Jurnal Biodjati 6(2):190–202, November 2021
e-ISSN : 2541-4208
p-ISSN : 2548-1606

JURNAL BIODJATI

http://journal.uinsgd.ac.id/index.php/biodjati

THE PHYSIOLOGICAL RESPONSES OF *Zea mays* L. AND *Cucumis sativus* L. ON DROUGHT STRESS AND RE-WATERING

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Received : May 12, 2021
Accepted : October 11, 2021

DOI: 10.15575/biodjati.v6i2.12572

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Abstract. *Drought leads to deficit water availability and its detrimental effects seriously threaten plant growth. This study assessed the physiological, biochemical, and antioxidant adjustments in different types of photosynthetic plants between Zea mays L. (C4) and Cucumis sativus L. (C3 plant) under response to short-term drought stress. Analyses of relative water content (RWC), proline, and ascorbic acid (AsA) were performed to explore how these plants react to drought. Fifteen-day-old plants were subjected to full irrigation or gradual drought periods for 2-d, 4-d, 6-d, and 8-d following by recovery for 7-d. The results revealed that drought significantly reduces leaf RCW in both plants. Re-watered Z. mays after 8-d drought was higher than C. sativus and reestablished RCW by 23% of stressed plant although remained lower by 9% of the well-watered plant. While, proline and AsA contents in Z. mays were higher than those in C. sativus in drought treatment at 8-d (2.05 μmol/g FW) and 6-d (3174.60 AsA/100 g FW), respectively, that could demonstrate osmotic adjustment ability in this C4 species. The increased proline in both plants also indicates a good strategy for plants to recover. Rewatering gave a decrease AsA and could be expected that plants restore cellular activity after oxidative injury. Based on our study, proline is the most informative biochemical marker to differentiate plant response to drought and Z. mays adjusted defense mechanism to drought rather than C. sativus due to higher accumulation of proline, better antioxidant activity, and improved RCW after recovery.*

Keywords: *acorbic acid, Cucumis sativus* L.*, drought, proline, relative water content, Zea mays* L.

Citation
Meriem, S., Muliyah, E., Angio, M. H. & Triardati. (2021). The Physiological Respones of *Zea mays* L. and *Cucumis sativus* on Drought Stress and Re-Watering. *Jurnal Biodjati*, 6(2), 190–202..

INTRODUCTION

During the last decades, climate changes are being the key drivers to rise global warming. This elevates temperatures and water deficit leading to an increased drought that result in an imbalance of high transpiration loss and low water uptake that affects plant physiological systems (Jalota et al., 2018; Lamaoui et al., 2018). This abiotic stress is considered a prominent threat that inhibits and also alters metabolism pathways, evenly reduces yields and plant productivity (Caser et al., 2019; Heinemann et al., 2017;

Lei et al., 2020). Prolonged drought highly reduces soil water availability that plays a critical factor in limiting water uptake and nutrients for plants (da Cunha Cruz et al., 2020; Huang et al., 2020). Clearly, drought-stress plants are facing photosynthetic capacity problems as a consequence of the reduction in stomatal conductance which caused a reduced intercellular carbon concentration and leads to a decreased carbon assimilation, and also disrupt photolysis caused by low xylem water potential (Albert et al., 2011; Chen et al., 2020).

Plant responses to drought reveal varied mechanisms to survive and adapt during water stress periods depending on different plant types. Moderate drought in C4 plants withstands photosynthetic carbon assimilation rate, reduces cumulative transpiration, and defends water use efficiency, neither for C3 plant (Hamim, 2005; Hartzell et al., 2018). This is in accordance with compatibility of C4 plants to preserve mesophyll carbon-concentrating mechanism when stomatal conductance is reduced in drought. As a damage result of drought, reactive oxygen species (ROS), C4 plants adapts to upregulate antioxidant systems that are intended to suppress toxic ROS but insufficient in C3 plants (Uzilday et al., 2012). Ascorbic acid (AsA) acts as the main antioxidant related to environmental stress by preventing cell from oxidative damage (Conklin & Barth, 2004) and was reported to improve growth under water deficit (Hamama & Murniati, 2010). To improve osmotic imbalance and against oxidative damage during water deficiency, plants increase osmolyte concentrations, for instance, soluble sugars and amino acids including proline (Shimpl et al., 2019; Szabados & Savouré, 2010). Proline not only plays as an osmoprotectant by maintaining cell homeostasis and scavenging ROS but also encompasses enhancingplant tolerance to stress damage (Meena et al., 2019).

The understanding of physiological processes in C3 and C4 plants in mitigating drought stress still needs further exploration. Previous studies on physiological processes of C4 plants toward drought stress have been conducted on *Echinochloa crusgallii, Amaranthus caudatus*, and *Z. mays* that sustained net CO_2 assimilation even stomatal conductance was reduced without enhanced carbon dioxide concentration (Hamim, 2005; Vanaja et al., 2011), *Z. mays* also experienced high antioxidant activities followed with damage reduction to counter water stress (Nayyar & Gupta, 2006), and *Pennisetum centrasiaticum* indicated water use efficiency and rapid recovery (Luo et al., 2020). The studies have investigated that C4 plant exhibited endured physiological responses under drought stress. However, how endogen proline and AsA are able to withstand drought and recovery periods still needs to be evaluated so far. Here, we assessed physiological aspect (relative water content), biochemical (proline content), and antioxidant (ascorbic acid content) in *Zea mays* (C4) and *Cucumis sativus* (C3) at different levels of drought periods relative to full irrigated control plant and also evaluated measured properties changes under rewatering conditions. Results of this study provide a basis plant physiology response of C4 and C3 plants, in particular, focused on osmoprotectant and antioxidant accumulation to manage adapted breeding programs in the frequent drought of future climate changes.

MATERIALS AND METHODS

Plant Growth and Treatment Experimental Set-up

The study was conducted from May to August 2017. Seeds of *Z. mays* var. Bonanza 9 and *C. sativus* var. Etha 87 were planted in polybags, which contained a mixture of ¾ sand and ¼ compos. Four seeds of each polybag were germinated and irrigated twice a day in a plastic greenhouse. Fifty days after emergence, seedlings were treated with full irrigation, water stress, and rehydration, with three replications of each trial. Water stress was carried out by retained irrigation for 2-d, 4-d, 6-d, and 8-d and followed by rewatering during 7 days for recovery. Young leaves were used to measure the biochemical analysis for each replication.

Relative Water Content Analysis

Leaf relative water content (RWC) was measured according to Jeon et al. (2006). Fully developed leaves were holed using a cod borer with 0.5 cm in diameter. They were weighed as fresh weight (FW), followed by placed in a petri dish containing aquadest for 24 h to attain turgid weight (TW). Subsequently, leaves were dried in an oven at 60°C for 48 h to gain dry weight (DW). Data of RWC was measured as Equation 1:

$$\text{RWC} = \frac{FW\,(g) - DW\,(g)}{TW\,(g) - DW\,(g)} \times 100 \qquad (1)$$

Proline Analysis

The method of Bates et al. (1973) was used to determine the proline concentration. Fresh young leaves of 0.5 g were extracted with 3% (w/v) sulphosalicylic acid and centrifuged. Reagent mixture of ninhydrin was prepared by dissolving 1.25 g of ninhydrin with 30 mL of glacial acetic acid, 20 mL of warm 6 M H_3PO_4, air-dried then kept at 4°C. A 2 mL of supernatant was mixed with 2 mL of ninhydrin solution and 2 mL of glacial acetic acid in a test tube and boiled in a water bath at 100°C for 1 h. After termination in the ice bath, 4 mL of toluene was added, followed by homogenized with vortex for 15s. The toluene phase was collected and the absorbance was read at 520 nm using a spectrophotometer. Toluene was used as a blank and proline concentration (μmol.g^{-1} FW) was calculated using the standard curve.

Ascorbic Acid Analysis

Free ascorbic acid (AsA) was determined according to the method of Reiss (1993). Young leaves (0.5 g) were extracted with 5% (w/v) metaphosphoric acid and filtered with filter paper of Whatman No.1. An extract was titrated with 0.8 g/L of dichlorophenol-indophenol (DCIP). The DCIP solution was standardized early by titrating a solution containing1 mL of ascorbic acid (4.0 mg/L) and 9 mL of metaphosphoric acid (5%). The pink color was defined as the endpoint of titration.

Statistical Analysis

The data were analyzed using The Independent Sample t-test to compare parameters between C3 and C4 plants. A one and two-way analysis of variance were used to determine the effect of time and treatments in each plant. If significant differences were presented, Tukey HSD was tested among treatments. All data represented the value of Mean ± SE. In the case of ascorbic acid, data presented as Mean ± CI (Confidence Interval at 5%). All the analyses were conducted using IBM SPSS software (version 25.0. Armonk; IBM corp.; NY) with a $p = 0.05$.

RESULTS AND DISCUSSION

Relative Water Content

The leaves RWC in *Z. mays* were significantly higher than those in *C. sativus* at 2-d and 8-d after rehydrated exposure, while a higher value was otherwise recorded in *C. sativus* at drought stress of 2-d (Table 1). Repeated-measure of ANOVA revealed that the effect of time F=4.69), treatment (F=85.29), and their interaction (F=3.88) significantly (P=0.01) decrease the RWC in *Z. mays* (Fig. 1). In *C. sativus*, the test of repeated ANOVA also showed a significant (P=0.01) effect of time (F=6.69), treatment (F=207.46), and their interaction (F=4.22) which showed a declined RWC (Fig. 1). Both plants showed that dropped RWC in which remained lower than control plants was detected in water-deficit plants. Even rewatering did not reverse to control values. The deficit of water tended to decrease along with increase of time. RWC decreased by 16% and 29% in *Z. mays* compared to control at 2-d and 4-d after drought time, respectively, and decrease by 30% at 6-d and 8-d (Fig. 1a). Recovery effect was lately showed after 8-d rehydrated in *Z. mays* that higher than drought stress. Subsequently, *C. sativus* showed a fluctuate decrease of RWC by 20%, 10%, 31%, and 15% along with the increased time of drought stress. *C. sativus* represented a divergent pattern in which rewatering did not rectify the RWC of leaves even persisted lower than water-stress and control plants (Fig. 1b).

Table 1. Relative water content (RWC) between *Zea mays* and *Cucumis sativus* in each treatment

Treatments		***Z. mays***	***C. sativus***
Full irrigation		1.09 ± 0.04 ^{ns}	1.06 ± 0.03 ^{ns}
2-d	water stress	0.92 ± 0.08 ^{ns}	0.85 ± 0.01 ^{ns}
	water stress + rehydrated	0.82 ± 0.04 *	0.65 ± 0.01 *
4-d	water stress	0.77 ± 0.04 **	0.96 ± 0.00 **
	water stress + rehydrated	0.77 ± 0.01 ^{ns}	0.74 ± 0.02 ^{ns}
6-d	water stress	0.73 ± 0.01 ^{ns}	0.73 ± 0.05^{ns}
	water stress + rehydrated	0.74 ± 0.02 ^{ns}	0.66 ± 0.03 ^{ns}
8-d	water stress	0.77 ± 0.05 ^{ns}	0.90 ± 0.02 ^{ns}
	water stress + rehydrated	0.95 ± 0.04 **	0.62 ± 0.02 **

Note. Abbreviation: * $p < 0.05$, ** $p < 0.01$, n.s - not significant, Independent Sample t test. Values show Mean ± SE

Drought caused a range variation of physiological mechanisms between *Cucumis sativus* (C3) and *Zea mays* (C4) plants observed in our study. Our study revealed that drought exposure strongly reduced leaves RWC in both C3 and C4 plants that were similar to other recorded species such as *Periploca angustifolia* (Dghim et al., 2018). Drought reduces soil water availability which resulted in the reduction of water supply to leaves through xylems. This declines leaf hydraulic conductance that could reduce water potential and detracts turgor pressure which is displayed by a low leaf RWC. A decreased leaf water potential and a high transpiration rate greatly lower RWC value in water deficit conditions. In *Z. mays*, leaf hydration decreases parenchyma cells conductance and declines xylem path pressure in bundle sheath (Kim et al., 2018). Plant adaptation to prolonged water shortage is likely can be further explained by the changes in the root system morphology (Feng et al., 2016) rather than leaf structure. An increased period and severity of drought cause the decrease of leaves RWC in *Z. mays* that continue dropping to a lower level compared with control and rewatering. This significant reduction was affected by the longest period at 8-d drought exposure. Type of C4 plants responded to water stress by re-established their RWC after rewatering at 8-d which increased up to 28% from last treatment of 6-d. However, this response was not recorded in C3 plant as has been reported that *C. sativus* require a considerable water supply to reduce water deficit (Song et al., 2020). This different pattern was supported by the positive recovery effect of RWC after rewatering in *Z. mays* that were 50% higher than that in *C. sativus* at 8-d in the present study. An increased RWC might be due to the increased of water potential that resulted from an increase of stomatal closure and reduction of transpiration (Mathobo et al., 2017; Merlaen et al., 2019). Although plant uptakes the water through the xylem to restore the loss of leaves' water potential by drought, this could not rapidly reach a similar value to that of control plants. In line with other studies, rewatering the water-stressed plants, however, could not resolve the declined RWC due to the drought effect (Dikšaitytė et al., 2019; Pourghayoumi et al., 2017). Such reduction of RWC may be caused by increase absorption or translocation of water to other parts of plants as a physiological mechanism to suppress drought impact. However, several studies have reported that apparent reduction in water status under water deficiency leads to inhibition in stages of plant growth (Okunlola et al., 2017).

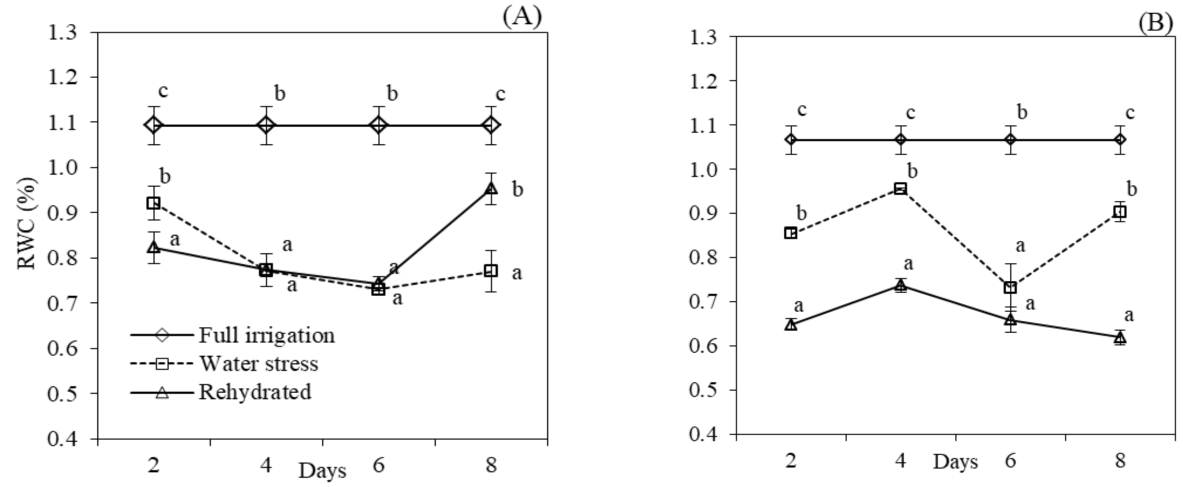

Figure 1. Changes in RWC of (A) *Z. mays* and (B) *C. sativus* through treatment periods in full irrigation (◊), water stress (□), and rehydrated (Δ). Values show Mean ± SE. ($P < 0.05$, Tukey HSD test). Different letters indicate significant differences between treatments ($p < 0.05$, Tukey HSD test)

Proline Content

Exposure of water deficit to *Z. mays* showed higher proline significantly than those in *C. sativus* along with the increased stress periods (4-d, 6-d, and 8-d) (Fig. 2). Leaves of 2-d water stress did not affect significantly between plants. The rehydrated treatment presented the changes of proline in both leaves' plants, which showed a significantly higher concentration in *Z. mays* at 4 and 8 days and higher in *C. sativus* at 2 and 6 days after rehydration.

Repeated measurement by ANOVA showed that time (F=383.52), treatment (F=365.82), and their interaction (F=148.52) significantly (P=0.01) affected proline concentration in *Z. mays* (Fig. 3). As indicated in Fig. 3a, treated plants of *Z. mays* demonstrated increased proline compared with control plants, which had the highest value after 8-d of drought stress and 4-d of rehydrated treatment (increased by 20% and 21% respectively). A low proline of *Z. mays* after 2-d rehydration compared to control plants showed a highly increased proline after 4-d and was significantly declined then recovered after re-watered at 6-d and 8-d, respectively. In *C. sativus*, repeated measures of ANOVA showed a significant effect on proline (P=0.01) with time (F=74.86), treatment (F=722.28), and their interaction (F=147.63) (Fig. 4). There was a decrease in proline compared with control plants at 2-d, 6-d, and 8-d excluding 4-d of drought stress (Fig. 3b). Long exposure of water deficit at 8-d showed the lowest proline, decrease by 8% and 13% compared with control and rewatering plants respectively. Rehydration in *C. sativus* showed a recovered value of proline at 2-d (15% increase compared with control plants) but 4-d after re-watered showed a significantly dropped proline by 11% decrease. However, irrigation after water deficit showed an increase proline compared with control plants and drought treatments.

Our data showed that higher proline was accumulated in *Z. mays* during water stress than *C. sativus*. These amino acids were increased after the recovery period of 4-d and 8-d at drought stress of *Z. mays* and produced higher of those compared with control plants. It is likely shown that *Z. mays* may induce a high endogenous proline synthesis during a long period of drought conditions. This status significantly resulted in a response of plants to resolve the reduced RWC of leaves in our experiments which is similar to other studies (Chaeikar et al., 2020). It is considered why proline increased at the end of the drought period is a strategy in *Z. mays* to improve root absorption for water intake. *C. sativus* exhibited an adverse effect due to a dropped proline during water stress and this solute was significantly lower compared with control, although had an increased by 4-d. It was found that *C. sativus* depended on rewatering after drought stress to recovery and increase proline which was consistently higher compared with control, whereas *Z. mays* synthesized much proline during drought periods. Our study is in aggrement with different plant species, those were *Sorghum bicolor* cv Sugargraze (Nxele et al., 2017), as well as *Matthiola incana* (Jafari et al., 2019) and *Oryza sativa* (Khan et al., 2019) which demonstrate an increased proline that was more pronounced during drought stress and accounted for increase osmotic adjustment. Withholding water rises glutamate dehydrogenase activity that involves as an important precursor in proline biosynthesis under environmental stresses (Hessini et al., 2019) which is positively up-regulated by Δ1-pyrroline-5-carboxylate synthetase (P5CS) expression (Ming et al., 2017; Wang et al., 2019). It has been confirmed that proline accumulation is found to act as an osmoprotectant by maintaining cell membrane structure and foliar water status to sustain chlorophyll synthesis and consequently protect plants from detrimental impacts of water deficiency (Semida et al., 2020). Furthermore, the accumulation of high internal proline plays a pivot core in the reduction of oxidative stress and adjusting redox balance.

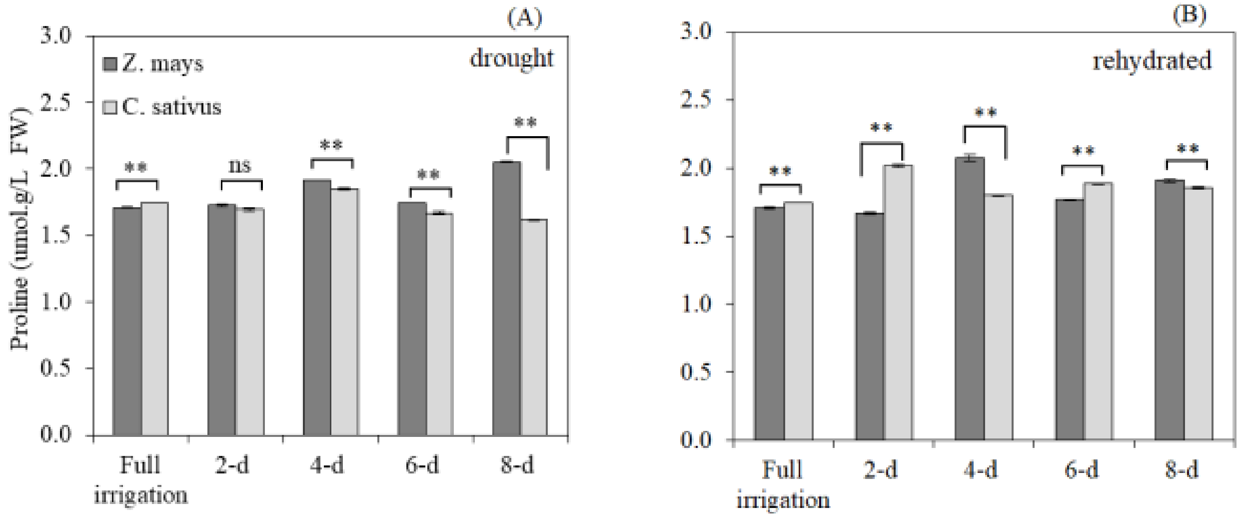

Figure 2. Proline concentration of (a) water stress exposure and (b) rehydrated. Values show Mean ± SE. Abbreviation: * $p < 0.05$, ** $p < 0.01$, n.s = not significant, Independent Sample t test

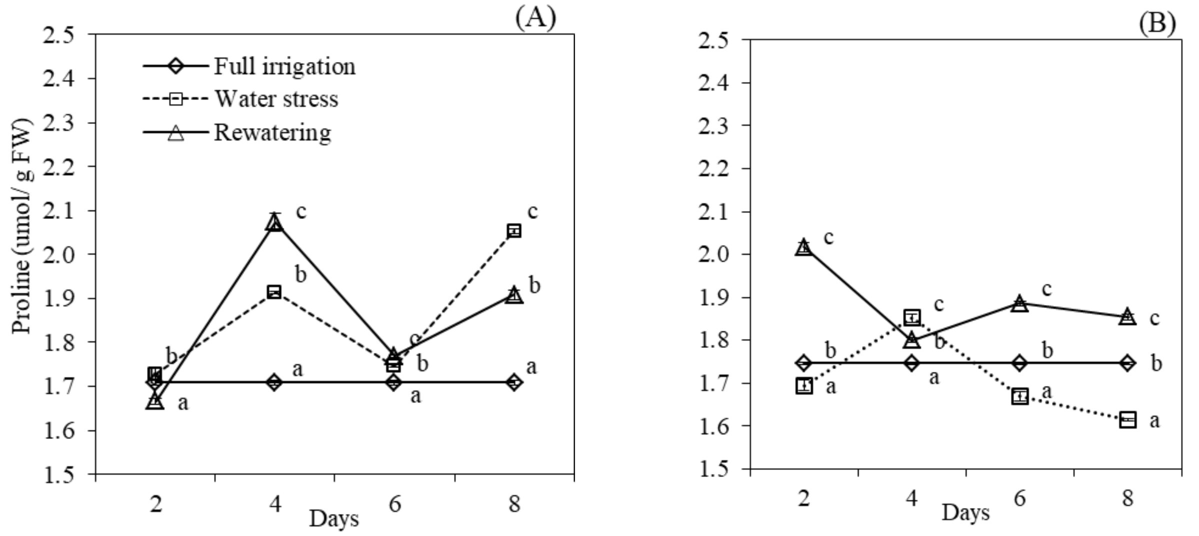

Figure 3. Changes in proline concentrations for (a) *Z. mays* and (b) *C. sativus* through treatment periods in full irrigation (◊), water stress (□), and rehydrated (Δ). Values show Mean ± SE. Different letters indicate significant differences between treatments ($p < 0.05$, Tukey HSD test)

Ascorbic Acid Content

Ascorbic acid (AsA) in *Z. mays* showed higher value along with increase period treatments than that of *C. sativus* excluding at 2-d and 8-d after rewatering (Fig. 4). Increasing period showed a decreased proline in *Z. mays* after 8 days rehydrated and these values were similar with *C. sativus*. As shown in Fig. 5a, AsA of *Z. mays* showed the highest contents at 6-d after stress of drought among trial experiments (increase by 47% and 31% compared with control and rehydrated plants respectively). Reduced AsA were showed by rehydrated (50%) followed by drought stress (40%) compared with control plants. Indeed, fluctual changes of AsA in *Z. mays* showed that plants exposed with water stress tend to accumulate high proline at 6-d. A trend of AsA in *C. sativus* diverged from that in *Z. mays* (Fig. 5b). Re-watered plants just showed the accumulation of high AsA at 2-d, increase by 50% and exceed 100% in control and water-stressed plants respectively. Drought just showed a similar value with control plants at certain time of 6-d, but tended to decrease again at 8-d in which similar with rehydrated plants. However, drought-stress plants of *C. sativus* did not showed an accumulation of AsA exceeding of control plants along with increasing periods.

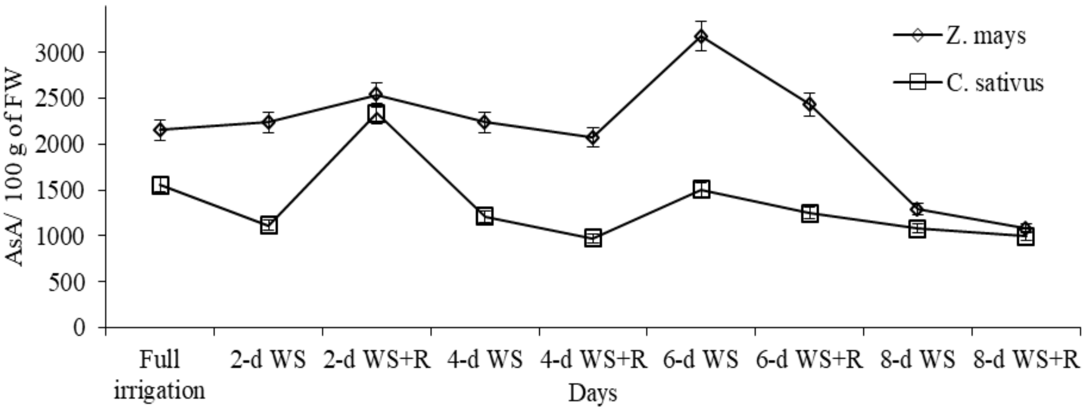

Figure 4. Changes in Ascorbic Acid (AsA) through treatment periods. Values show Mean ± CI (Confidence Interval at 5%). WS (Water Stress); R (Rehydrated)

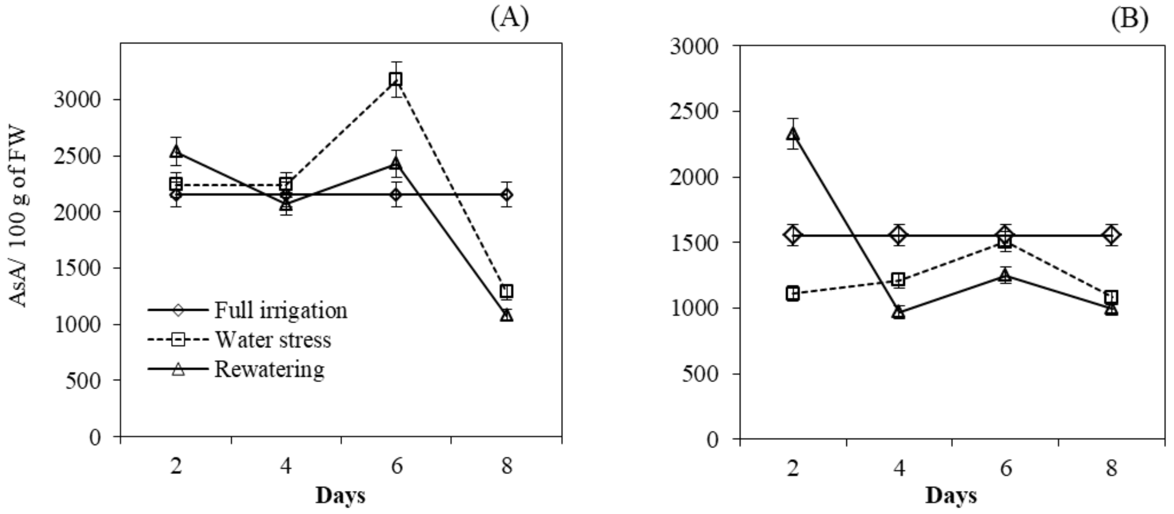

Figure 5. Changes in Ascorbic Acid (AsA) through treatment periods for (a) *Z. mays* and (b) *C. sativus* in full irrigation (◊), water stress (□), and rehydrated (Δ). Values show Mean ± SE (Confidence Interval at 5%)

In addition to response drought and recovery after rewatering, AsA is considered as an important antioxidant by oxidized enhanced oxygen radicals of ROS thus have been underlined as a main indicator of abiotic stresses (Franzoni et al., 2019). Water status foliar tightly correlates with soil water deficit caused by oxidative stress which in turn induced higher AsA concentration that leads to reduce stomatal aperture in dry season

(Figueiredo-lima et al., 2018). In our study, although *Z. mays* contained higher AsA than those in *C. sativus*, a significant increased AsA was only found in a certain period of 6-d of drought. Otherwise, *C. sativus* did not have tolerance in this drought situation through AsA mechanism. This antioxidant concentration during drought could be varied depending on species. Brunetti et al., (2019) obtained similar result that AsA concentration in *Celtis austalis* did not changed and affected by 2-wk-drought. In addition, scavenging antioxidant also did not differed between *Vigna unguiculata* genotypes (Carvalho et al., 2019). It is very likely *Z. mays* as C4 plant in this experiment showed a moderate symtom due to drought periods, thus also did not showed an increase endogenous hormone of abcisic acid which reported found in severe drought (Caser et al., 2019). For re-watering effect, both C3 and C4 plant in our study declined AsA contents during increased period which were lower compared with control. It could be assumed that reduce AsA was likely the result of recovery from drought damage. This was similar to *Prunus* hybrids (Sofo et al., 2005) that confirmed the reduction of not only AsA but also down-regulated of antioxidant enzymes. Rewatering may drive sufficent water need for plant to restore all celluler compartment and metabolism processes and to restrict damage caused by ROS production during water shortage, and those are expected to display similar condition with well-watered plants. Other studies highlighted positive effect after recovery, such as a increase capacity of photosynthesis (Sihem et al., 2020) and activated potential secondary metabolite compounds to adapt (Almeida et al., 2020).

It was recorded that evaluating proline in this study was suggested as a considered informative parameter to understanding drought response in different plant species. The results suggest that maintained proline in *Z. mays* (C4) provided with appropriate irrigation by rewatering over exceeded periods in our study is possible to reserve water with consideration of the growth adapt mechanism. Drought stress could have been severe effects depending on the plant species and the exposure periods of water stress.

ACKNOWLEDGMENTS

Great thanks are recorded to Biology Department, IPB University for supporting laboratory analysis in this experiment.

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