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OPINION

Importance of Timing of Dark Acclimation for Estimating Light Inhibition of Leaf Respiratory CO_2 Efflux

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1 | Introduction

Leaf respiratory CO_2 efflux (R_{CO_2}) is important to the carbon economy of plants (Amthor and Baldocchi 2001). Daytime non-photorespiratory leaf R_{CO_2} appears to be inhibited by light (Atkin et al. 2000a, 2000b; Buckley and Adams 2011; Tcherkez et al. 2017a, 2017b), and this too is important for the carbon economy of plants (Tcherkez et al. 2017b, but see also Kang et al. 2014) and ecosystems (Bruhn et al. 2011; Heskel et al. 2013a; Keenan et al. 2019). Variation in the degree of light inhibition of daytime leaf R_{CO_2} has been observed among species (Souza et al. 2021; Sun and Yao 2023), with position in a canopy (Tissue et al. 2002; Souza et al. 2021; Schmiege et al. 2023), with environmental conditions (Atkin, Evans, and Siebke 1998a; McLaughlin et al. 2014; Heskel et al. 2013b, 2014; Ayub et al. 2014; Schmiege et al. 2021; Sun and Yao 2023), time of day (Faber et al. 2022) and net photosynthesis (Atkin et al. 1998b; Schmiege et al. 2023), photorespiration (Heskel et al. 2013b; Ayub et al. 2014) and daytime respiration of darkened leaves (Ayub et al. 2014). This broad range of potential sources of variation has focused attention on how to estimate daytime leaf R_{CO_2} in the light, (often termed R_l, R_L, or R_{day}), (Villar, Held, and Merino 1994; Tcherkez et al. 2017a, 2017b; Peisker and Apel 2001; Yin et al. 2011; Berghuijs et al. 2019; Yin and Amthor 2024). Techniques for this include the Laisk method (Laisk 1977), the Kok method (Kok 1948), the $^{13}CO_2$ and mass spectrometry method (Haupt-Herting, Klug, and Fock 2001), the Yin method (Yin et al. 2011), the Gong method based on ^{13}C disequilibrium (Gong et al. 2018) and the reaction–diffusion model (Berghuijs et al. 2019). Recently, though, Tcherkez et al. (2024) proposed that we change the term to 'diurnal net decarboxylations' (using the symbol D_d) to acknowledge that the CO_2 efflux not only encompasses non-photorespiratory CO_2 production, but also many other types of leaf decarboxylations in daylight. We agree with this proposal, but further acknowledge that the daytime non-photorespiratory leaf CO_2 efflux also may encompass potential release of CO_2 that has been transported to the leaf from stem or roots (Stutz et al. 2017). Thus, to further emphasize that we here assign a term to not only the diurnal period, but in particular the light conditions at daytime as opposed to 'dark-adapted' leaves at daytime, we here use the symbol, D_{DL} (Table 1). To calculate a degree of light inhibition of leaf D_{DL} (i.e., the relative effect of light on non-photorespiratory leaf D_{DL}), it is for all techniques necessary to also estimate a daytime leaf R_{CO_2} (i.e., non-photorespiratory leaf R_{CO_2} without direct effects of light; here termed $R_{CO_2,DD}$, but not to be confused with night-time leaf R_{CO_2}; see Table 1) in darkened leaves for comparison with D_{DL}. This requires a 'dark adaption' (or perhaps more correctly termed 'acclimation') of the leaf during the daytime prior to measurement and this issue has not received much attention in recent decades (Atkin, Evans, and Siebke 1998a, 1998b). The question therefore remains: how long must one wait following the abrupt darkening of a leaf at daytime before assigning a measured CO_2 efflux rate to $R_{CO_2,DD}$ to compare with D_{DL} and thus estimate the degree of inhibition of $R_{CO_2,DD}$ by light (i.e., $[1 - D_{DL}/R_{CO_2,DD}]$*100%)?

2 | On the Time Course of Daytime Dark-Acclimated Leaf R_{CO_2}, $R_{CO_2,DD}$

Post-illumination leaf respiratory metabolism during dark acclimation during daytime differs from that in the illuminated leaf during photosynthetic and photorespiratory activity (Atkin et al. 2000b; Noguchi and Yoshida 2008; Buckley and Adams 2011; Tcherkez et al. 2017a, 2017b). Further, a consensus is that upon abrupt imposition of a dark period during

TABLE 1 | Different types of measurements of leaf (non-photorespiratory) respiratory CO_2 efflux (R_{CO_2}).

Definition of types of measurements of leaf R_{CO_2}	Symbol applied here	Interpretation	Comments
Night-time R_{CO_2}	$R_{CO_2,N}$	Represents leaf R_{CO_2} at night + potential leaf efflux of CO_2 transported to the leaf from the stem and roots. $R_{CO_2,N}$ can exhibit some LEDR[a] in the first period after onset of darkness.	Historically considered a measure of '*dark respiration*,' primarily reflecting mitochondrial activity, and naturally occurring at night due to the absence of light. Even at constant temperature, $R_{CO_2,N}$ exhibits a nocturnal variation (Bruhn et al. 2022).
Daytime R_{CO_2} in light	$D_{,DL}$	Represents leaf R_{CO_2} at daytime in light + other daytime metabolic decarboxylation + potential leaf efflux of CO_2 transported to the leaf from the stem and roots. At very low light, though, LEDR[a] may occur as part of $D_{,DL}$ (Gauthier et al. 2020), i.e. organic acid metabolism.	Historically termed R_L or R_d to indicate respiration in the light or day respiration. Tcherkez et al. (2024) suggested the term 'diurnal net decarboxylations' for the estimated CO_2 flux measured during the daylight hours and in the presence of light. Even at constant temperature, $D_{,DL}$ exhibits a diurnal variation (Faber et al. 2022).
Daytime R_{CO_2} in abruptly darkened leaves	$R_{CO_2,DD}$	Represents, in principle, $D_{,DL}$ minus any light effects on any component underlying $D_{,DL}$. $R_{CO_2,DD}$ can, depending on the timing upon dark-acclimation exhibit a PIB[b] and an LEDR.	Not a naturally occurring CO_2 flux. As discussed in this Opinion, this flux is highly dependent on timing. In C3 species, there can be a PIB[b] (ca. 15–60 s after darkening) associated with photorespiratory spillover effects into the dark period. LEDR[a] typically peaks at ca. 3 min after darkening, after which it decreases.

[a]Light-enhanced dark respiration;
[b]post-illumination burst.

the day the leaf CO_2 efflux first exhibits a post-illumination burst (PIB, in C3 species) due to photorespiratory spillover effects (Bulley and Tregunna 1971; Doehlert, Ku, and Edwards 1979) that lasts 15–60 s (Brown and Gracen 1972; Doehlert, Ku, and Edwards 1979; Atkin, Evans, and Siebke 1998a, 1998b; Hoefnagel, Atkin, and Wiskich 1998; Atkin et al. 2000a; Parys and Romanowska 2000; Igamberdiev, Romanowska, and Gardeström 2001), which is not observed in C3 species at low $[O_2]$ (Atkin, Evans, and Siebke 1998a, 1998b). There is also a consensus that after the PIB there is a period of light enhanced dark respiration (LEDR) (Stokes et al. 1990; Reddy, Vani, and Raghavendra 1991; Atkin, Evans, and Siebke 1998a, 1998b; Atkin et al. 2000b; Parys and Romanowska 2000; Noguchi and Yoshida 2008; Buckley and Adams 2011) that peaks within ca. 3 min after the light–dark transition (Cornic 1973, Atkin, Evans, and Siebke 1998a, 1998b), and further that for estimating a daytime leaf $R_{CO_2,DD}$, one needs to wait at least until after this LEDR to measure a steady $R_{CO_2,DD}$ (Atkin et al. 2000b), which is only achieved after the respiratory substrate level has stabilised (Atkin et al. 1998b). There is, however, no clear consensus about how long one in should wait after darkening a leaf before daytime leaf $R_{CO_2,DD}$ is measured (Figure 1). In practice, researchers have dark-acclimated leaves for periods ranging from 0 to 60 min after light off (Figure 1). This is despite the very few published studies investigating or showing the length of the LEDR period (Figure 2). Thus, in most studies hitherto on the degree of light inhibition of leaf D_{DL} the scientific community appears to have decided the length of dark acclimation more or less blindly (Figure 1). In most studies with measurements of the time course of leaf $R_{CO_2,DD}$ from immediately upon dark acclimation, $R_{CO_2,DD}$ after the PIB appears to decrease for longer than 60 min (Figure 2) which is longer than the period assumed (or used) in most studies (Figure 1). Therefore, in most previous studies it is likely that leaf $R_{CO_2,DD}$ would have continued to decrease after measurement of leaf $R_{CO_2,DD}$ for comparison with D_{DL} (Figures 1 and 2). The speed of the decrease in dark-acclimated daytime leaf $R_{CO_2,DD}$ (even when measured at constant temperature) can depend on the relative rate of R_{CO_2} at the time of darkening. For example, different initial respiratory rates may be related to temperature (Figure 2, Azcón-Bieto and Osmond 1983) and the different initial respiratory activities among species (Figure 2, Parys and Jastrzębski 2006). The same phenomenon is also known for night-time decrease in leaf $R_{CO_2,N}$ when measured at constant temperature (Bruhn 2023). Therefore, it would be interesting with more studies in order to understand the rather large

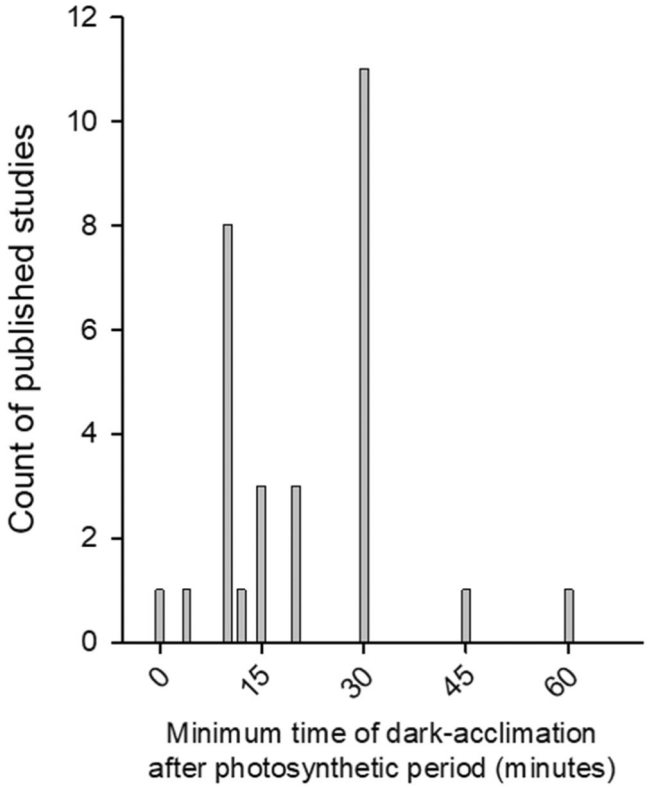

FIGURE 1 | Count of studies with varying length of the period of dark acclimation for measurements of leaf $R_{CO_2,DD}$ for comparison with estimates of leaf D_{DL}. A total of 36 studies were published between 1994 and 2024 (see Table S1), out of which 6 did not report the length of the dark acclimation period.

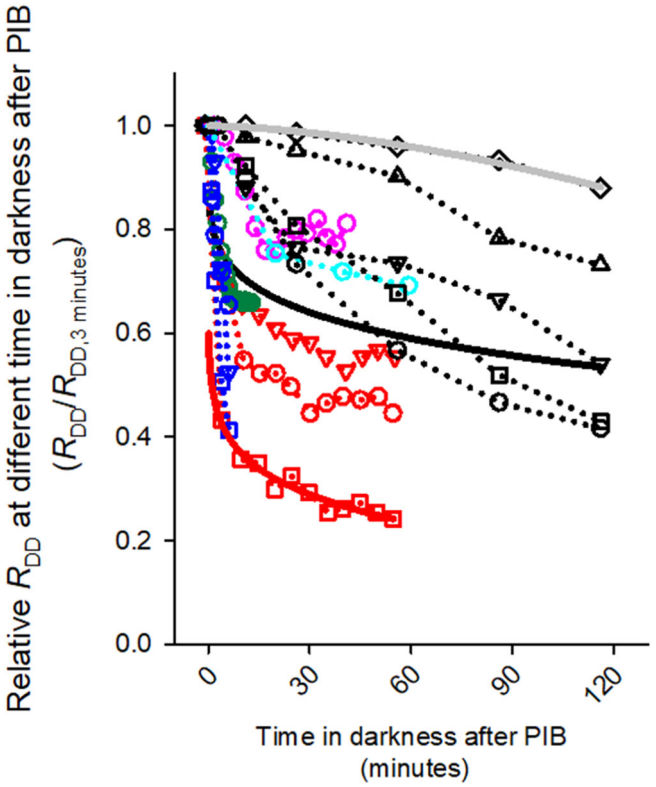

FIGURE 2 | All available published studies of the time course of dark-acclimated leaf $R_{CO_2,DD}$ measured after the 3 min period suggested in this Opinion. Both CO_2 efflux ($R_{CO_2,DD}$) and O_2 uptake ($R_{O_2,DD}$) are shown. List of studies and underlying data are available in Table S2. Red circles represent R_{CO_2} in *Zea Mays* (Parys and Jastrzębski 2006); red squares represent $R_{CO_2,DD}$ in *Panicum miliaceum* (Parys and Jastrzębski 2006); red triangles down represent $R_{CO_2,DD}$ in *Panicum maximum* (Parys and Jastrzębski 2006); green circles represent $R_{CO_2,DD}$ in *Nicotiana tabacum* L. cv. W51 (Atkin et al. 1998b); blue symbols represent $R_{O_2,DD}$ in *Pisum sativum* cv Arkel protoplasts (Reddy, Vani, and Raghavendra 1991) after 5 min (circles), 10 min (squares), or 15 min (triangles down) illumination prior to dark acclimation; pink circles represent $R_{CO_2,DD}$ in *Poa compressa* L. (Atkin et al. 1997); cyan circles represent *Festuca arundinacea* Schreb. (Parys and Romanowska 2000); black symbols represent $R_{CO_2,DD}$ in *Triticum aestivum* cv Gabo (Azcón-Bieto and Osmond 1983) measured at 30°C (circles), 27°C (squares), 24°C (triangles down), 20°C (triangles up), or 13.5°C (diamonds). Full black regression line ($f = y_0 + a^*x^b$, where $y_0 = 1$, $a = -0.1883 \pm 0.0315$ ($p < 0.0001$), $b = 0.1902 \pm 0.0486$ ($p < 0.0001$), $R^2 = 0.1873$, $n = 115$) represents all data. Full grey regression line ($f = y_0 + a^*x^b$, where $y_0 = 1$, $a = -0.0000539 \pm 0.0000313$ ($p = 0.1601$), $b = 1.6175 \pm 0.1255$ ($p < 0.0001$), $R^2 = 0.9927$, $n = 6$) represents the trace with least relative decrease in R_{DD}; full red regression line ($f = y_0 + a^*x^b$, where $y_0 = 1$, $a = -0.4959 \pm 0.0147$ ($p < 0.0001$), $b = 0.1059 \pm 0.0088$ ($p < 0.0001$), $R^2 = 0.9716$, $n = 12$) represents the trace with highest relative decrease in R_{DD}. [Color figure can be viewed at wileyonlinelibrary.com]

differences in the speed of the decrease in dark-acclimated daytime leaf $R_{CO_2,DD}$ (Figure 2).

3 | Mechanistic Considerations Underlying the Time Course of Daytime Dark-Acclimated Leaf $R_{CO_2,DD}$

During the LEDR (Figure 2) mitochondrial R_{CO_2} may be higher due to decarboxylation of organic acids accumulated during photosynthesis when some mitochondrial TCA cycle enzymes are inhibited by light (Atkin et al. 2000b; Noguchi and Yoshida 2008; Buckley and Adams 2011; Griffin and Turnbull 2012; Florez-Sarasa et al. 2012; Tcherkez et al. 2017a, 2017b). Upon the initial brief increase after the dark transition there is a rapid decrease in the concentration of malate, citrate and pyruvate (Figure 3). This may be the main reason for the continued decline over hours in leaf $R_{CO_2,DD}$ after abrupt darkening (Figure 2). On the other hand, the mitochondrial + cytosolic redox level (NADPH:NADP and NADP-MDH activation %) and energy level (ATP:ADP) both appear to decrease rapidly after the dark transition stabilizing at around a 50% level after 3 min (Figure 3). The decrease in the reduction level will immediately lead to a decrease in the thioredoxin-induced inhibition of TCA cycle enzymes (Daloso et al. 2015), i.e., to a stimulation of TCA cycle activity. Moreover, the potential for alternative oxidase engagement (AOXa1 relative expression) increases (Figure 3), meaning a reduced potential for mitochondrial ATP production. However, although AOX expression increases, the total AOX activity may actually decrease because of the decreased reduction level and the decreased pyruvate level. Together, these lines of evidence overall support the idea (Atkin et al. 2000b; Noguchi and Yoshida 2008; Buckley and Adams 2011; Tcherkez et al. 2017a, 2017b) that LEDR and the subsequent decrease in

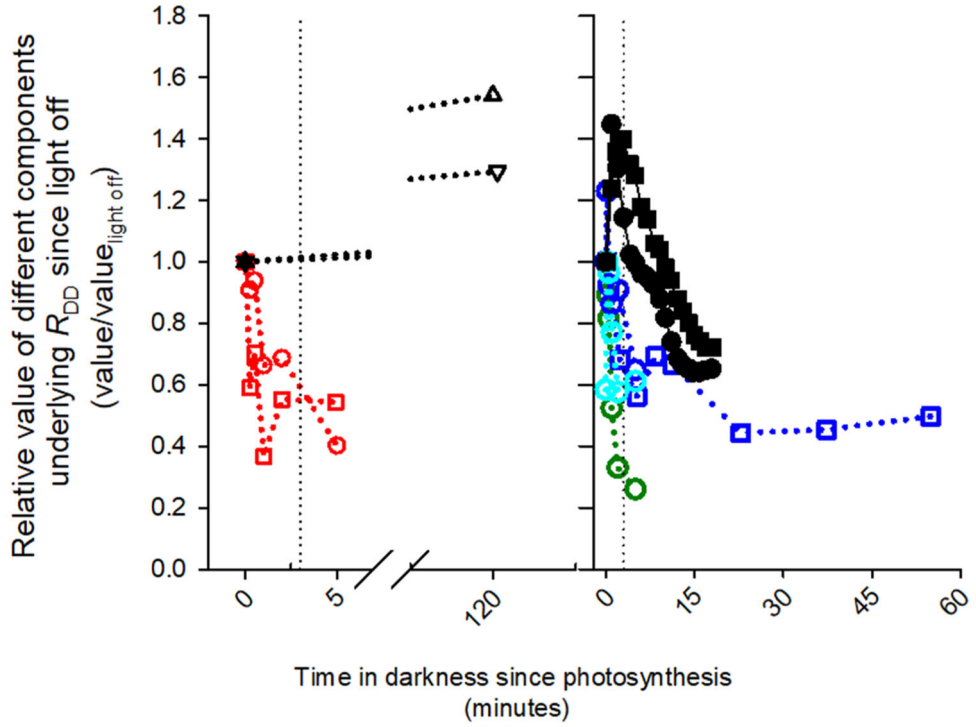

FIGURE 3 | Published studies of components underlying dark-acclimated leaf R_{DD} immediately after illumination expressed as values relative to value immediately at light–dark transition. For leaf cytosol + mitochondria in *Hordeum vulgare* L., cv. Gunilla, Svalöf, Sweden (Igamberdiev, Romanowska, and Gardeström 2001), ATP:ADP is represented by red circles, NADPH:NADP is represented by red squares, NADP-MDH activation % is represented by green circles, malate concentration is represented by blue circles, and citrate concentration is represented by cyan circles; malate concentration in *Ricinus communis* L. (Gessler et al. 2009) is represented by blue squares; AOXa1 relative expression in *Arabidopsis thaliana* (black symbols, Zhang et al. 2010) immediately after illumination with red (triangles down) or blue light (triangles up); filled black symbols represent pyruvate concentration (Lehmann et al. 2016) in *Halimium halimifolium* L. (circles) and in *Oxalis triangularis* A. St.-Hil. (squares). Vertical dotted reference lines indicate 3 min after darkening of leaves. [Color figure can be viewed at wileyonlinelibrary.com]

$R_{CO_2,DD}$ are largely functions of the fate of the organic acids (Figure 3) accumulated during photosynthesis prior to the dark acclimation. In experiments with *Ricinus communis* L. ^{13}C enrichment of the $R_{CO_2,DD}$, indicating use of organic acids, in the first 20 min of the dark period coincides with the LEDR (Barbour et al. 2007).

4 | Suggestion of a Fixed Timepoint for Measurement of Daytime Dark-Acclimated Leaf $R_{CO_2,DD}$ in the Absence of Own Measurements of the Length of LEDR

In contrast to all prior literature (see Table S1), we would as an alternative viewpoint argue that, since LEDR is considered to be a metabolic condition depending on the photosynthetic activity during illumination prior to abrupt darkening (Atkin, Evans, and Siebke 1998a, 1998b; Atkin et al. 2000b; Buckley and Adams 2011; Tcherkez et al. 2017a, 2017b), the 'best' estimate of non-photorespiratory leaf $R_{CO_2,DD}$ will be obtained by measuring the leaf R_{CO_2} during LEDR rather than avoiding it (Table 2). This is because, to study the degree of light inhibition (e.g., $[1 - D_{DL}/R_{CO_2,DD}]$*100%) at daytime, the basis for comparing D_{DL} should be conditions that are as close as possible to the conditions during photosynthesis in light prior to dark acclimation, bar the photorespiratory R_{CO_2} (i.e., Rubisco-mediated decarboxylation). Therefore, we suggest that leaf $R_{CO_2,DD}$ (for comparison with leaf D_{DL}) be measured as soon as possible after the PIB, but during the peak of LEDR (see Cornic 1973, Atkin, Evans, and Siebke 1998a for an example of high temporal resolution of leaf $R_{CO_2,DD}$ during daytime dark acclimation). This would mean that daytime leaf $R_{CO_2,DD}$ in C3 species should to be measured ca. 3 min after abrupt darkening (Atkin, Evans, and Siebke 1998a) in the absence of own measurements of the length of LEDR. In our opinion, following this new suggestion, would enhance the chance that the leaf $R_{CO_2,DD}$ during LEDR (after the PIB) is close to the maximum when organic acid catabolism is at a maximum. The further one waits for the measurement of leaf $R_{CO_2,DD}$ after the peak of LEDR, the less likely it is that the calculated apparent inhibition by light reflects the potential respiratory metabolism occurring in light, immediately prior to the abrupt darkening of leaves during daytime.

Experiments with *Helianthus annuus* var. Bashful indicate that even under photosynthetic conditions at low light an increased respiratory use of organic acids occurs in contrast to high light conditions (Gauthier et al. 2020). Thus, LEDR can occur even prior to an abrupt darkening of leaves (i.e., during D_{DL}), a condition we typically ascribe to the period after the darkening of leaves (i.e., during $R_{CO_2,DD}$). However, this does not alter our suggestion that, in order to compare with D_{DL}, $R_{CO_2,DD}$ should be measured during the assumed peak of LEDR (after the PIB) to ensure that conditions (during $R_{CO_2,DD}$) are as close as

TABLE 2 | Consequences of different timepoints for measuring leaf $R_{CO_2,DD}$.

Point in time in relation to the abrupt darkening of the leaf during daytime[a]	Consequences
0–3 min	CO_2-efflux is likely influenced by PIB, and leaf $R_{CO_2,DD}$ would be exaggerated when compared to the respiratory metabolism in the light
Ca. 3 min	Leaf $R_{CO_2,DD}$ is at or close to the maximum rate (after PIB) during LEDR and hence the closest possible comparison to respiratory metabolism in the light
After ca. 5 min	Leaf $R_{CO_2,DD}$ is likely affected by decreasing LEDR, and the calculated degree of inhibition by light is underestimated

[a]These considerations are guidelines in the case that the investigator has not established the precise time-course of leaf R_{CO_2} at daytime, in the light and upon abrupt darkening of the leaf during PIB, LEDR and post LEDR.

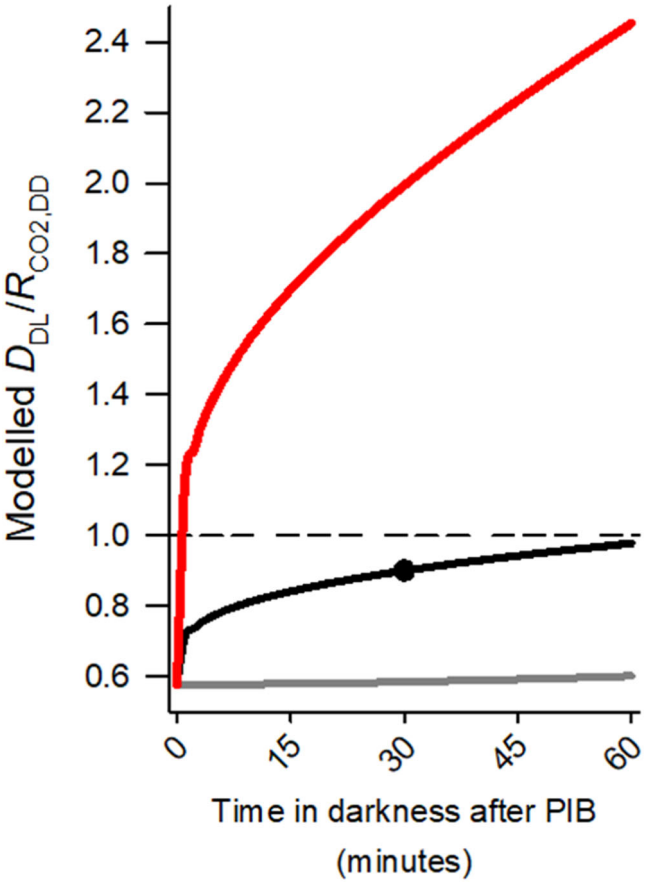

FIGURE 4 | Modelled $D_{DL}/R_{CO_2,DD}$ as a function of time. Underlying this modelling is a typical modest 10% observed light inhibition of leaf $R_{CO_2,DD}$, when $R_{CO_2,DD}$ most commonly is measured after 30 min of dark acclimation (see Figure 1) and therefore $D_{DL}/R_{CO_2,DD} = 0.9$ at 30 min (indicated with a black dot). Note that "0.9" here denotes a "modest" 10% inhibition by light. Black line represents the calculated time course in $D_{DL}/R_{CO_2,DD}$ using the full black regression through all data of R_{DD} (see Figure 2); grey line represents the calculated time course in $D_{DL}/R_{CO_2,DD}$ using the full grey regression through only the trace of least relative decrease in R_{DD} (see Figure 2), red line represents the calculated time course in $D_{DL}/R_{CO_2,DD}$ using the full red regression through the trace of highest relative decrease in R_{DD} (see Figure 2). Note that the$R_{CO_2,DD}$ is here assumed to have a value of 1.0 at time zero before the temporal decrease during dark acclimation and D_{DL} is assumed to be 0.9 during the entire dark acclimation period as it is typically estimated during illumination prior to dark acclimation. [Color figure can be viewed at wileyonlinelibrary.com]

possible to the conditions during photosynthesis in light prior to dark acclimation. Only in this way can the effect of light vs. darkness (e.g., $[1 - D_{DL}/R_{CO_2,DD}]$*100%) be studied. Furthermore, photorespiration is known to affect both protein phosphorylation and mitochondrial redox poise and can lead to the inactivation of TCA cycle enzymes (Møller, Rasmusson, and Van Aken 2020, 2021). However light inhibition of the CO_2-producing PDC completely disappears within 5 min of turning off the light (Gemel and Randall 1992), indicating that the inhibitory effects of photorespiration during the preceding light period have also disappeared within 5 min of darkness. This dynamic regulation of mitochondrial enzyme activities in response to darkness highlights the need to standardize the timing of flux measurements if we hope to elucidate the biochemical and physiological mechanisms controlling of $R_{CO_2,DD}$ and/or the biological and ecological consequences of the light inhibition.

5 | Consequences of a Fixed Timepoint for Measurement of Daytime Dark-Acclimated Leaf $R_{CO_2,DD}$

After the first ca. 3 min of dark acclimation, leaf $R_{CO_2,DD}$ is likely continuously decreasing (Figure 2) due to a decrease in the concentrations of TCA substrates (Figure 3). Beyond this time, the level of $R_{CO_2,DD}$ increasingly diverges from that during illumination leading to a change in the calculated light inhibition of D_{DL} (i.e., $[1 - D_{DL}/R_{CO_2,DD}]$*100%, Figure 4, Table 2) which likely accounts for much of the variation among studies. This depends on both speed of relative decrease in $R_{CO_2,DD}$ (Figures 2 and 4) and the timing of measurement of $R_{CO_2,DD}$ to compare with D_{DL} (Figures 1 and 4). In the 'typical' case of 30 min dark acclimation (Figure 1) and a mean rate constant of the temporal relative decrease in $R_{CO_2,DD}$ (Figure 2) a previously reported modest calculated degree of light inhibition (i.e., $[1 - D_{DL}/R_{CO_2,DD}]$ *100%) of 10% (Figure 4) will result in $[1 - D_{DL}/R_{CO_2,DD}]$ *100% = 58% instead, if $R_{CO_2,DD}$ was estimated 0 min after the PIB (i.e., ca. 3 min after start of dark acclimation) (Figure 4), which is a substantial change in the calculated degree of light inhibition. Here we have chosen to show the example of ca. 10% inhibition by light, because (i) it is close to average morning values obtained with the Kok-method (corrected for changes in C_i) across 13 deciduous, 4 evergreen and 4 herbaceous species from humid continental and humid

subtropical climates in the field using 30 min of dark-acclimation (Faber et al. 2022) and (ii) it illustrates the potential size of the change in calculated degree of inhibition by light simply by changing the timing of the measurement of $R_{CO_2,DD}$ from the normal 30 min. A further complication could be time of day as leaf D_{DL} and dark-acclimated leaf $R_{CO_2,DD}$ exhibit different diurnal variation when measured at constant temperature (Florez-Sarasa et al. 2012; Faber et al. 2022) and the cellular organic acid content appears to increase severalfold during the daytime (Flis et al. 2019).

6 | Concluding Remarks and Perspectives

We suggest that in the future daytime leaf $R_{CO_2,DD}$ is measured 3 min after dark acclimation (in the absence of own measurements of LEDR, Table 2) to avoid the problems illustrated in Figure 4 when investigating the degree of light inhibition of leaf D_{DL}. This will most likely result in higher degrees of calculated light inhibition of daytime leaf D_{DL} than reported previously (Figures 1 and 4), speed up future surveys, and hopefully result in less inter-study variation in the calculated degree of light inhibition of leaf D_{DL}. It is even possible that earlier reports of a light stimulation of leaf D_{DL} (i.e., $D_{DL}/R_{CO_2,DD} > 1$; Zaragoza-Castells et al. 2007; Atkin et al. 2013; Crous et al. 2017; Faber et al. 2022) to some extent have been a consequence of the issues illustrated in Figure 4. Finally, we hope that our suggestion will lead to better agreement between leaf level and ecosystem level studies of light inhibition of leaf D_{DL} for certain biomes (Keenan et al. 2019).

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data are available in the listed publications (Tables S1 and S2) underlying Figures 1 and 2 and in publications listed in the figure texts.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.