Comparative Analysis of Primary Photosynthetic Reactions Assessed by OJIP Kinetics in Three *Brassica* Crops after Drought and Recovery

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Abstract: Plant drought tolerance depends on adaptations of the photosynthetic apparatus to changing environments triggered by water deficit. The seedlings of three *Brassica* crops differing in drought sensitivity, *Brassica oleracea* L. var. *capitata*—white cabbage, *Brassica oleracea* L. var. *acephala*—kale, and *Brassica rapa* L. var. *pekinensis*—Chinese cabbage, were exposed to drought by withholding water. Detailed insight into the photosynthetic machinery was carried out when the seedling reached a relative water content of about 45% and after re-watering by analyzing the OJIP kinetics. The key objective of this study was to find reliable parameters for distinguishing drought−tolerant and drought-sensitive varieties before permanent structural and functional changes in the photosynthetic apparatus occur. According to our findings, an increase in the total performance index (PI_{total}) and structure–function index (SFI), positive L and K bands, total driving forces (ΔDF), and drought resistance index (DRI) suggest drought tolerance. At the same time, susceptible varieties can be distinguished based on negative L and K bands, PI_{total}, SFI, and the density of reaction centers (RC/CS_0). Kale proved to be the most tolerant, Chinese cabbage was moderately susceptible, and white cabbage showed high sensitivity to the investigated drought stress. The genetic variation revealed among the selected *Brassica* crops could be used in breeding programs and high-precision crop management.

Keywords: drought tolerance; chlorophyll *a* fluorescence; structure–function index; drought resistance index

1. Introduction

Brassica crops originate from an area with mild winters and warm, dry summers. Numerous species of the *Brassica* genus were modified and domesticated and are ranked among the top five main vegetable crops worldwide. However, most of these vegetables, including white cabbage, Chinese cabbage, and kale, are grown in the Mediterranean region [1,2]. According to the Organization for Food and Agriculture of the United Nations (FAO) report, between 2000 and 2020, the share of cabbages was almost halved [3]. However, brassicas have received much attention in the last few years. High levels of nutrients and health-promoting phytochemicals (phenolic compounds, vitamins, β-carotene, lutein, glucosinolates) have classified them as a "functional food" [4–6]. Kale has frequently been promoted as a "superfood," but no scientific evidence exists to confirm its superiority over other cruciferous vegetables [7]. The advantages of kale are reflected in its tolerance to unfavorable environmental conditions [8,9].

In the last few decades, drought has been recognized as one of the most severe stresses adversely affecting plant development and yield. Significant economic losses have been recorded in recent years, and the situation is expected to worsen in the near future. Hence, climate change will undoubtedly affect the human food chain [10–12]. Plants have evolved a variety of complex metabolic pathways to ensure survival in harsh environments [13–17]. Still, to cope with the new challenges, plant changes and adaptations are required over and over. Scientists aspire to explore and improve the structural and functional properties of the photosynthetic apparatus to enable the more efficient and effective use of water and mineral resources [10,13,18,19].

Photosynthesis is one of the essential physiological processes directly related to plant growth and biomass accumulation. Plants must adjust their photosynthetic apparatus according to environmental stresses [20,21]. The inhibition of photosynthesis induced by the lack of water correlates well with the decrease in the leaf water content [22] and stomatal conductance [23,24]. Stomatal closure is the earliest response that prevents additional water loss from transpiration pathways at mild and moderate drought stress [15,25]. An insufficient supply of CO_2 drives the oxygenase function of Rubisco [26], consequently leading to the loss of ATP [27]. Inhibited light energy utilization causes the disturbance of the electron transport chain [28], promotes reactive oxygen species production [29], changes the ratio of the photosynthetic pigments, and also affects the disorganization of thylakoid membranes [30,31]. Permanent metabolic and structural changes were recorded after extreme or prolonged drought stress [32,33]. Further, the effect of drought on the photosynthetic apparatus depends on the species, genotypes, stress intensity, and duration.

Many papers have described an adverse impact on the plant's photosynthetic apparatus [33–37]. To summarize the main findings, drought causes changes in the redox state of PSI, impairs an electron transfer at the donor and acceptor side of PSII, affects the oxygen-evolving complex (OEC), decreases the efficiency of both PSII and PSI and the energetic connectivity between them, and inhibits the overall electron transfer capacity [38]. The damage resulting from the lack of CO_2, called photoinhibition, occurs primarily on PSII [39–41]. However, compared to PSI, PSII is less sensitive to water deficit, and permanent adverse effects occur only in extreme drought conditions [42,43].

Chlorophyll *a* fluorescence measurement (Chl) is a commonly used technique that provides a large amount of information on the physiological state of plants and also enables the early detection of invisible changes in the photosynthetic apparatus function and structure affected by certain environmental conditions [44–47]. Previous research indicates the potential possibility of using this method for screening sensitive and tolerant genotypes of different plant species [48–51].

While many papers describe the influences of water deficit on photosynthesis in food crops growing worldwide [52–55], limited studies have been conducted on *Brassica oleracea* varieties and other cabbages growing in the arid and semi-arid Mediterranean environment. The influence of drought length on *Brassica oleracea* var. *capitata* seedlings was investigated in the forest-savannah transition zone in Ghana on a morphological and physiological level, without insight into photosynthesis [56]. The impact of water deficit on photosynthetic performance and growth was explored in aeroponically grown Tuscan kale (*Brassica oleracea*) in a tropical greenhouse [57] and also in juvenile Chinese kale (*Brassica oleracea* var. *alboglabra*) and Caisin (*Brassica rapa* subsp. *parachinensis*) grown in southeast Asian countries [58]. Changes in the photosynthetic performances of white cabbage, Chinese cabbage, and kale, crops that usually grow in Croatia and other Mediterranean countries, were investigated under soil salinity conditions. While kale was pointed out as the most tolerant, Chinese cabbage was the most sensitive to salt stress [8]. The correlations between phytohormones and drought were investigated in the same crops [9]. A recent investigation into numerous kale accessions (*Brassica oleracea* var. *acephala*) highlighted the photosynthetic parameters PI_{ABS} and Fv/Fm as among the most informative variables in the drought tolerance definition [59].

To upgrade the previous knowledge of drought-stress physiology, with particular emphasis on the photosynthetic apparatus, three *Brassica* crops differing in drought tolerance were selected for this study: white cabbage (*Brassica oleracea* L. var. *capitata*), kale (*Brassica oleracea* L. var. *acephala*), and Chinese cabbage (*Brassica rapa* L. var. *pekinensis*). The main goal was to find reliable photosynthetic parameters that could distinguish drought-tolerant and drought-sensitive varieties before the appearance of irreversible structural and functional changes leading to permanent damage. Certain biophysical parameters, such as Fv/Fm, PI_{ABS}, PI_{total}, and variable fluorescence at the K and L bands, stand out as the key ones for detecting the drought impact on plants. However, we hypothesized that this research will point out some other crucial parameters describing specific changes in primary photochemistry events in Brassicas affected by drought stress. Screening the field-grown crops by certain Chl *a* fluorescence parameters could be a useful tool for the fast determination of the varieties in terms of their sensitivity or tolerance to stress and, thus, for the selection of appropriate breeding strategies.

This is the first research work on photosynthesis under drought conditions conducted on selected cabbage varieties usually grown in Croatia and other Mediterranean countries. According to our expectations, the results will highlight the photosynthetic performance of drought-tolerant *Brassica* crops and promote these easy-to-grow food crops with great health benefits. Further, the insight into photosynthetic efficiency, using chlorophyll *a* measurements as a phenotyping tool, could be useful in breeding programs and high-precision crop management. Improving photosynthesis could contribute to developing effective strategies for protecting the health of the global population.

2. Materials and Methods

2.1. Plant Material

The seedlings of Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis* (Lour.) Hanelt cv. Cantonner Witkrop), white cabbage (*Brassica oleracea* var. *capitata* cv. Varaždinski), and kale (*Brassica oleracea* var. *acephala* cv. IJK9) were grown as described in Pavlović et al. [9]. Briefly, after the germination in 1% agar plates, each few-day old seedling was transferred into the individual plastic pots filled with the commercial substrate Stender A240 (Stender GmbH, Schermbeck, Germany). The seedlings were grown in a growth chamber (115 µmol m^{-2} s^{-1}, 21 °C, photoperiod 16/8 h). Four-weeks old seedlings were subjected to drought by withholding water, while the control plants were watered regularly. When the drought-stressed plants reached an RWC of 45 ± 10%, they were recovered by re-watering for 24 h, while the RWC reached 86, 80, and 86% in the Chinese cabbage, white cabbage, and kale respectively Each variety reached about 45% RWC at different paces: Chinese cabbage after about 7 days, white cabbage after 10 days, and kale after about 15 days. For details and figures, see [9].

2.2. Measurements of Photosynthetic Parameters

The photosynthetic efficiency was measured in three groups of plants: control, drought-stressed, and recovered ones in each *Brassica* crop. Chlorophyll *a* (Chl) fluorescence measurements were measured *in vivo* using a Plant Efficiency Analyzer (PEA, Hansatech, Norfolk, UK). Chl measurements were performed on seven (*n* = 7) dark-adapted plants (30 min) per group of each cultivar. Chl transients (OJIP) were induced by applying a pulse of saturating red light with a maximum intensity at 650 nm and a photon flux of 3000 µmol m^{-2} s^{-1}. Changes in fluorescence were measured over one second, and the obtained data were used to calculate JIP-test parameters (Table 1) [47,60].

Table 1. Definitions of measured and calculated JIP-test parameters [47,60–63].

Recorded Data and Technical Parameters	
F_0	Fluorescence intensity at 20 µs, when all PSII RCs are assumed to be open
F_m	Maximal fluorescence intensity when all PSII RCs are closed
S_m	Normalized total area above the OJIP curve, reflecting multiple-turnover events
V_t	Relative variable fluorescence at time *t*
V_K/V_J	Indicator of PSII donor-side limitation, a relative measure of OEC inactivation
p	Overall connectivity parameter; $p = [p_{2G}(Fm/F_{50}\,\mu s - 1)]/[1 + p_{2G}(F_m/F_{50}\,\mu s - 1)]$
RC/CS_0	Measure for Q_A^- reducing RCs per excited leaf cross-section (CS)
Quantum Efficiencies and Flux Ratios	
$\phi_{P0}= TR_0/ABS$	Maximum quantum yield of primary photochemistry; the probability that an absorbed photon will be trapped by the PSII RC and will reduce one Q_A
$\psi_{E0}= ET_0/TR_0$	Probability that an absorbed photon will enter the electron transport chain; electron transport efficiency
$\phi_{E0}= ET_0/ABS$	Quantum yield for electron transport
$\delta_{R0}= RE_0\text{-}ET_0$	Probability that an electron is transported from the reduced PQ to the electron acceptor side of PSI
$\phi_{R0}= RE_0/ABS$	Quantum yield of electron transport from Q_A^- to the PSI end electron acceptors
ABS/RC	Effective antenna size of an active reaction center (RC). Expresses the total number of photons absorbed by Chl molecules of all RCs divided by the total number of active RCs
ET_0/RC	Electron transport per active RC
TR_0/RC	Maximal trapping rate of PSII. Describes the maximal rate by which an excitation is trapped by the RC
DI_0/RC	Effective dissipation per active RC
RE_0/RC	Electron flux reducing end electron acceptors at the PSI acceptor side per RC
Performance Index and Driving Forces	
SFI	Structure–function index on an absorption basis; $(RC/ABS) \times \phi_{P0} \times \psi_{E0}$
PI_{ABS}	Performance index (potential) for energy conservation from photons absorbed by PSII to the reduction of intersystem electron acceptors; $[\gamma_{RC}/(1 - \gamma_{RC})][\phi_{P0}/(1 - \phi_{P0})][\psi_{E0}/(1 - \psi_{E0})]$
PI_{total}	Performance index (potential) for energy conservation from photons absorbed by PSII to the reduction of PSI end acceptors; $[\gamma_{RC}/(1 - \gamma_{RC})][\phi_{P0}/(1 - \phi_{P0})][\psi_{E0}/(1 - \psi_{E0})][\delta_{R0}/(1 - \delta_{R0})]$
$DF_{total} = \log PI_{total}$	Total driving forces for the photosynthesis of the observed system, created by summing up the partial driving forces for each of the several bifurcations
DRI	Drought resistance index; $\log[(PI_d/PI_c)(PI_r^2/PI_c^2)]$

OJIP transients represent the mean values of seven measurements for each treatment and variety, where every treatment was normalized to their corresponding control. The fully watered seedlings of each variety were served as controls, and they were used as referent values. Specific events of the OJIP transient in the OK, OJ, JI, and PI phases were shown as differences in the variable fluorescence and presented as ΔV_{OP}, ΔV_{OK}, ΔV_{OJ}, ΔV_{JI}, and ΔV_{IP}, normalized to corresponding controls [61]. Each curve represents the average of seven measurements ($n = 7$) per treatment and variety. The total driving forces (DF_{total}) of the total photosynthetic electron transport, shown as $\log PI_{total}$, were summed up by the corresponding partial driving forces: $\log \gamma_{RC}/(1 - \gamma_{RC})$, $\log \varphi_{P0}/(1 - \varphi_{P0})$, $\log \psi_{E0}/(1 - \psi_{E0})$, and $\log \delta_{R0}/(1 - \delta_{R0})$ [62]. The difference for the control and the drought and/or recovery, respectively, ΔDF_{total}, was calculated as $\Delta DF = DF_{drought/recovery} - DF_{control}$.

The drought resistance index (DRI) was calculated for each *Brassica* variety, as described for heat stress and recovery [63], with a modification for drought stress, based on the principle defined in [50], by using the driving force (DF) of PI_{ABS} (log PI). The recovery outcome was doubled compared to the one measured after the drought (Table 1).

2.3. Statistical Analysis

Statistical differences between seedlings subjected to drought followed by recovery and their corresponding controls were evaluated for each *Brassica* crop separately. Analyses were subjected to analysis of variance (ANOVA) followed by Tukey's HSD *post hoc* test using Statistica software (ver. 13.1., Tibco Software Inc., Palo Alto, CA, USA). The data are presented as the means $\pm$ standard deviation (SD) of seven biological replicates ($n = 7$). The differences were considered significant at $p \leq 0.05$. Correlations between Chl *a* parameters for all three *Brassica* crops, as well as the correlation between RWC and the performance index, energy fluxes, performances, and probabilities in all three *Brassica* crops, were performed using the correlation matrix of average values after autoscaling, using XLSTAT Statistical software for Excel (ver.2021, Addinsoft, Paris, France). Linear correlations between the selected variables were determined by Pearson coefficients. Each point represents the mean value of seven replicates ($n = 7$), while the difference was considered significant at $p \leq 0.05$. The PCA Variable contributions (loadings) of parameters are shown in Table 2.

3. Results and Discussion

The previous reports on these three selected *Brassica* crops distinguished kale as the most drought-tolerant variety, while the Chinese cabbage was shown to be the most sensitive one, which was correlated with changes in phytohormones and their metabolic pathways [9,59]. In addition, the same *Brassica* crops exposed to short-term salt stress showed identical responses, with Chinese cabbage being the most sensitive and kale being the most tolerant variety [8]. Moreover, it was shown that the salt susceptibility of Chinese cabbage was associated with the decline in the photosynthetic capacity for efficient energy conversion. For a better understanding of photosynthetic apparatus adaptations to drought and subsequent recovery by re-watering in selected *Brassica* crops, we described specific reactions and events during the electron flow from PSII to PSI by analyzing prompt Chl transients and distinctive JIP-test parameters.

3.1. Changes in OJIP Transients after Drought and Recovery

Prompt Chl fluorescence transients (Figure 1) between drought-stressed and -recovered *Brassica* crops revealed obvious ΔV_{OP} differences among varieties. Chinese cabbage showed a positive ΔV_{OP} amplitude after drought. Upon re-watering, the amplitude turned negative, with noticeable J and I bands (Figure 1a). White cabbage showed positive amplitudes (Figure 1f) after drought and recovery, with higher amplitudes observed in drought-stressed seedlings. In kale, both transients were negative (Figure 1k), with more pronounced curves observed after recovery. The OJ phase reflects the reduction in Q_A and the partial reoxidation of Q_A^- [64]. Positive ΔV_{OP} amplitudes are usually reported for various species, such as maize [38], barley [34], sorghum [33], perennial ryegrass [65], and linden trees [37], exposed to drought, and they are often reported in genotypes/cultivars described as sensitive ones. The ΔV_{OP} curve reveals additional inflections which correspond to L, K, H, and G bands that describe specific events of primary photochemistry [66].
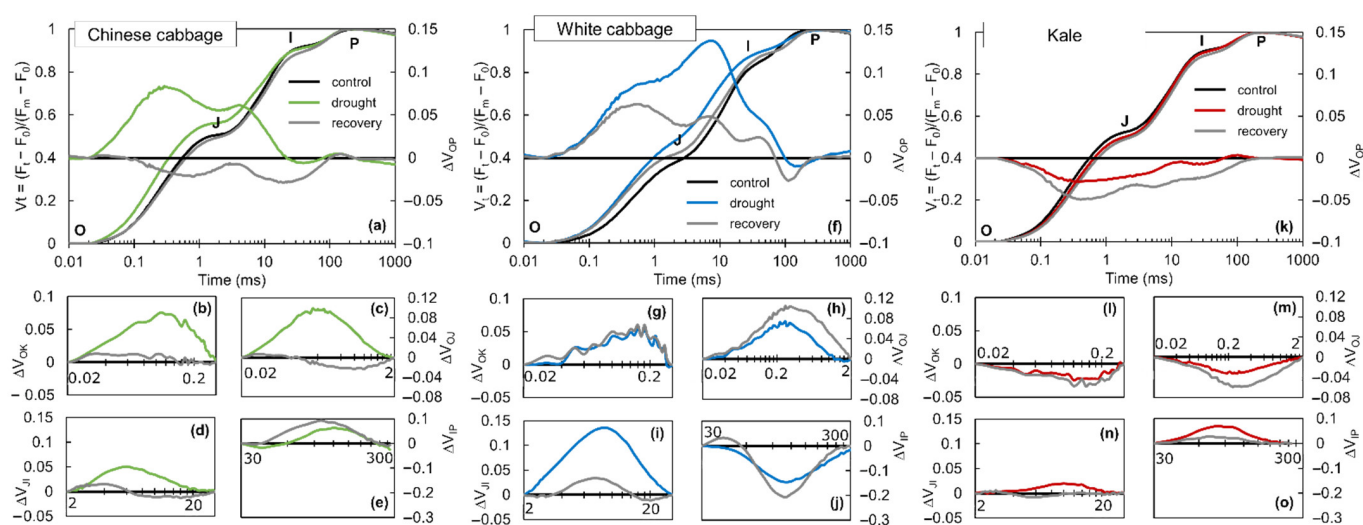
Figure 1. Shapes and amplitudes of OJIP transient curves determined in three *Brassica* seedlings after exposure to drought and subsequent recovery are shown as kinetics of relative variable fluorescence Vt and as difference kinetics ΔVOP (**a,f,k**). Difference kinetics ΔVt for the individual bands L (**b,g,l**), K (**c,h,m**), H (**d,i,n**), and G (**e,j,o**) are plotted at different time ranges. The O, J, I, and P steps are indicated in V_t curves.

The L band (ΔV_{OK}) was shown to be a reliable parameter describing the energetic connectivity between photosynthetic units [61,67], while the K band was recognized as a great indicator of stress in plants, especially drought [33,34,36,38,50,63,68,69]. All three *Brassica* crops revealed similar results for both L and K bands. In Chinese cabbage, there were positive band amplitudes (Figure 1b,c), while after recovery, the amplitude was similar to the control one. White cabbage showed positive amplitudes (Figure 1g,h), while kale revealed negative amplitudes (Figure 1l,m) of L and K bands in both drought-stressed and re-watered seedlings. Positive L bands were reported frequently in drought-stressed plants [34,38,50,68], and they are a sign of low energetic connectivity and lower system stability [61]. Connectivity and PSII stability could be disturbed in water-deprived plants due to the degradation of PSII proteins, causing the reorganization of and reduction in thylakoid membranes' stability [70,71]. The negative L band is associated with the efficient utilization of excitation energy, since PSII units are more connected, forming a better stability of the system [61]. The positive K band is connected with decoupling the oxygen-evolving complex (OEC) and/or an increased size of functional antennae [37,61], while a negative amplitude usually indicates tolerance to stressful conditions of the investigated genotype [33,34,50] as well as increased adaptability [72], suggesting the tolerance of kale seedlings to drought. Drought-stressed Chinese cabbage showed a higher K band amplitude compared to white cabbage. However, white cabbage showed that the inactivation of OEC was severe, and it could not recover for running functional reactions. Similar behavior was reported for the sensitive barley genotype after re-watering [33,50]. The negative K band amplitude in Chinese cabbage after recovery suggested that the accessibility of proline [9], as an alternative electron donor to OEC, replaced a sufficient number of electrons to drive efficient photosynthetic reactions.

The H band is related to the reduction in and oxidation of the plastoquinone (PQ) pool, and it is used to describe multiple-turnover events [65,73–75]. Slower reoxidation causes an increased accumulation of reduced electron carriers that form a positive H band [67,76]. In Chinese cabbage (Figure 1d) as well as in kale (Figure 1h), drought stress induced the appearance of positive H band inflection. Upon recovery, the H band revealed a higher positive amplitude compared to that in stressed Chinese cabbage, while in kale, the recovery curve was more similar to the control, with almost no visible inflections. White cabbage also showed a positive H band (Figure 1i), but it was more pronounced in drought-stressed seedlings than it was in re-watered ones. Drought stress induced a reduction in the PQ

pool size, causing a faster reduction in PQ in all three *Brassica* crops. Upon recovery, the size of the PQ pool changed from smaller to bigger at different rates for three *Brassica* varieties. The reduction rate of the PSI acceptor side from the PQ pool is shown as a G band, and it depends on the available $NADP^+$ molecules [37,75]. Chinese cabbage showed a positive G band in both drought-stressed and recovered seedlings (Figure 1e), while drought-stressed kale (Figure 1o) showed a positive amplitude that returned to control values after recovery. However, in white cabbage (Figure 1j), both curves were shown to be negative. A positive G band indicated a decrease in the PSII acceptors pool, accompanied by reduced electron transport and a subsequent reduction in the PSI reduction rate [75]. On the other hand, the negative G band seen in white cabbage was suggested to be a compensatory mechanism developed by plants exposed to inadequate and stressful conditions [37,77]. In such conditions, the number of available $NADP^+$ molecules increased, which, in turn, increased the activity of PSI [37,75]. Additionally, it was recently suggested that drought-induced changes in the I-P amplitude, corresponding to the G band, could be associated with the build-up of the cyclic electron flow [78], which would protect PSI from the overreduction of its acceptor side.

3.2. Changes in JIP-Test Parameters after Drought and Recovery

Spider plots (Figure 2) revealed the differential response of the three selected *Brassica* crops to drought and subsequent recovery. Drought stress induced a reduction in PI_{total}, ϕ_{P0}, ϕ_{E0}, and ψ_{E0} in Chinese and white cabbage, while in kale, the observed changes were not significant. Since PI_{total} includes events involving PSI, it was recognized as a more sensitive and reliable parameter for drought detection than PI_{ABS}. In addition, ϕ_{P0} was also reported as an insensitive indicator of drought since it declines only in severe drought conditions [74,79]. Nevertheless, in some cases, it could be used as a good indicator of drought stress [80]. Based on our results (Figure 2a,b), it could also be used as such, especially for drought-sensitive *Brassica* crops. The PI_{total} decrease in drought-stressed plants is usually associated with the downregulation of electron transport [81]. This is corroborated by the decrease in ϕ_{P0} and ψ_{E0}, together with its quantum yield, ϕ_{E0}, in drought-stressed Chinese and white cabbage, indicating the decline in electron transport further than Q_A^- [37], most probably due to the induction of Q_B-non-reducing RCs upon the exposure to drought [82]. In addition, the drought-induced decrease in ϕ_{R0} and δ_{R0} in white cabbage suggested a decrease in the electron flow rate between reduced intersystem electron acceptors and PSI [83]. Re-watering caused the recovery of most parameters in Chinese cabbage to control values (Figure 2a). Surprisingly, δ_{R0} significantly increased after recovery compared to the control, indicating a higher efficiency of PSI electron transport [84]. The recovery in white cabbage (Figure 2b), however, did not reach the control value, as reported in Chinese cabbage. It was suggested that the partial recovery of certain parameters, especially PIs, upon re-watering in sensitive barley accession was associated with early leaf senescence [33]. The fully recovered ϕ_{R0} and δ_{R0} to the control values in white cabbage corroborated the increase in the electron flow rate at the PSI acceptor side (negative G band). Such results suggested the formation of functional adaptations as a response to drought in white cabbage [77,84,85].

The energy fluxes per active reaction centers (RCs) revealed an increase in absorption (ABS), trapping (TR_0), and dissipation (DI_0) upon exposure to drought in Chinese and white cabbage, while the electron transport (ET_0) and electron flux that reduce the final electron acceptors and the PSI acceptor side (RE_0) showed no change. Re-watering induced the recovery of all of the abovementioned parameters to the control values in Chinese cabbage. White cabbage, however, showed no recovery for ABS/RC, TR_0/RC, and DI_0/RC after re-watering, but ET_0/RC increased compared to the control. Kale showed no changes during the treatments of any parameter. An increase in the specific fluxes ABS/RC, TR_0/RC, and DI_0/RC is a usual response of plants exposed to drought [33,34,37,81]. Such increase is usually the result of damaged OEC and impaired electron transport further than Q_A [71,86]. A parallel decrease in ϕ_{P0} confirmed the possibility of RC inactivation

by drought, resulting in lower electron feeding from OEC [61]. It was also recently suggested that an increase in the named parameters due to a prolonged exposure to stress could diminish the energetic connectivity by detaching light-harvesting antennae from PSII [37]. In addition, the decrease in ET_0/RC upon the drought exposure, accompanied by the increase in ABS/RC and TR_0/RC, suggested the transformation of active RCs into dissipative ones [61,87]. This is corroborated by the increased DI_0/RC in drought-stressed seedlings, suggesting that this could be a useful protective mechanism in drought-stressed seedlings of white cabbage. Moreover, the unchanged RE_0/RC indicated low pressure on PSI, preventing its overreduction [83]. Since Chinese cabbage recovered most of the parameters to the control level, it could be assumed that primary reactions were reversibly downregulated and that all trapped energy could be efficiently utilized in the electron transport chain. In white cabbage, however, the damages were more severe, as judged from the high values of energy fluxes, even after recovery, suggesting that white cabbage was the most drought-sensitive variety compared to Chinese cabbage and kale.
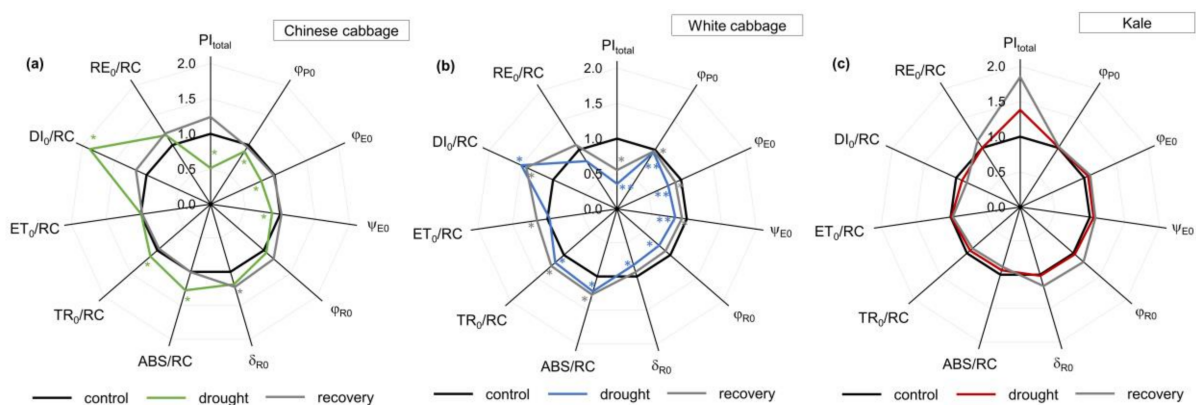

Figure 2. Spider plots represent selected JIP-test parameters that characterize PSII functioning in three *Brassica* seedlings: Chinese cabbage (**a**), white cabbage (**b**) and kale (**c**), subjected to drought followed by recovery. Each dataset is normalized to the respective controls (watered seedlings) separately for each variety (control = 1). Asterisks (*) signify differences between the treatments and the corresponding control, while double-asterisks (**) represent significant differences between both the control and recovery at $p \leq 0.05$ (ANOVA, HSD).

Minimal fluorescence (F_0, Figure 3a) was shown to be a reliable indicator of the PSII state during stress [88]. An increase in F_0 was observed in Chinese and white cabbage after the drought, while kale showed no significant change. Recovery induced a decrease in the parameter to the control level in Chinese cabbage; however, in white cabbage, the value remained as high as in drought-stressed seedlings. As a parameter associated with the primary photochemistry of PII, its increase suggests that plants subjected to stressful conditions downregulate PSII activity [70,89]. It was reported recently that the increase in F_0 was the result of disconnected light harvesting antennae from the PSII core complex in chilling stressed sugarcane [90]. However, it has been recently reported that stabile F_0, in combination with stable TR_0/RC (Figure 2c), as observed in kale, reflects constant trapping by active RCs that are able to reduce Q_A [64]. The energetic connectivity between PSII complexes is an important feature for evaluating specific energy fluxes, and plants with higher connectivity utilize light energy more efficiently than plants with lower connectivity [86]. The connectivity parameter (*p*) is related to the initial phase of Chl transients and can be used to estimate the redox state of PSII electron acceptors [91] and the PQ pool [92]. A low connectivity between PSII units lowers the excitation pressure and thus protects PSII in stressful conditions. Our investigation revealed a significant increase in *p* only in white cabbage after drought stress compared to the control, while recovery-induced connectivity dropped to the control value (Figure 3f). Increased PSII connectivity was also reported in young grapevine leaves [93] as well as in *Phalenopsis*

plants at low light conditions [86]. Even though the F_0 increased, the positive L band (Figure 1g) in drought-stressed white cabbage suggested the lower connectivity of PSII units. However, an increase in the connectivity parameter suggested a possible defense mechanism that prevents the overreduction of the PQ pool and thus lowers the damage to PSII [37].
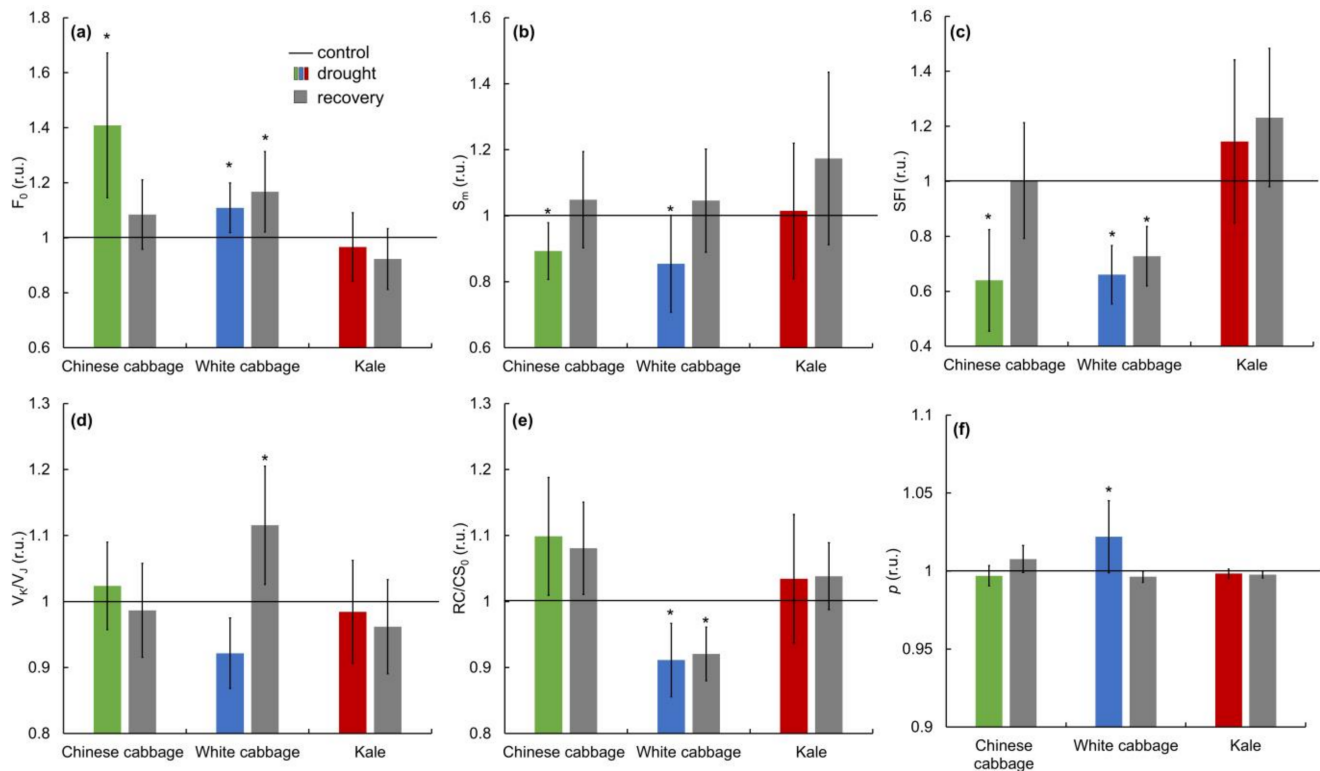

Figure 3. Chlorophyll *a* fluorescence parameters characterizing the PSII functioning: minimal fluorescence intensity, F_0 (**a**), normalized area, S_m (**b**), structure – function index, SFI (**c**), fraction of inactivated OEC, V_K/V_J (**d**), density of reaction centers per excited cross section, RC/CS_0 (**e**) and overall connectivity parameter, *p* (**f**) measured in three *Brassica* seedlings subjected to drought and subsequent recovery. Normalized data are presented as the mean $\pm$ SD; $n = 7$; asterisk (*) represents a significant difference at $p \leq 0.05$ (ANOVA, HSD).

It was suggested that S_m, together with PI_{total} (Figure 2), could reflect the vitality of the plants subjected to certain environmental stress [94]. The S_m parameter describes the normalized total area above the OJIP curve, reflecting multiple-turnover events. It is also an equivalent measure for the number of electrons transported by PSII to reach the maximum fluorescence intensity and close all RCs [94]. There was a decrease in S_m in Chinese and white cabbage (Figure 3b) upon the drought, while in kale, there was no difference compared to the control. After recovery, the S_m values in all varieties reached the control values. The decrease in S_m upon drought, as well as the positive H band (Figure 1d,i), suggested that Q_A could be reduced but not re-oxidized, since it cannot perform multiple turnovers as fast as the control seedlings due to the limited electron transport [95,96].

The strength of the inner factors that endorse reactions in PSII is described as the SFI or the structural and functional index [44,64]. Drought induced a decrease in SFI in Chinese and white cabbage (Figure 3c) compared to the control. While the SFI increased to the control values in Chinese cabbage after recovery, in white cabbage, the SFI remained as low as it was in drought-stressed seedlings. Various stressful conditions, such as salinity [97], PEG−induced drought stress, and salt stress [98–100], provoked changes that caused the instability of photosystems. An SFI decrease reflects the limited electron transport and

diminished overall photosynthetic activity. It was reported recently that, in sensitive sunflower hybrids, the SFI decreased upon severe drought stress, while in tolerant hybrids, the SFI remained at the control level [101]. An SFI decrease in white cabbage, therefore, suggests that stressful conditions lower the influence of internal factors, thus diminishing reactions in PSII.

The ratio of variable fluorescence at times 0.3 and 2 ms (V_K/V_J) is a reliable indicator of the restrictions at the PSII donor side [81,99] and can be used as a relative measure of OEC inactivation [102]. Drought stress did not induce significant changes in V_K/V_J in any *Brassica* variety (Figure 3d). After recovery, there was a significant increase in the white cabbage compared to the control, while the Chinese cabbage and kale showed no significant changes. An increase in V_K/V_J is usually the result of the uncoupling of OEC [81,102]. It was reported recently that UV-B radiation could induce an increase in V_K/V_J in sensitive and moderately tolerant populations of Scots pine seedlings [81], in rice seedlings exposed to high light, salinity, and PEG−induced drought [99], as well as in *Actinidia* plants deprived of nitrogen [103]. Our results suggested that drought caused OEC damage in white cabbage, since V_K/V_J increased after recovery. The fact that the K-band (Figure 1h) showed a positive amplitude corroborated this assumption. Moreover, an increase in RC/CS_0 (Figure 3e) in combination with increased ABS/RC and TR_0/RC (Figure 2b) confirmed the impairment of OEC in drought-stressed white cabbage. The density of active PSII RCs per excited cross-section (RC/CS_0) decreased in white cabbage upon drought, and after recovery, it remained as low as it was during stress. On the other hand, Chinese cabbage and kale showed no difference compared to their controls. Differential stressful conditions usually induce the lowering of RC/CS_0 in various species [33,99,103,104], suggesting a lower tolerance to such conditions. Therefore, a decrease in RC/CS_0 suggests an increased susceptibility of white cabbage to drought compared to Chinese cabbage and kale.

3.3. Total Driving Forces for Photosynthesis

The differences in driving forces for photosynthesis (ΔDFs) give us insight into changes in partial driving forces between treatments and the corresponding control (Figure 4). Partial driving forces describe events for energy conservation from the exciton to the reduction in the PSI end acceptor [62,67]. They describe the contribution to the DF_{total} due to the PSII antenna size and/or the density of RC as $\log \gamma_{RC}/(1 - \gamma_{RC})$, that due to light reactions for primary photochemistry as $\log \phi_{P0}/(1 - \phi_{P0})$, that due to dark reactions as $\log \psi_{E0}/(1 - \psi_{E0})$, and the contribution of reduction events of PSI as $\log \delta_{R0}/(1 - \delta_{R0})$ [60,67]. By calculating ΔDFs, the negative contributions of $\log \gamma_{RC}/(1 - \gamma_{RC})$, $\log \phi_{P0}/(1 - \phi_{P0})$, and $\log \psi_{E0}/(1 - \psi_{E0})$ were observed in drought-stressed Chinese cabbage. After recovery, the DF_{total} turned positive due to the less negative $\log \phi_{P0}/(1 - \phi_{P0})$ and the positive contributions of $\log \psi_{E0}/(1 - \psi_{E0})$ in addition to the stabile $\log \delta_{R0}/(1 - \delta_{R0})$. White cabbage revealed a negative DF_{total} in both seedlings (drought-stressed and after recovery); however, after recovery, the DF_{total} was lessened by half the value compared to drought due to all four partial DFs being less negative. Kale revealed the completely opposite response. A positive DF_{total} was observed for both treatments due to the positive contributions of all partial DFs with higher values observed in the kale seedlings after recovery, with most of the contributions from $\log \psi_{E0}/(1 - \psi_{E0})$ and $\log \delta_{R0}/(1 - \delta_{R0})$. Such results suggested that the higher contribution of all partial driving forces led to DF_{total} restoration after the recovery in Chinese cabbage and kale. Several drought-sensitive barley genotypes showed negative ΔDFs due to the lower precipitation during anthesis. Increased precipitation at the grain filling stage triggered the recovery of ΔDFs in most genotypes, except for one that was signified as a sensitive one [34]. Moreover, it was suggested that drought-sensitive barley varieties showed the highest reduction in DFs [50], which agrees with our results, showing the most negative ΔDFs in white cabbage.
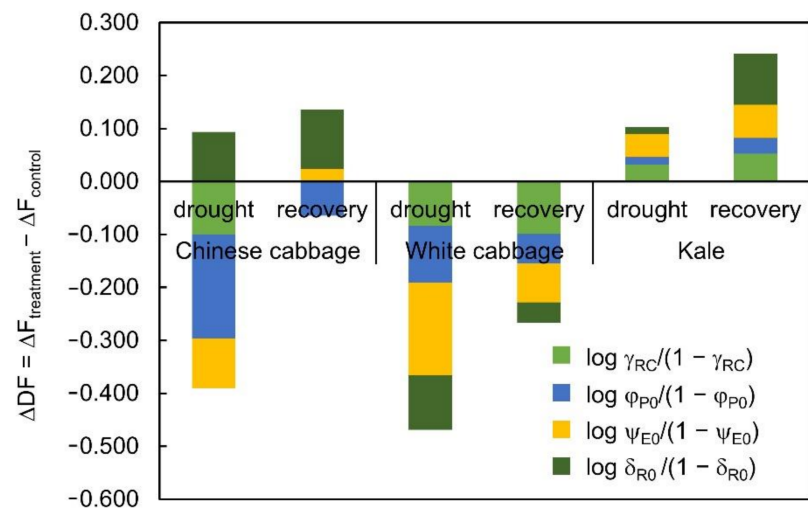
Figure 4. Difference in the driving forces (ΔDF) of three *Brassica* seedlings after exposure to drought and subsequent recovery. Stacked columns represent differences in DFs in treated seedlings minus the corresponding control separately for each variety. Each DF is calculated by summing up their partial driving forces.

3.4. Linear Model of the Relative Performance Index and the Yield of Electron Transport

The correlation between the logarithms of the relative yield of electron transport (ET_0/ABS, ϕ_{E0}) and performance index at the absorption basis (PI_{abs}) represents the capacity of energy utilization and the overall performance of the plant [34,105,106]. The linear relationship between logarithms indicates that any change in ϕ_{E0} would induce a change in relative PI_{abs} in plants exposed to drought and after recovery [50]. To emphasize the reactions of each variety, the data were normalized to their corresponding control. Our results revealed that the seedlings after recovery generally had a better vitality than those after the drought stress (Figure 5). The linear relation showed the strongest relationships in kale ($R_d^2 = 0.983$, $R_r^2 = 0.959$; Figure 5c) compared to Chinese and white cabbage (Figure 5a,b). It is proposed that, generally, tolerant cultivars show positive values, while the susceptible ones exhibit lower values [87,105,107]. However, consistency between treatments was also identified as a major factor in the determination of the overall performance of the plant [34]. White cabbage was shown to be the variety with the lowest consistency (Figure 5b), which was also supported by the formation of positive L and K bands (Figure 1g,h), suggesting a considerable sensitivity to drought. Moreover, the absolute value of the slope between ϕ_{E0} and the log PI_{abs} can be used to quantify the tolerance to the drought of certain *Brassica* variety [70]. Based on that, compared to Chinese cabbage and kale, white cabbage, with the lowest absolute slope values, could be signified as the most sensitive to drought.
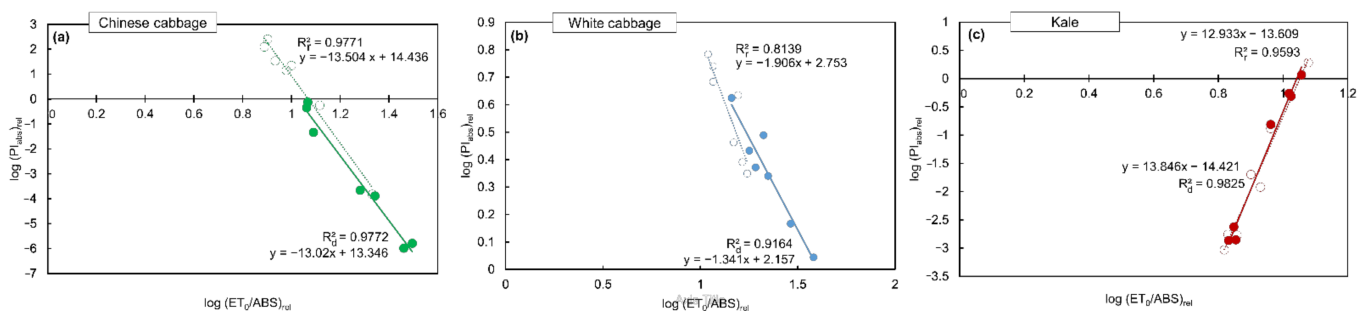

Figure 5. Linear model between logarithms of relative ET_0/ABS and PI_{ABS} in three *Brassica* seedlings: Chinese cabbage (**a**), white cabbage (**b**) and kale (**c**), subjected to drought (filled circles) and subsequent recovery (empty circles) relative to corresponding controls.

3.5. Drought Resistance Index and Principal Component Analysis

The drought resistance index (DRI) was calculated for each *Brassica* variety separately in order to quantify the reactions of seedlings to drought and subsequent recovery. Our results reveal positive DRI values for kale, while Chinese and white cabbage showed negative ones (Figure 6a). DRI could be used as a measure for the energy conservation potential of a variety in stressful conditions [63]. The calculation of the stress factor index was introduced as a practical tool for the classification of stress tolerance [108]. It is usually calculated as a relative performance index at crucial points of plant exposure to stress by taking into account and emphasizing the importance of the duration of exposure and the ability of plant to recover [80,109]; however, it could be modified depending on different setups of experiments, including the extent of stress exposure or the recovery from it [63]. The performance index, drought factor index or drought stress/resistance indexes are extensively used indicators used to classify drought tolerance in various plants as well as phenotypic plasticity [110–112]. They have often been used to screen varieties, cultivars, or genotypes that have been subjected to various stresses, mostly drought [48,50,80,109,113,114] and temperature stress [63,108]. It has been suggested recently that this could be a useful parameter in identifying genotypes differing in their response to drought [80,115]. The ability of a specific variety to recover after stressful conditions—in this case, after the drought—was shown to be genotype-specific [34,107]. Therefore, drought-sensitive genotypes show a higher decrease in PI_{ABS} and, consequently, lower DRI values [50]. Similar results were shown for soybean genotypes sensitive to dark chilling [108], barley cultivars [50], sesame lines [48], and sunflower hybrids [101] sensitive to drought, as well as for wild barely [36] sensitive to heat stress.
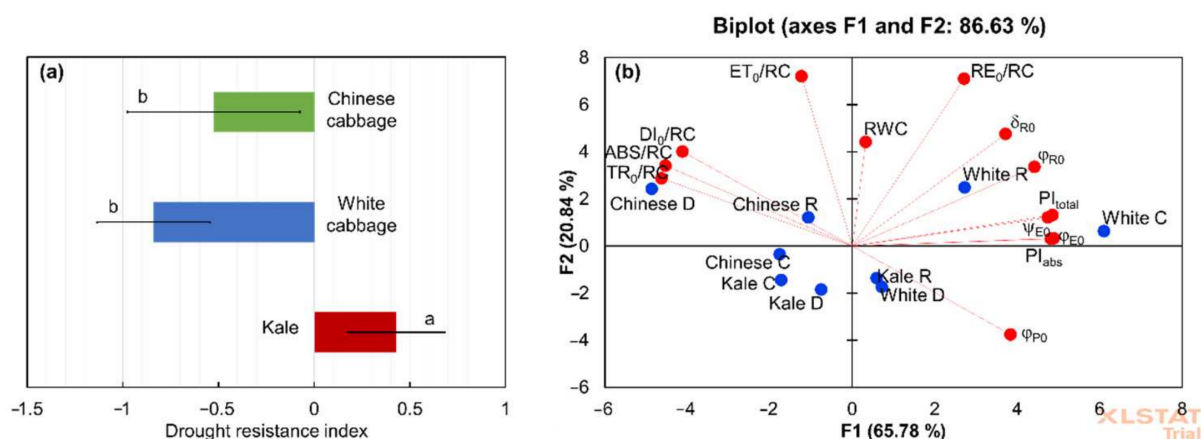

Figure 6. Drought resistance index (DRI) (**a**) of three *Brassica* seedlings subjected to drought relative to corresponding controls. Bars represent the means ± SD of seven measurements (*n* = 7); different letters represent significant differences at $p \leq 0.05$ (ANOVA, HSD). Principal component analysis (PCA) (**b**) shows variation within and among three *Brassica* seedlings (blue dots) in the control (C) and after drought (D) and recovery (R) in relation to the PSII functioning parameters, performance index, quantum efficiencies, and flux ratios shown as red dots.

Therefore, our results corroborate the fact that kale is the most resistant *Brassica* variety, while white cabbage is the most sensitive one.

The PCA (Figure 6b, Table 2) was performed based on a matrix of Pearson's correlation coefficients ($p \leq 0.05$) to compare correlations among *Brassica* varieties in the control, drought, and after-recovery conditions in relation to selected photosynthetic parameters and RWC (data not shown; see Pavlović et al. [9]). Before the analysis, the autoscaling of the average values was performed to standardize the parameters. The first two components explained 86.63% of the variability. Chinese cabbage and kale in the control and drought-stressed kale positioned in the fourth quadrant showed a negative correlation with RWC and parameters describing quantum efficiencies and probabilities, as well as

with PI_{total} and RE_0/RC. However, the same parameters contributed the most to the reactions of white cabbage in the control and drought stress conditions. Energy flux ratios, especially those describing absorption, trapping, and dissipation, contributed negatively to reactions in drought-stressed white cabbage and in kale after recovery. However, the same parameters contributed the most to reactions in Chinese cabbage exposed to drought. Kale showed the lowest variability in performance, suggesting a higher efficiency due to a better tolerance to drought. On the other hand, white cabbage showed the highest variability in performance, suggesting that the decrease in photosynthetic efficiency was the result of its higher sensitivity to drought compared to the other two *Brassica* varieties.

Table 2. Variable contributions (loadings) for the principal component analysis model in Figure 6b.

Parameter	F1	F2	F3
RWC	0.066	0.502	0.782
ϕ_{P0}	0.776	−0.428	0.434
ϕ_{E0}	0.988	0.038	0.108
ψ_{E0}	0.982	0.150	0.012
ABS/RC	−0.916	0.388	−0.037
TR_0/RC	−0.935	0.326	0.116
ET_0/RC	−0.249	0.820	0.349
DI_0/RC	−0.832	0.456	−0.259
PI_{ABS}	0.974	0.035	0.025
ϕ_{R0}	0.894	0.382	−0.208
δ_{R0}	0.753	0.541	−0.354
RE_0/RC	0.549	0.809	−0.158
PI_{total}	0.960	0.138	−0.048

4. Conclusions

Our results for the selected *Brassica* crops determined kale to be the most tolerant, Chinese cabbage as moderately tolerant, and white cabbage as the most sensitive to drought stress. Drought stress induced no visible signs of damage on kale seedlings, the primary photochemistry was not disturbed, and the electron flow was not blocked at the PSII level, as well as intersystem electron carriers. Moreover, the better stability of the system led to the enhanced conservation of energy through electron transport compared to Chinese and white cabbage. However, the slight drought-induced disturbances observed at PSI recovered completely after re-watering, corroborating its high drought tolerance. As for Chinese and white cabbage, drought induced significant disturbances in PSII photochemistry. In addition to the lower connectivity of PSII units and the decoupling of OEC, the inactivation of reaction centers and the reduced electron flow rate between Q_A and Q_B decreased the ability to efficiently utilize absorbed and trapped light energy. Nevertheless, the higher dissipation of excess light reduced the capacity for photochemical Q_A reduction by increasing the PQ pool in both Chinese and white cabbage. In white cabbage, less pressure on PSI consequently caused a slower transfer of electrons to the PSI acceptor side, which could be a compensatory mechanism for protecting PSI from overreduction and, thus, coping with drought stress. The fully recovered PSII photochemistry in Chinese cabbage suggested the reversible downregulation of PSII reactions. However, the re-watering of white cabbage did not induce a full recovery of most of the parameters, suggesting more severe damage to the photosynthetic units and corroborating its higher drought sensitivity.

The biophysical interpretation of Chl *a* fluorescence parameters offers a convenient framework for distinguishing drought-tolerant and -sensitive *Brassica* crops. To detect the downregulation of specific events in primary photochemistry before harmful and irreversible consequences occur, it is necessary to identify specific parameters that would indicate the possible sequence of events. In the present study, positive L and K bands, an increase in the PI_{total} and the SFI factor, as well as positive ΔDF and DRI could suggest drought tolerance. On the other side, negative L and K bands, in addition to a lower

PI_{total}, SFI, and RC/CS_0, could distinguish a variety with a lower tolerance or even a higher sensitivity to drought-induced stress. Detecting the drought-resistant varieties by noninvasive methods such as Chl *a* fluorescence is a useful tool for the fast screening of crops exposed to drought in the field to anticipate the response and to develop efficient strategies for protection, thus keeping the yield stable.

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