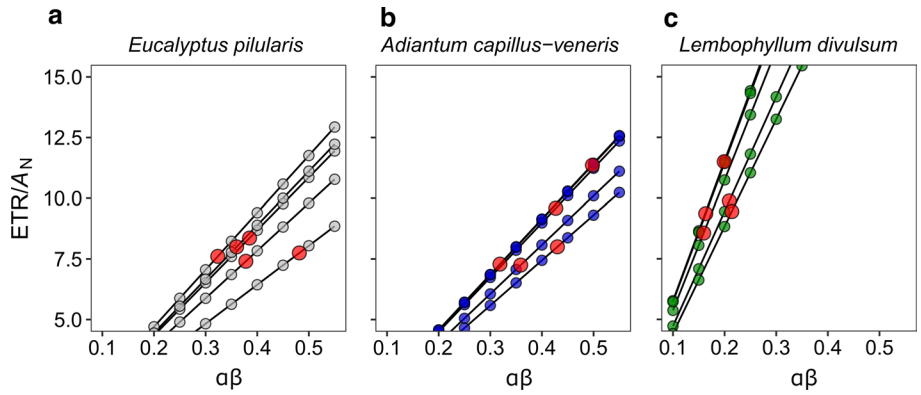


Fig. 3 Sensitivity analysis for the ETR/A_N ratio with the variation of absorptance of PSII ($\alpha\beta$) for: **a** the angiosperm *Eucalyptus pilularis* (averaged $A_N = 13.8 + 1.3$ μmol m^{-2} s^{-1}, $\phi_{PSII} = 0.187 \pm 0.027$, PPFD = 1500 μmol m^{-2} s^{-1}, ETR = 109.1 ± 12.5 μmol m^{-2} s^{-1}, $\alpha\beta = 0.38 \pm 0.02$, values are mean ± SE); **b** the fern *Adiantum capillus-veneris* (averaged $A_N = 1.6 \pm 0.2$, $\phi_{PSII} = 0.120 \pm 0.014$, PPFD = 400, ETR = 14.2 ± 0.7, $\alpha\beta = 0.40 \pm 0.03$); and **c** the moss *Lembophyllum divulsum* (averaged $A_N = 1.05 \pm 0.06$, $\phi_{PSII} = 0.136 \pm 0.007$, PPFD = 400, ETR = 10.2 ± 0.6, $\alpha\beta = 0.18 \pm 0.01$). The fives replicates were obtained from Perera-Castro et al. (2022). Red points indicate real measured values of αβ measured from Valentini et al. (1995) and ETR/A_N ratio. The rest of coloured points follow the legend of Fig. 2

the emission of PSII (see revision of Kalaji et al. 2017). However, some studies suggest a much larger contribution of PSI at F_s without any contribution at F_m', which would result in large errors in the estimation of a 'true' ETR (Agati et al. 2000; Franck et al. 2002). While these observations may deserve further studies, we believe that this potential bias is not particularly important in many cases, owing to the often-observed tight correlations between A_N and ETR measured concurrently by the same device at the same area and time (Fig. 2 but, especially, see in the original papers how much tighter the correlations are in most of the cases for each particular study included in the data set). Moreover, chlorophyll fluorescence is often retrieved from a few layers of cells, while gas exchange is retrieved from the whole set of cell layers in the mesophyll. The estimation of ϕ_{PSII} from a layer of cells differing from those where carbon fixation mainly occurs can lead to bad estimates of 'whole leaf' ETR. For instance, if ETR is measured in the abaxial side in hypostomatous leaves, this would likely result in an overestimation of ETR, especially when using an actinic light of a wavelength that is mainly absorbed in the abaxial layers, i.e., blue light (Vogelmann 1993; Vogelmann and Han 2000; Evans 2009; Evans et al. 2017; Lichtenberg et al. 2017). In fact, a positive relationship can be observed between ETR/A_N and leaf mass per area (LMA) for the compiled species (Fig. 2c), probably resulting from a larger contribution to the amplification of the aforementioned error in species with a wider palisade tissue, whose chlorophyll fluorescence is mostly the only one detected by the instrument (Buckley and Farquhar 2004), and/or by a higher photorespiration in sclerophyllous leaves (Huang et al. 2019). Notice that the intercept of ETR/A_N–LMA relationship is 7.29. Nevertheless, in some cases, different types of pigments accumulate particularly in the adaxial side, leading to unreliable ETR/A_N ratios. In such cases, sometimes, measuring ETR from the abaxial surface results in improved estimation of ETR, although this should be evaluated in each particular case (personal observation).

Source of variation of the ETR/A_N ratio

From all the above, methodological biases in the variance of the ratio ETR/A_N must be considered, especially if α and β are assumed, blue light is used or species with higher LMA are studied. Despite this, ETR/A_N values between 7.5 and 10.5 are most frequently observed in non-stressed C_3 plants (Fig. 4a), with an average of 10.3 ± 4.9 (mean ± standard deviation) for C_3 angiosperms. This average value does not considerably changes when a stricter criterion is used for the calculation of ETR, i.e., when including only data with known absorptance of leaves (α) (ETR/A_N of 10.9 ± 5.6) or even when including only data with known leaf absorptance and partitioning of photosystems ($\alpha\beta$) obtained after Valentini et al. (1995) (ETR/A_N of 12.6 ± 6.7). We nevertheless strongly recommend performing independent estimations of α or $\alpha\beta$, especially if the data are intended to be used for, e.g., estimating mesophyll conductance or photosynthetic limitations (see, e.g., Pons et al. 2009). Nevertheless, on average, LMA is explaining more variability of ETR/A_N than is the method used for obtaining $\alpha\beta$. For C_4 species, this ratio is sensibly different, 4.7 ± 1.2, although this is obtained from a lower number of compiled studies (15 for C_4 as compared with 68 studies included for C_3 plants). The lower ETR/A_N value of C_4 plants is explained by the enhancement of RuBP carboxylation at the expense of its oxygenation

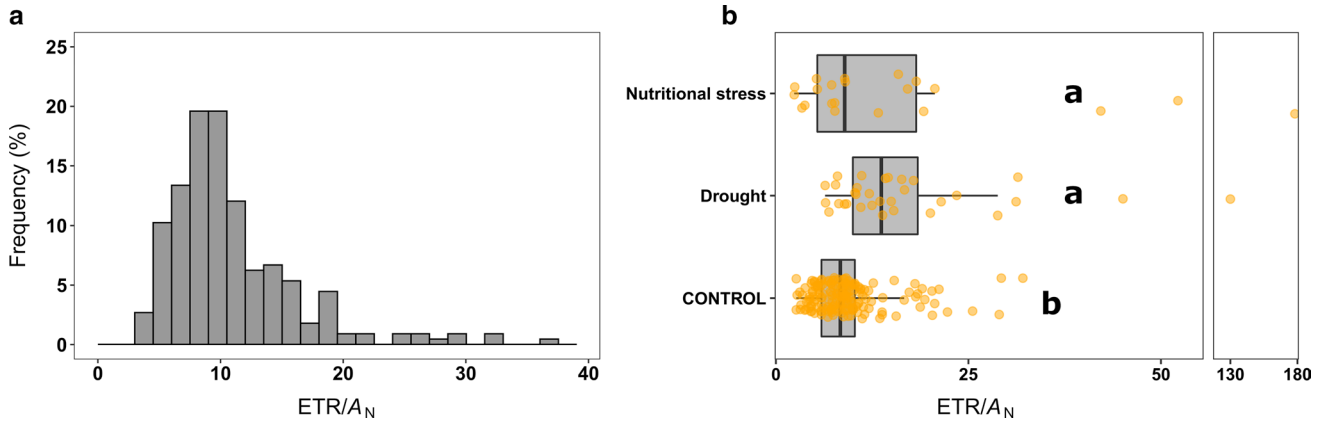


Fig. 4 Values of ETR/A_N compiled for C_3 plants. **a** Frequency chart of ETR/A_N (% of entries from a total of 224 entries, including 66 studies of 157 different species) of non-stressed plants, grouping the observations by discrete intervals of ETR/A_N of 1.5. **b** Comparison of ETR/A_N values of non-stressed plants with compiled values for nutritionally or drought stressed plants (see references in Supplementary Excel File). Letters mean significant differences by Kruskal–Wallis test ($P = 0.001$)

by Rubisco thanks to a far much higher CO_2/O_2 ratio at the carboxylation site of the enzyme (Peterson 1994; Zhou et al. 2020). The ETR/A_N value of 4.7 is still higher than the theoretical limit of 4, which can be explained by the extra energy requirements of C_4 species for returning carbon skeletons from bundle-sheath to mesophyll cells (Furbank et al. 2000) and by the contribution of the mitochondrial respiration to the total A_N.

These averaged ETR/A_N ratios can vary under several conditions, reflecting relevant physiological information about the photosynthetic process and the status of the studied plants. Variations in light and CO_2 concentration seem not to strongly affect the balance between electron transport and CO_2 fixation, except for very low CO_2, when photosynthesis is limited by CO_2 diffusion (ETR/A_N increases) or when photorespiration is severely inhibited (ETR/A_N decreases) (Sharkey et al. 1988; Aranda et al. 2020). However, factors such as water availability and—sometimes—nutritional stresses promote a significant increase of ETR/A_N (Fig. 4b), which is also often reported for salt stress (Naidoo et al. 2002; Uzilday et al. 2014) and non-optimal temperatures (Flexas et al. 1999; D'Ambrosio et al. 2006). Some examples for other stresses such as salinity, ozone, flooding or abiotic factors have also been included in the compiled dataset. This behaviour of ETR/A_N can be explained by a different sensitivity to stress of both processes. For instance, Flexas et al. (2002) showed that ETR only responded to the drop of water potential during severe drought, but not to stomatal closure under moderate stress, thus promoting an increase of the ETR/A_N ratio as drought progressively developed. A similar response should be expected for measurements under very high vapor pressure deficit that can affect stomatal conductance and A_N more strongly than ETR (Kaiser et al. 2020; Inoue et al. 2021). Under this scenario, when A_N declines, photorespiration and, perhaps, other alternative electron sinks, such as nitrogen and sulphur assimilation and the Mehler-peroxidase cycle (Fig. 1) may play a protective role, since these processes permit continued operation of the photosystems while preventing over-reduction of the electron acceptor pools (Sunil et al. 2019). In fact, several attempts for determining the possible magnitude of alternative electron sinks (alternative to acceptors CO_2 or O_2) have also been done for non-stressed and stressed plants by the combined use of chlorophyll fluorescence and gas exchanges measurements (Loreto et al. 1994; D'Ambrosio et al. 2006; Driever and Baker 2011; Ivanov et al. 2012; Yi et al. 2014), yet these have not been conclusive thus far.

On the other hand, a non-expected deviation of ETR/A_N from 'standard' values in putatively non-stressed plants can also be observed, in some cases, reaching values lower than 4—i.e., totally unreliable according to photosynthetic stoichiometry—or values higher than 20, i.e., those more typically associated with stressed plants. Especially for these extremely higher or lower values of ETR/A_N, we must consider that these cases are revealing technical problems with the measurements of ETR and/or A_N. This variation of ETR/A_N seems to follow a trade-off with A_N (Fig. 5a), so that a boundary line between both parameters can be observed, so that the highest ETR/A_N are found exclusively when A_N values are lower than 15. This boundary line is not found for the relationship between ETR/A_N and ETR (Fig. 5b). This is a relevant clue for explaining the source of variation of ETR/A_N. The highest values of this ratio correspond to mosses and liverworts, group of plants that are characterized by their poikilohydry, i.e., their incapacity to control the water content of their tissues. Therefore, they are frequently in equilibrium with the relative humidity of the environment (Proctor and

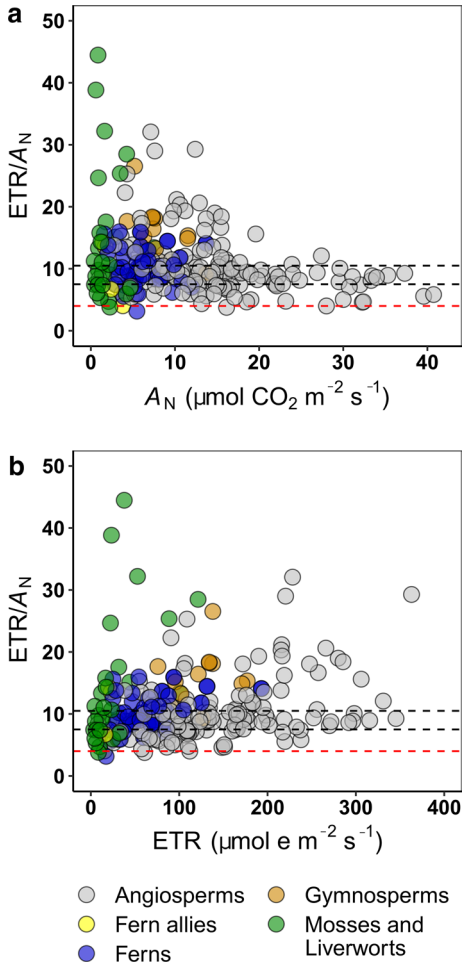


Fig. 5 Variation of ETR/A_N of non-stressed C_3 plants according to each of its components: **a** net CO_2 assimilation rate (A_N), and **b** electron transport rate (ETR). Red dashed line indicates the theoretical minimum value of 4 for ETR/A_N. Black dashed lines indicate the interval 7.5–10.5 of ETR/A_N

Tuba 2002), which can complicate gas-exchange measurements. Actually, the accidental desiccation of mosses or liverworts during their measurement typically results in the increase of ETR/A_N ratio (Perera-Castro et al. 2022). Measurements of unintentionally stressed plants are something that cannot be dismissed even for angiosperms, since stressed plants (and therefore with higher ETR/A_N ratio, as explained above) are more probably showing low values of A_N, partly explaining the boundary line of Fig. 5a. Another source of error of ETR/A_N is related to the LMA, as previously exposed. In our dataset (see Supplementary Excel file), species with the highest LMA (> 180 g m^{-2}), including *Quercus* spp., *Rhododendron delavayi*, *Camelia japonica* and gymnosperms, also present photosynthesis lower than 15 µmol m^{-2} s^{-1}. Therefore, the observed boundary line of ETR/A_N can partly be inherent to the leaf economics spectrum (Wright et al. 2004), by which high LMA (and high ETR/A_N, Fig. 2c) is unlikely to be found in species with high A_N.

Measuring unintentionally stressed plants and/or plants with high LMA can only explain the largest observed ETR/A_N ratios. However, for the very low values of ETR/A_N, especially those lower than 5–6, another source of error must be considered. This kind of error is related to any possible technical problems that devices can have, including calibrations, signal problems or protocols of measurements. Hence, we propose that checking this ratio can become a useful routine test for the proper functioning of both instruments, the fluorometer and the IRGA.

ETR/A_N ratio as a routine test for instruments and plants

The usefulness of checking ETR/A_N ratio is likely well known for most experts, but its use for internal data quality control has not been widely explicit. Since the formulation of ETR by Krall and Edwards (1992), a few studies have reported the value of this ratio. Flexas et al. (2002) used the ETR/A_N ratio as an indicator of the stages of the photosynthetic regulation under drought in vine, latterly endorsed (de Souza et al. 2003, 2005) and extended for other species (Medrano et al. 2002). To the best of our knowledge, after 2 decades, this ratio was only explicitly mentioned as a reliability criterion for detecting inconsistencies in the data by Martins et al. (2013), Carriquí et al. (2019) and Perera-Castro et al. (2022). Here, we propose that the quick measurement of ETR/A_N ratio in a non-stressed plant should be another must-do routine test for verifying the proper functioning of the equipment. The authors have read too many papers where a quick check reveals fully unreliable ETR/A_N ratios in the reported data. Even a researcher who has strong experience in the field can sometimes misleadingly take bad data when suffering, e.g., time restrictions and/or weather inclemency—as we ourselves have done many times. The problem arises when such inconsistent data are nevertheless published. Hence, ideally, we aim every photosynthetic researcher use this ratio as a rapid checkpoint as to whether the measurements are likely to be on an acceptable range or not, and to explicitly report the result of such checking. This practice would result in decreasing the publication of data which are, for whatever of the reported reasons, misleading and wrong, avoiding reaching conclusions that are not supported by reliable data.

The intervals of ETR/A_N most frequently observed for non-stressed C_3 plants are 7.5–10.5 (Fig. 4a). Higher or lower values of ETR/A_N can still result from the natural variation of this ratio, with more or less contribution of photorespiration and mitochondrial respiration to the total A_N, and f_{cyclic}, $f_{pseudocyclic}$ and chlororespiration to the total

electron 'consumption'. The variation of ETR/A_N values may be also linked to the factors limiting photosynthesis under optimum conditions, since a predominance of CO_2 diffusional limitation is expected to result in a higher ETR/A_N, as occurs for very low CO_2 (Sharkey et al. 1988). However, ETR/A_N ratios consistently differing from this interval may reveal either the existence of some pitfall in the measurements or point towards the use of not totally unstressed control plants.

Both ETR/A_N ratios extremely lower or higher than 7.5–10.5 in unstressed plants may be a symptom of a wrong calibration or malfunction of the device. Proper calibrations should be done not only for gas analysers, but also for light sensors and flowmeter. Normally, light sensors are acquired with a certificate of calibration. This certificate normally includes a date of expiration or a recommendation about the required frequency of recalibration after field deployment. If such sensors do not have this certification—sometimes this happens, especially for internal sensors—this calibration is expected to be done by users, correcting any possible rim effect of a light sensor located in a border of the leaf chamber. Regarding gas analysers, manuals frequently contain a routine maintenance protocol for checking the absolute signal of gases, which includes storing not only the zero, but also the span of CO_2 and H_2O by the use of a reference gas and a gas cooler, respectively, which in turn provide a known concentration of CO_2 and H_2O. Zeroing the flowmeter can also be easily done by the user following instructions of the manual. However, any other problem with the flowmeter or air pump would need specialized technical support.

Even with a good calibration, the choice of unsuitable settings can also affect the correctness of measurements. For example, measuring ϕ_{PSII} requires the recording of fluorescence basal signal at steady state (F_s or F') and the maximum fluorescence peak (F_m') reached during a saturating pulse. Therefore, if the gain of fluorescence signal is too low, i.e., a low F_s level, the difference between the two points of fluorescence, F_s and F_m', would present small resolution, affecting to the replicability of the measurement and the noise of both ϕ_{PSII} and ETR. The optimal level of F_s can be determined by checking which minimum gain guarantees the maximum F_s level that does not result in oversaturation of the F_m' signal. This is something that should be evaluated for each species and treatment. Something similar applies for the saturating pulse intensity and duration. Users usually settle for default values. However, a weak or short pulse can result in non-saturation of photosystems and, thus, an underestimated F_m', ϕ_{PSII} and ETR. To avoid this problem, a chart visualization of the course of fluorescence during the saturating pulse can help to detect or reject this problem. If complete saturation is not possible, the use of the multiphase flash or the intercept method is highly recommended instead (Earl and Ennahli 2004; LI-COR 2008, 2020; Loriaux et al. 2013). However, even when saturating, during a pulse some devices can present problems when recording F_m' due to a decrease of measuring light provoked by an unavoidable heating of the LEDs (Heinz Walz GmbH 2019). This also leads to an underestimation of F_m', ϕ_{PSII} and ETR. These problems can be detected by applying pulses to a fluorescence standard and annotating the undesirable variation of fluorescence signal. Any detected decrease of fluorescence signal during pulse should be considered for correcting real F_m' of the measured leaf. Assuming that these factors associated with F_m' are fixed is not recommended (Perera-Castro et al. 2014).

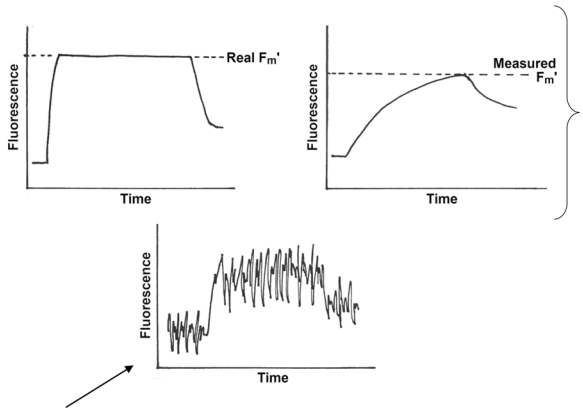
Other kind of slips are more related with the user experience and good practices during measurements rather than with the state of the equipment or the suitability of chosen settings. It is widely established that optimum measurements can only be achieved in well light-adapted plants. Stomatal conductance and the activation of photosynthetic enzymes take longer times to fully respond to light than ϕ_{PSII}. Thus, precipitated measurements of combined gas exchange and fluorescence would result in overestimated ETR/A_N ratios. Even under a proper light-adapted condition, errors can be originated if measurements of non-stabilized A_N are done. Stable A_N usually takes longer times than chlorophyll fluorescence (in terms of minutes vs. seconds) as a consequence of the need of the gas system to purge every time that a new leaf is introduced in the chamber. In this sense, it can be useful to record technical replicates in order to be sure of the invariance of the ETR/A_N ratio along time. This would be helpful also for detecting undesirable drops of A_N due to suboptimal conditions during measurements. For instance, measurements under very high vapor pressure could result in quick stomatal closure even for non-stressed plants, compromising CO_2 diffusion and A_N (Grossiord et al. 2020). If automatic control of humidity is not possible, users should pay attention to this, just as they are routinely paying attention to the effects of CO_2 leakage through gaskets of the measured head, since this was warned by Flexas et al. (2007b) and Rodieghero et al. (2007). For field measurements at CO_2 levels closed to the external one, leakages of CO_2 do not represent a significant problem and are frequently neglected. In non-ventilated indoor measurements, instead, or when measuring at CO_2 concentrations different to ambient, exit/entrance of external CO_2 to the chamber occurs, which is especially intensified when several users are briefing in the same room. The way to avoid this problem is ventilation of indoors and considering apparent A_N measured in an empty chamber or in a dead leaf (after, e.g., immersion in boiling water for a few minutes until maximum quantum yield of PSII (F_v/F_m) is zero, as explained by Flexas et al. 2007b).

Non-stressed C_3 plants, preferentially of low LMA, with no visual chlorosis or pubescence

ETR/A_N >> 7.5-10.5	ETR/A_N << 7.5-10.5

Is there an overestimation of ETR?

- **Check calibration of light sensors**. An accurate recording of light intensity during measurements is relevant for calculation of ETR.



- **Check saturating pulse**. A weak pulse can result in non saturation of photosystems and in an underestimated F_m', ϕ_{PSII} and ETR. Check saturating chart to detect problems with the pulse. Technical replicates are highly recommended.
- **Check fluorescence signal during pulse.** Even when saturating, during a pulse some devices can present problems when recording F_m' due to an unavoidable heating of the LEDs. This also leads to an underestimation of F_m', ϕ_{PSII} and ETR.
- **Check the fluorescence signal**. A very low fluorescence basal signal (F_s or F') can lead to low variable fluorescence levels ($F_q = F_m'-F_s$) and, therefore, noisy ϕ_{PSII} and ETR. Check the manual of the device for seeing the recommended basal level of fluorescence signal.

Is there an underestimation of A_N?

- **Did the gas exchange signal stabilize?** In light-adapted leaves, fluorescence signal is stabilized more quickly than gas exchange (in terms of second *vs*. minutes). A precipitated measurement of A_N would lead to both under- and overestimation of this parameter.
- **Were measurements done after enough light adaptation?** In non-light adapted leaves, ϕ_{PSII} can achieve steady state more quickly than A_N, the latter delayed by stomatal conductance. In this case, precipitated measurement of A_N would result in its underestimation.
- **Were measurements done under non optimal conditions?** Even for non-stressed plants, measurements under very high vapor pressure could result in quick stomatal closure, compromising CO_2 diffusion and A_N. If automatic control of humidity is not possible, users should pay attention to this.

Is there an overestimation of A_N?

- **Check leaks.** For field measurements at CO_2 levels closed to the external one, leakages of CO_2 do not represent a problem and are frequently neglected. In non-ventilated indoor measurements, entrance of external CO_2 to the chamber can occurs, especially when several users are briefing in the same room. Check this leak by measuring apparent A_N in an empty chamber.

- **Check IRGAs calibration.** Analyzers need periodically calibrations for both CO_2 and H_2O, which include zero and span of the absolute signals. Avoid rapid, non-careful storage of calibrated constants, which can result in problems in dCO_2 and dH_2O (the difference between reference and sample analyzers). The use of pure, certificated gases is highly recommended.
- **Check flowmeter.** Even though frequently underestimated, errors in the recordings of the actual flow can also results in important errors in the measurement of A_N. Zeroing the flowmeter can easily be done by the user, although more serious problems needs a specialized technical support.

◂**Fig. 6** Checklist of the possible errors of measurements that are explaining ETR/A_N rations above or below the normal most common range of 7.5–10.5 for a non-stressed C_3 plant

In Fig. 6, we summarize the above points as a checking set-list when ETR/A_N values above or below the standard values are recorded. After following all check points shown in Fig. 6 in one or several control plants, ETR/A_N ratios out of the range can still appear for the plants aimed to be measured. In this case, other plant-intrinsic factors must also be considered, including the cases of being subjected to any kind of stress or presenting a higher LMA. An additional method for confirming the occurrence of stress with independence of the type of metabolism has been described by Flexas et al. (2022). This method is based in the calculation of the ratio between A_N and the maximum net CO_2 assimilation rate under high CO_2 concentration (A_{max}), measured by gas-exchange measurements in contrasted control non-stressed plants or calculated from theoretical assumptions as described in Flexas et al. (2022). Therefore, the concurrence of high ETR/A_N and low A_N/A_{max} ratios can be interpreted as stress symptoms that should be avoided in control plants. Regarding leaf structure, high LMA, especially for monostomatous leaves or the presence of non-photosynthetic pigments in the adaxial side of the leaf, can perturb ETR/A_N, as explained above. For that reason, this check list should be ideally done for one or several well-known plants, especially if their photosynthetic type and absorptance are known, and considering also their LMA, stomatal distribution and pigment accumulations for interpreting the obtained ratios.

Notice that all the points of this checking list are referred to stable values of ETR/A_N. If both parameters are attempted to be measured in plants with very low photosynthetic rates or using very small areas inside the gas-exchange cuvette, A_N more than ETR will oscillate due to low precision, and this will result in a large variation of ETR/A_N within technical replicates (but perhaps not in a consistently extreme average value). If the measured area cannot be increased, increasing the number of technical replicates may result in a more precise averaged A_N and ETR/A_N.

Conclusion

The ETR/A_N ratio is presented as a useful tool for detecting inconsistencies in the simultaneous measurements of chlorophyll fluorescence and gas exchange provided by most current commercial photosynthesis devices. Most studies reported values of ETR/A_N between 7.5 and 10.5 for non-stressed C_3 plants, and around 4.7 for C_4 plants. The method used for estimating the absorbance of PSII is, on average, not affecting considerably this range. On the contrary, high LMA is associated with higher ETR/A_N ratios. Knowing this, a non-stressed plant of known metabolism and low LMA can be routinely used for checking the correct performance of both devices, fluorometer and gas-exchange system, as well as to detect the presence of stress in plants of unknown status. All possible errors associated with both methods are summarized in a checklist that pretends to be useful for most users to detect them. We urge users to explicitly refer to this ratio in future studies as a proof for data internal quality control.

Author contribution statement JF and AVPC conceived and designed the review. AVPC wrote the first draft of the manuscript. All authors read and approved the final version of the manuscript.

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Data availability The data that support the findings of this study are available in the supplementary material of this article.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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