SCIENTIFIC ARTICLE

Analyses of OJIP transients in leaves of two epiphytic orchids under drought stress

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Abstract

The tolerance to low water availability is a decisive factor for growth and survival of orchids in their natural environment. The objective of this study was to characterize the photochemical traits of two epiphytic orchids (*Cattleya warneri* and *Miltonia spectabilis*) under water deficit (WD). Chlorophyll *a* fluorescence signals were recorded from young and fully expanded leaves of 5 plants/species after dark-adaption for 60 minutes, between 6-9 a.m. after 0, 30, 60, and 90 days of WD, using a Handy-PEA fluorometer (Hansatech, UK). Increases of O-J and J-I phases and L and K-bands and decreases of I-P phase were observed after 30 days of WD, especially in *C. warneri*. Decreases in the capacity to photochemically reduce quinone A (Q_A) and the kinetic properties required for redox reactions of the plastoquinone pool, the loss of energetic connectivity between units of PSII, inactivation of the oxygen evolution complex, and decrease of the overall rate of reducing the electron acceptor pool of photosystem I were observed in *M. spectabilis*, a more tolerant species. The greater ability of this species to maintain higher relative water content (RWC) in photosynthetic tissues allows greater photochemical activity.

Keywords: *Cattleya warneri*, *Miltonia spectabilis*, chlorophyll *a* fluorescence, K-band, photosystem II.

Resumo

Análise dos transientes OJIP em folhas de duas orquídeas epífitas submetidas ao estresse hídrico

A tolerância à baixa disponibilidade hídrica é fator decisivo para o crescimento e sobrevivência das orquídeas em seu ambiente natural. O objetivo deste estudo foi caracterizar os atributos fotoquímicos de duas orquídeas epífitas (*Cattleya warneri* e *Miltonia spectabilis*) sob déficit hídrico (WD). A fluorescência da clorofila *a* foi registrada em folhas jovens e totalmente expandidas de 5 plantas/espécie adaptadas ao escuro por 60 minutos, entre 6 e 9 h da manhã, a 0, 30, 60 e 90 dias de WD, usando um fluorômetro Handy-PEA (Hansatech, Reino Unido). Aumentos nas fases O-J e J-I, bandas L e K e diminuição da fase I-P foram observados após 30 dias de DW, principalmente em *C. warneri*. A diminuição da capacidade de redução da quinona A (Q_A) e das propriedades cinéticas requeridas para reações redox do *pool* de plastoquinona, a perda de conectividade energética entre unidades de fotossistema II (FSII), a inativação do complexo de evolução do oxigênio e a manutenção da taxa global de redução do *pool* aceptores de elétrons do fotossistema I foram observadas em *M. spectabilis*, espécie evidentemente mais tolerante. A maior habilidade dessa espécie em manter o conteúdo relativo de água nos tecidos fotossintéticos permite maior atividade fotoquímica.

Palavras-chave: *Cattleya warneri*, *Miltonia spectabilis,* fluorescência da clorofila *a*, Banda K, fotossistema II.

Introduction

Among abiotic factors, low precipitation is among the environmental factors with the greatest negative effect on the survival of epiphytic plants. Rainfall can be highly variable in space and time, thus affecting plant-water relationships (Grzesiak et al., 2019; Guevara-Perez et al., 2019). Overall, the low growth of plants in environments with low precipitation is probably due to physiological stress factors, such as low photosynthetic rate of chlorotic leaves.

Previous studies have often found diminished fitness of plants growing under low precipitation in relation to well-watered plants. Among the main physiological disturbances caused by water deficit are lower cell turgidity, variations of stomatal opening and chloroplast structure, and decreased transpiration and CO_2 assimilation rate (Lang et al., 2018). Reduced CO_2 assimilation can

result in damage to photosystem II (PSII) reaction centers (RC), and consequently upset the equilibrium between photochemical activity of PSII and electron requirement for photosynthesis, generating over-excitation of the photosynthetic apparatus and photoinhibitory damage to PSII RC, in turn reducing the functional state of the photosynthetic apparatus (Pollastrini et al., 2017). Several research groups have reported the negative influence of water deficit on the physiological and photosynthetic properties of orchid plants (Guevara-Pérez et al., 2019; Li and Zhang, 2019; Tay et al., 2019).

Short rainless periods are common in Brazil, such as in northern Espírito Santo State, which typically has 3-4 months of drought every year. In this period, epiphytes are subject to changes in ecological conditions, such as increased temperatures and reduced air humidity. Nevertheless, an evolutionary advantage of epiphyte plants is the capacity to utilize alternative sources of water such as fog and dew. According to Wu et al. (2018), fog contributes 20% or more of the total water input in cloud forests, including the water used by orchids. However, these water sources are usually not enough to maintain positive water balance in plants during low precipitation seasons.

In vivo chlorophyll *a* fluorescence (Chl*a*F) measurements have been widely used to evaluate the functional state of the photosynthetic apparatus, especially PSII, providing useful physiological indicators to study plants' sensitivity to abiotic stress (Borawska-Jarmulowicz et al., 2020). It is possible to differentiate between drought-resistant and drought-sensitive species using Chl*a*F measurements as well as to identify the action site of stress on the photosynthetic electron transport chain (Oukarroum et al., 2016; Falqueto et al., 2017; Kalaji et al., 2018).

Considering that water availability is the abiotic factor that most affects the physiological traits of epiphytes, in this study we assumed that relative water content and *in vivo* Chl*a*F would be important traits to differentiate adaptive strategies of the photosynthetic apparatus in orchid plants growing under water stress conditions. Finally, we expected the regulation of specific steps of photosynthetic electron transport to indicate different levels of drought resistance among species. Our objective was to characterize the photochemical traits of two epiphytic orchids (*Cattleya warneri* T. Moore. and *Miltonia spectabilis* Lindl.), both evolved to cope with water deficit. We were interested in obtaining new information to better understand the adaptive strategies of epiphytic orchids to water deficit.

Material and Methods

Plants, growth conditions, and drought treatment

The experiment was performed using plants of two orchid species [*Cattleya warneri* T. Moore. and *Miltonia spectabilis* Lindl., CAM and C$_3$ species, respectively, according to Adelberg et al. (1998) and Pires et al. (2012)], which are very common epiphytic orchids in Brazil. The plants were obtained from a commercial nursery and were planted in 2-liter plastic pots (one plant per pot) containing pine bark + carbonized rice husk, mixed at the ratio of 1:1 and maintained in greenhouse conditions (18°43'0''S; 39°51'34''W) for acclimation. The photoperiod (light/dark) and relative air humidity in the greenhouse were 14/10 h and 75%, respectively. The plants were subjected to two different irrigation treatments: 50% of the plants were maintained under water deficit (non-irrigated plants) and 50% were maintained in well-watered conditions (control, manually irrigated daily by adding 300 mL of water). All plants were protected against bacterial pathogens and weed competition. Water deficit was imposed by completely removing irrigation. The plants remained under persistent drought for 90 days. All measurements were performed in the morning (between 6-9 a.m.) every 30 days up to 90 days of water deficit (0, 30, 60, and 90 d of WD).

Leaf relative water content (RWC)

Leaf RWC was measured as previously described by Barrs and Weatherley (1962). Five leaf discs per plant (diameter 0.6 cm) were sampled and immediately weighed (fresh mass-FM). Then they were immersed in distilled water in Petri dishes for 24 h at 25 ºC under a photon flux density of 40 μmol m^{-2} s^{-1} on filter paper and the turgid mass (TM) was determined. Next, the discs were dried in an oven at 70 ºC for 48-h, until reaching constant weight, showing no water in the tissue, to obtain the dry mass (DM). RWC was calculated using the following equation: RWC = (FM − DM)/(TM − DM) × 100. The results were expressed in percentage (%).

Chl *a* fluorescence transient (Chl*a*F)

Chl*a*F measurements were conducted on young and fully expanded leaves (second leaf from the apex) at room temperature using a plant efficiency analyzer (Handy-PEA, Hansatech, King's Lynn, Norfolk, England) as described by Kalaji et al. (2018). Before the measurements, the leaf samples were dark-adapted using leaf clips (Hansatech, UK) for 60 minutes to promote total oxidation of the photosynthetic system. The fast Chl*a*F kinetics (Fo to Fm) was induced by 1 s pulses of red light [650 nm, 3000 μmol(photons) m^{-2} s^{-1}], which was sufficient to generate maximal fluorescence for all the treatments. "O" refers to the initial minimal fluorescence level (F$_0$); P (≈300 ms) is the peak; and J (≈2 to 3 ms) and I (≈30 ms) are inflection points between the O and P levels. All normalizations of the Chl*a*F transients were carried out according to Yusuf et al. (2010). The variable relative fluorescence between O (50 μs) and P (300 ms) steps was normalized as V$_t$ = (F$_t$ − F$_0$) / (F$_P$ − F$_0$) and ΔV$_t$ = [V$_{t\ (treatment)}$ − V$_{t\ (control)}$]. The I–P phase was analyzed utilizing two different normalization procedures: V$_{OI}$ ≥ 1 [normalization between O- and I-steps, only the part with V$_{OI}$ ≥ 1] and V$_{IP}$ [normalization between I- and P-steps]. The kinetic difference ΔV$_{IP}$ was calculated as ΔV$_{IP}$ = V$_{IP(treatment)}$ − V$_{IP(control)}$ and is shown on a linear scale between 30 and 180 ms.

Experimental design and data analyses

The experimental design was completely randomized using two orchid species submitted to WD during 90 days. The experiment was performed with five replicates per treatment. The Biolyser 4HP software (Biolyser© R. M. Rodriguez, Bioenergetics Laboratory, University of Geneva, Geneva, Switzerland, version 3.06) was used to obtain the OJIP transients, and Excel 2013 was used to plot the transient graphs. RWC data were submitted to analysis of variance to compare the days of DW for each species. For significant values, means were compared by the Tukey test at P < 0.05 and expressed followed by the standard error (±SE) using the Sisvar® program (Ferreira, 2011).

Results

The RWC values decreased in both orchid species, from 92.7% and 93.3% to 45.8% and 54.4% after 90 days of water suppression in *C. warneri* and *M. spectabilis*, respectively, representing reductions of about 50.5% and 41.6% compared to WD-0 (Figure 1). Reductions of RWC occurred from WD-30 in *C. warneri* and only from WD-60 in *M. spectabilis*, which maintained higher values from WD-30 to WD-90.
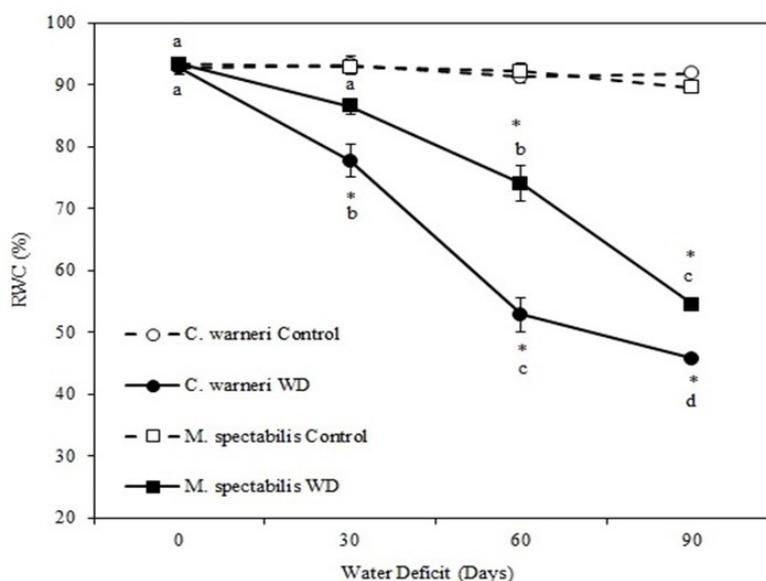
Figure 1. Relative water content (RWC) in *Cattleya warneri* and *Miltonia spectabilis* leaves subjected to water deficit (WD). Vertical bars indicate the standard error (±SE). The letters indicate difference between the days of WD and the asterisks indicate difference between treatment and control by the Tukey test at 5% probability (n = 5).

Increases of the J-step were observed at 30 days of water deficit (WD-30) in both species and persisted until WD-90 (Figures 2A and 2B). However, the J-step values were higher in *C. warneri*. Also, a slight increase of the I-step occurred in *C. warneri* (Figure 2A). Furthermore, the analyses of ΔV_t evidenced the appearance of positive bands between the O-J and J-I phases (Figures 2C and 2D). This occurred from 30 days of water deficit (WD-30) onward in both orchid species. However, the amplitude of the curves was more positive in *C. warneri* (approximately 20% at WD-90) compared to *M. spectabilis* (≈10%) (Figures 2C and 2D). In *M. spectabilis*, the WD effects observed on ChlaF at WD-90 (maximum values obtained for this species) were similar to those observed at WD-30 in *C. warneri* (Figures 2C and 2D).
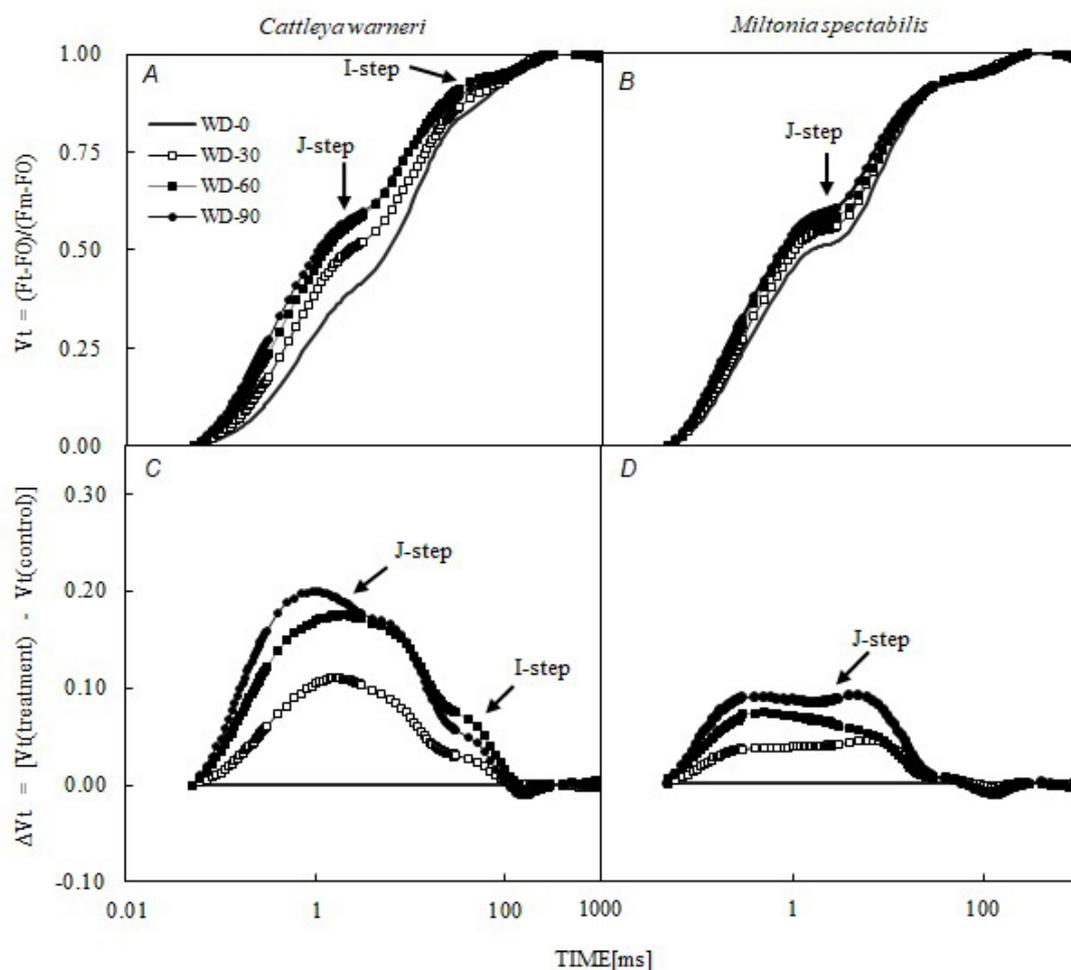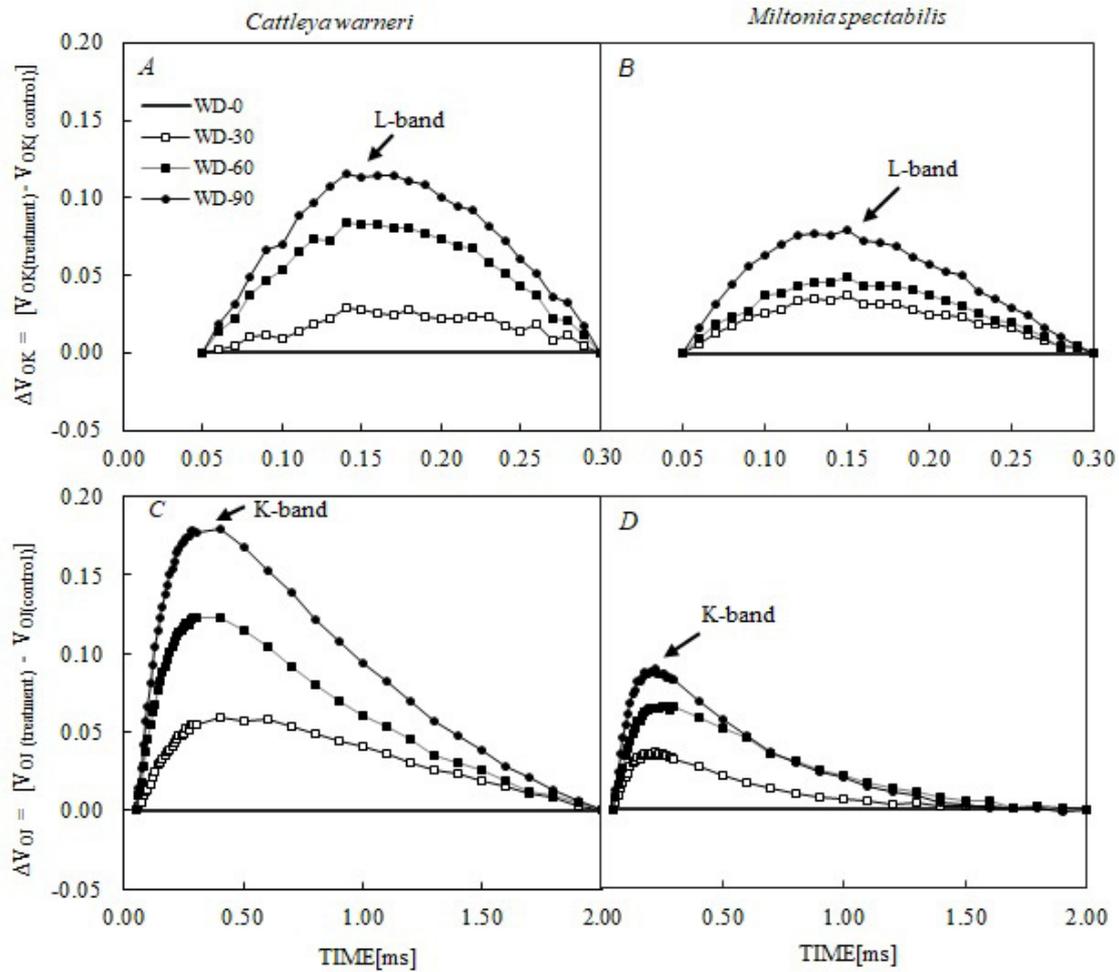
Figure 2. Kinetics of chlorophyll *a* fluorescence normalized between steps O-P in *Cattleya warneri* and *Miltonia spectabilis* submitted to water deficit (WD) for 90 days. Before the measurements, leaves were dark-adapted for 1 h. Relative variable fluorescence $V_t = (F_t - F_0)/(F_P - F_0)$ (A and B), and kinetic difference of V_t[$\Delta V_t = [V_t(\text{treatment}) - V_t(\text{control})]$ (C and D). The values are means, n = 5.

In both species, the energetic connectivity (L-band) and stability of the oxygen evolution complex-OEC (K-band) decreased after WD-30 and reached a minimum on the day of maximum stress (approximately 12% and 18% in *C. warneri* and ≈ 8% and ≈9% in *M. spectabilis* for the L- and K-bands, respectively) (Figure 3). However, in *C. warneri*, the increment observed in both L- and K-bands occurred gradually from WD-30 to WD-90 (Figures 3A and 3C). For *M. spectabilis*, the effects of water deficit on the energetic connectivity were grouped at two levels: 1- from WD-30 to WD-60 (characterized by similar L-band values) and 2- WD-90 (where the maximum L-band value was recorded).

Figure 3. Kinetic differences of V_{OK} and V_{OJ} obtained after double normalization between the steps O–K $[\Delta V_{OK} = V_{OK(treatment)} - V_{OK(control)}]$ (A and B), showing the L-band, and between the steps O–J $[\Delta V_{OJ} = V_{OJ(treatment)} - V_{OJ(control)}]$ (C and D), showing the K-band, respectively, in *Cattleya warneri* and *Miltonia spectabilis* submitted to water deficit (WD) for 90 days. Before the measurements, leaves were dark-adapted for 1 h. The values are means, n=5.

For *C. warneri*, the normalized curves of $V_{OI} \geq 1$ decreased with WD, reaching minimum values at WD-60 and WD-90 (Figure 4A). Conversely, no change in $V_{OI} \geq 1$ was recorded in *M. spectabilis* during the study (Figure 4B). The data normalized as V_{IP} and ΔV_{IP} declined in both species in relation to WD-0. At WD-90, the V_{IP} and ΔV_{IP} values were similar between species (Figures 4C-F).
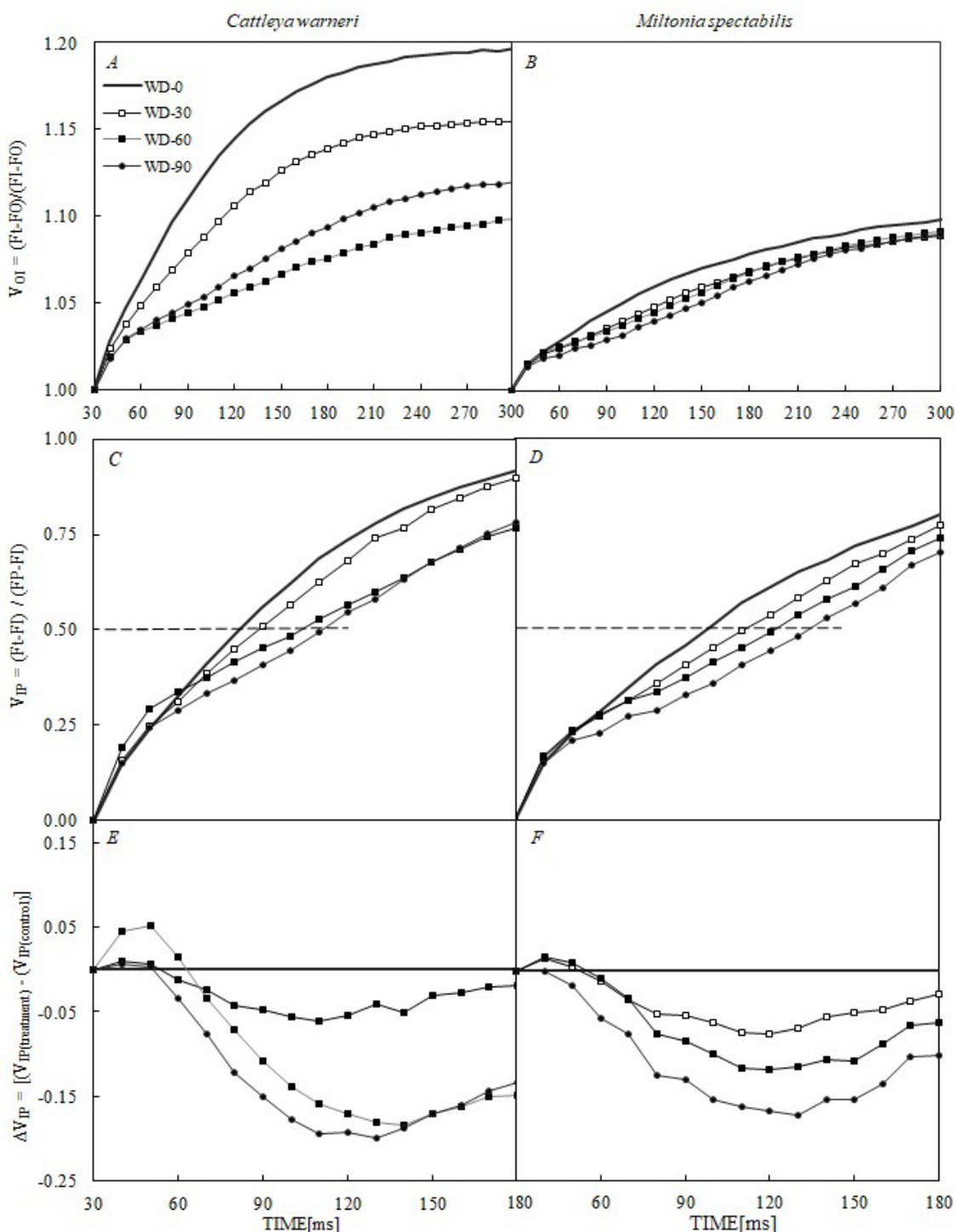
Figure 4. Kinetics of chlorophyll *a* fluorescence normalized between steps O-I and I-P of *Cattleya warneri* and *Miltonia spectabilis* plants submitted to water deficit (WD) for 90 days. Before the measurements, leaves were dark-adapted for 1 h. Relative variable fluorescence between the steps O-I [$V_{OI} = (F_T - F_0)/(F_I - F_0)$] in the interval between 30 and 300 ms (A and B), relative variable fluorescence between the steps I-P [$V_{IP} = (F_t - F_I)/(F_P - F_I)$] (C and D). The dashed lines indicate the half-life, the inverse of the time to reach $V_{IP} = 0.5$, as described by the Michaelis-Menten equation), and kinetic differences of V_{IP}: [$\Delta V_{IP} = V_{IP\ (treatment)} - V_{IP(control)}$] (E and F). The values are means, n=5.

Discussion

In this study, we compared the photochemical responses of leaves of two orchid species to WD in order to identify the action site of WD in the electron transport chain on the acceptor side of PSII. It is known that water stress results in damage to the photosynthetic apparatus and reductions of stomatal aperture, light absorption and biochemical reaction of CO_2 fixation, as reported previously by Falqueto et al. (2017) and Martins et al. (2017). In this study, the Chl$a$F technique proved to be an efficient tool to identify the differences between species.

During the experiment, the plants submitted to WD showed common characteristics of this type of stress, such as wilting of leaves, which occurred around 60 days after water deprivation, reaching a maximum at around the 90 days in both orchid species. Also, leaf abscission occurred in *M. spectabilis* after 60 days of drought. Leaf abscission is considered an effective mechanism to prevent drought damage during periods of low water availability (Naidoo and Naidoo, 2018). Before abscission, senescence occurs. Senescence is initially deleterious, but is an important evolutionary process in plants' life cycle that contributes to their fitness, ensuring survival in certain niches. Furthermore, senescence and abscission can be considered important processes to maintain the relative water content, contributing to mobilization of water, as well as promoting exportation of reserve compounds from wilted and old leaves to young leaves after catabolic conversion of the cell materials accumulated during the leaf growth phase, as described by Yang et al. (2016). These compounds serve as nutrients to maintain young leaves.

The increases observed at the J-step in *C. warneri* and *M. spectabilis* and the I-step in *C. warneri*, with consequent appearance of a positive band in the O-J and J-I phases, are an indication of limited electron flux resulting from the accumulation of PSII acceptors (Q_A, Q_B) and PQ pool in reduced forms (Zushi and Matsuzoe, 2017) (Figure 1). Increases in the J-step were also the main typical characteristics of the Chl$a$F transient OJIP in *Augea capensis* Thunb. and *Zygophyllum prismatocarpum* E. Meyer *ex* Sond, growing in the arid Namib Desert (Heerden et al., 2007). This increase of the J-step is attributed to the peak concentration of $Q_A^-Q_B$ and $Q_A^-Q_B^-$ originated from the electron transport chain, where electrons are transferred from Q_A to Q_B (Goltsev et al., 2016). This appears to be a common response of several species to environmental stress. As proposed by Redillas et al. (2011), this occurs because PSI is less tolerant to drought than PSII, so the efficiency of electron transport to PSI is lower and causes the accumulation of reduced forms ($Q_A^-Q_B^-$). In the present study, we observed that starting at 30 days of WD there was accumulation of reduced forms of the intermediaries of the electron transport chain, limiting the photochemical flow between PSII and PSI in both species (Xin et al., 2013). However, this limitation was more clearly observed in *C. warneri*, despite the characteristic leaf of this orchid species. This result is surprising, since higher tolerance is expected of CAM plants compared to C_3 plants when exposed

to water stress (Niechayev et al., 2019). Those authors reported that plants with both C_3 and CAM metabolism are susceptible to the effects of water deficit, but the influence of water deficit on the photochemical activity was revealed previously to be stronger in C_3 than CAM plants. Moreover, the similar values of ΔV_t observed between WD-90 for *M. spectabilis* and WD-30 for *C. warneri* revealed that *M. spectabilis* is better able to photochemically reduce Q_A and maintain the kinetic properties required for reduction/oxidation of plastoquinone under WD. This fact may be related to the better ability to preserve the relative water content of the leaves for a longer period in *M. spectabilis*.

Also, the analyses of the Chl$a$F transient allowed us to identify the appearance of L- and K-bands. According to Oukarroum et al. (2016), under drought stress, the appearance of both L- and K-bands should be a good indicator of physiological disturbances before the manifestation of visual damage. The L-band (150 µs) is an indicator of energetic connectivity or grouping between the subunits of PSII and is related to maintenance of the structure/stacking of thylakoids in chloroplasts (Rosa et al., 2018). The L-band can assume positive or negative values, being more positive with lower energetic connectivity and less grouping of subunits (Yusuf et al., 2010). The results presented in Figures 3A and 3B show decreases in the energetic connectivity (positive L-bands) due to the effect of WD starting at 30 days (WD-30) in both *C. warneri* and *M. spectabilis* plants. This disturbance occurred gradually and was more pronounced in *C. warneri*, reaching values approximately 12% higher after 90 days of water deficit. On the other hand, in *M. spectabilis*, there was higher stability of energetic connectivity with water deficit, since the L-band values at 30 and 60 days were similar and only 5% higher compared to the control conditions (WD-0). Only at WD-90 did the L-band rise (≈8%) in relation to the control. These results show that the effect of WD on the stability and energetic connectivity in the C_3 species is more effective only after 60 days.

The presence of the K-band, which occurred around 300 µs, indicates the inactivation of OEC and/or increased antenna size of PSII (Yusuf et al., 2010). The pronounced K-band can be explained by energetic imbalance between the electron donor and acceptor sides in PSII, where the electron flow from OEC to P_{680} on the donor side of PSII is slower than the electron flow from PSII to Q_A on the acceptor side (Yusuf et al., 2010; Santos et al., 2020). In this study, the increases observed in the K-band in both species can be attributed to the inactivation of the OEC, considering that the water stress caused the appearance of positive bands starting from WD-30. Regarding the K-band values, the effects of WD were more pronounced in *C. warneri*, in which a gradual increase of the K-band values was recorded during the WD period, reaching values ≈ 18% higher than those observed at WD-0 (Figure 3C).

Holland et al. (2015) reported that WD also reduced the energetic connectivity and caused inactivation of the OEC in plants of *Agave salmiana* and *Quercus pubescens*, respectively. In this study, the higher stability and energetic connectivity between subunits of PSII and the better OEC

performance as well as the invariability of photochemical flow of electrons between PSII and PSI in *M. spectabilis* can be explained by the high RWC values found in this species. Another possible explanation is the occurrence of photorespiration in *M. spectabilis* (C_3 species). Photorespiration is characterized by increased oxygenation rates of Rubisco and decreased carboxylation rates, resulting in decreased photosynthesis. The photorespiratory pathway stimulates the utilization of photons when the CO_2 supply is limited by WD, consuming ATP and reducing energy use, protecting the photosynthetic apparatus against oxidative damages (Zhang et al., 2016).

The normalization between the I- and P-steps permits deducing the behavior of the electron transfer from the intersystem to the end electron acceptors of PSI. As described by the Michaelis-Menten equation, $V_{IP} = 0.5$ (highlighted with dashed lines in Figures 4C and 4D) is the half-life, the inverse of the time to reach $V_{IP} = 0.5$, defined as an estimate of the global rate of reduction of the end electron acceptors of PSI (Yusuf et al., 2010). In this study, we observed decreased capacity of adjustment of the reduction process of the end electron acceptor of PSI, which can be directly responsible for the accumulation of reduced Q_A^- in both species (*C. warneri* and *M. spectabilis*), as previously observed through analysis of the J-step.

Furthermore, these differences in adjustment of the reduction process associated with the end electron acceptor of PSI between days of WD can be observed more accurately by analyzing the kinetic differences ΔV_{IP} (Yusuf et al., 2010). Figures 4E and 4F show similar behavior between species, with progressive decrease of the regulation rate of the end electron acceptor reduction until WD-90 in *C. warneri* and *M. spectabilis*. Reductions in ΔV_{IP} were described by Jiang et al. (2008) in *Citrus grandis* plants under aluminum (Al) stress and by Yusuf et al. (2010) in transgenic *Brassica juncea* plants submitted to salt, heavy metal and osmotic stress. As previously noted by Yusuf et al. (2010) and Redillas et al. (2011), the regulation of the global reduction rate of the electron acceptors (V_{IP} and ΔV_{IP}) (Figures 4C and 4F) occurred independently of the regulation of the end electron pool size on the PSI acceptor side (V_{OI}) (Figures 4A and 4B). While in *C. warneri* there was decreased regulation of end electron acceptor reduction of PSI and the regulation of end electron pool size (Figures 4A, 4C and 4E) under WD, *M. spectabilis* had decreases only of the regulation of end electron acceptor reduction of PSI (Figures 4B, 4D and 4F).

Conclusions

In conclusion, the photosynthetic apparatus of *M. spectabilis* was more tolerant to WD than that of *C. warneri*, as shown mainly through of activities related to PSII and the intersystem (ΔV_T curves, K and L-bands). Although the regulation of end electron pool size differed between the species, the similarity of regulation of end electron acceptor reduction of PSI showed that the distinct behavior between the species was restricted specifically to the behavior of PSII. The result found in this study can be attributed to the photoprotective role of photorespiration in the photosynthetic apparatus, but especially was due to the better ability of C_3 species to maintain higher relative water content (RWC) during the experiment. More water in photosynthetic tissues allows greater photochemical activity.

Author Contribution

JBZ: investigation, writing - original draft, formal analysis. **CLP:** investigation, writing original draft, formal analysis. **VFS:** investigation, writing - original draft, formal analysis. **PCSB:** Writing - original draft, formal analysis. **JPRM:** review, editing. **DMS:** review, editing, conceptualization, methodology. **ARF:** writing, review, editing, conceptualization, methodology.

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